APPROACHES TOWARDS INCREASING DISEASE RESISTANCE IN PLANTS USING GENETIC ENGINEERING

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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August 1993

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"If you can write a PhD thesis, you can do anything in life."

Gary Lyon,

Scottish Crop Research Institute,

May 1992.

Acknowledgements

I would like to thank both my PhD supervisor, Dr. John Draper, and my present supervisor, Dr. Keith Lindsey, for reading this thesis and offering helpful comments and suggestions. I would also like to thank the following people who have offered me their specialised knowledge during the course of the project: Dr. Garry Whitelam for advice and discussion on the activities of superoxide dismutase; Dr. Chris Hadfield for help on all the yeast work and comments on the results; and Drs. Gary Lyon and Hugh Barker at the Scottish Crop Research Institute for help and advice concerning *Erwinia carotovora* and Potato Virus Y respectively, and especially to Gary Lyon for introducing me to Belhaven beer. Particular thanks go to the 'Dalgety Boys', Drs. Ken Siggins and Alan Mileham who introduced me to the wonderful world of PCR while I was working in the Dalgety lab as part of my CASE responsibility. I would also like to thank John Draper once again for sustaining this project throughout its three years.

I particularly want to thank Dr. Simon Warner (or plain old Simon as he was then) for practical advice on techniques and also Rob Darby for giving me asparagus RNA and numerous other things when requested. I also want to thank all those members of the Botany Department for the little things that really made a difference.

Finally, I thank all my friends, both past and present, and my family, especially my parents, for their constant support and belief in me.

And really finally, thanks to lan for everything.

Work contained within this thesis has been presented at the following conferences:

Sept 1990	Poster at the 5th International Symposium on the Molecular Genetics of Plant-
	Microbe Interactions, Interlaken, Switzerland. Abstract title: "Identification of a
	group of wound-induced polypeptides in Asparagus officinalis".
Apr 1991	Poster at the Society of Experimental Biology meeting, University of
	Birmingham. Abstract title: "Identification of wound-induced genes in
	Asparagus and application in engineering plants for resistance".
Oct 1991	Poster at the International Society of Plant Molecular Biology meeting,
	Tucson, Arizona. Abstract title: "Identification of wound-induced superoxide
	dismutase in Asparagus officinalis".

Apr 1992 Paper presented at the Society of Experimental Biology meeting, University of Lancaster. Abstract title: "Behaviour and molecular analysis of superoxide dismutase in Asparagus single cell suspensions".

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ABBREVIATIONS

AMPS	ammonium persulphate
АТР	adenosine 5' triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
рр	base pairs
BSA	bovine serum albumin pentax V fraction
cDNA	complementary DNA
cpm	counts per minute
CTAB	hexadecyltrimethylammonium bromide
2-D	two-dimensional
dA/C/G/TTP	2' deoxyadenosine/cytidine/guanosine/thymidine 5' triphosphate
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-sulphonic acid
IPTG	isopropyl-β-D-thiogalactoside
kh	kilobases
kDa	kilodaltons
NDa	NIGAROIS
min	minute(s)
MOPS	3-(N-morpholino) propane-sulphonic acid
4-MU	4-methylumbelliferone
MUG	4-methylumbelliferyl glucuronide

NBT	nitro blue tetrazolium
nt	nucleotide
PEG	polyethylene glycol
pfu	plaque forming unit
poly (A ⁺) RNA	polyadenylated RNA
PVP	poly vinylpyrolidone
QH2O	Millipore filtered, double distilled water
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylenediamine
Tris	Tris-(hydroxymethyl)-methylamine
UV	ultraviolet
v /v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-GLUC	5-bromo-4-chloro-3-indolyl-β-D-gulcuronide

Other abbreviations used are explained in the text or are found in Sambrook et. al. (1989).

CHAPTER 1

INTRODUCTION

The interaction between plants and pathogenic microorganisms is highly complex. Study of this interaction and also of the response of the plant to stress in general, may reveal insights into methods by which, through the use of genetic engineering, we may improve and/or supplement this response to provide increased resistance to diseases. This introductory chapter outlines elements of the response of the plant to pathogens and wounding, and leads into the thesis aims which are the exploitation of aspects of the plant's response to stress to increase resistance in plants to bacterial infections.

1.1 Stress-inducible gene expression in plants

In their natural environment plants are unable to move to escape or avoid environmental adversity. Instead, they have evolved a highly complex response to protect against the stresses caused by, for example, weather conditions, wounding and attack by various pests and pathogens. The initial responses by the plant serve to either repair or seal off damaged tissue to prevent dessication and invasion by opportunistic pathogens. Proteins are then synthesised which serve to prevent further spread of any invading pathogens, while signals are sent to the rest of the plant resulting in the expression of proteins that confer systemically acquired resistance (SAR) with long-term, wide range protection from pathogen attack.

Many pathogens are opportunistic and are only able to infect plants through damaged tissue. For example, many viruses can only be transmitted through aphids which pierce the outer plant tissue layers enabling viral particles to enter. It is therefore unsurprising that the response of the plant to both wounding and pathogen attack is very similar in many respects. The initial response of sealing the wound site to prevent desiccation also acts to prevent entry and spread of pathogens. One can therefore envisage that similar signalling processes are in operation in activating the genes involved in both wounding and pathogen attack.

1.1.1 The wound response

Since the response to both wounding and pathogen invasion are similar, study of the wound response may give insights into the defence mechanism of plants. Examination of the wound response may also reveal possible approaches for engineering resistance.

According to Bostock and Stermer (1989) a wound is "any external or internal injury that breaches the outer protective layers of the plant.....Implicit in this definition is the physical rupturing of cells with concomitant loss of compartmentation". Wounds therefore include the obvious physical damage caused, for example, by severe weather conditions and the feeding of insects and herbivores. The definition also includes damage originating internally such as that resulting from the growth and spread of pathogens, leading to the appearance of visible symptoms.

The sealing of wound sites varies according to plant species (reviewed by Bostock & Stermer, 1989). In general, monocotyledonous plants show little or no wound-induced cell division with the cells at the wound surface undergoing autolysis and death. Cell walls immediately adjacent to the wound site are then infused with lignin or other polyphenols to seal the wound. In dicotyledonous woody and herbaceous plants, cells at the wound site also undergo autolysis but, in contrast to monocotyledonous plants, parenchyma cells adjacent to the wound site (at least in larger wound sites) redifferentiate to form a lignosuberinized boundary zone, and meristematic activity of cells next to this boundary layer form a suberinized wound periderm. Both responses involve an increase in transcriptional activity with concomitant increase in metabolic activity and upregulation of genes associated with the defence response.

1.2 The defence response

Genes induced as a consequence of pathogen invasion or wounding (reviewed by Gianinazzi, 1984; Fritig *et. al.*, 1987; Bowles, 1990; Bol *et. al.*, 1990) lead to the synthesis of proteins that initially serve to modify the cell wall, leading to the formation of a physical barrier to prevent spread of any invading pathogen from the entry site. These wall-strengthening polymers include lignin, callose, extensins and glycine-rich proteins. Deterrents, such as the phytoalexins, hydrolytic enzymes, and digestive enzyme inhibitors, are also synthesised to prevent pathogen multiplication and spread from the infected area. Signalling molecules such as salicylic acid and ethylene are produced, causing the induction of the defence response in areas of the plant far removed from the initial site of wounding or infection. Enzymes are also induced as a consequence of the oxidative stress caused by wounding and pathogen invasion.

1.3 The phenylpropanoid biosynthesis pathway

Many of the products formed as a consequence of pathogen attack are the products of a single pathway, the phenylpropanoid biosynthesis pathway. Branches of this pathway lead to the production of flower pigments, UV protectants, insect repellents, and signal molecules involved in plant-microbe interactions (reviewed in Hahlbrock & Scheel, 1989). In the defence response, the pathway is important in leading to the synthesis of phytoalexins, which are low molecular weight compounds with antimicrobial activity (reviewed by Kuc & Rush, 1985), to the synthesis of the precursors of lignin, suberin and other wall-bound phenolics (reviewed by Lewis & Yamamoto, 1990), and to the possible synthesis of the signalling molecule, salicylic acid (Ward *et. al.*, 1991). The role of salicylic acid will be discussed further in section 1.8.3.

1.3.1 Phenylalanine ammonia-lyase

The first enzyme of the phenylpropanoid biosynthesis pathway is phenylalanine ammonia-

lyase (PAL) which catalyses the deamination of L-phenylalanine to yield trans -cinnamic acid (reviewed by Hahibrock & Scheel, 1989; Bowles, 1990). Phenylalanine itself is one of the end products of the shikimate pathway, the first enzyme of which, 3-deoxy-D-arabino heptulosonate-7-phosphate synthase, was found to be wound-inducible in potato (Dver et. al., 1989). PAL itself is also wound-inducible in potato (Shaw et. al., 1990; Rickey & Belknap, 1991) but only under aerobic conditions (Rumeau et. al., 1990). The study of PAL genes in parsley (Lois et. al., 1989) and bean (Cramer et. al., 1989) suggests that the enzyme is coded for by a family of genes. Three of the parsley genes, PAL1, PAL2, and PAL3, were found to be induced by wounding, fungal elicitor and by UV irradiation with induction by fungal elicitor being more rapid (2 hours) than induction by UV irradiation (4 hours). By using labelled antisense PAL RNA as a probe, Schmelzer et. al. (1989) determined that PAL is expressed very rapidly following infection by Phytophthora megasperma, with expression being highly localised to the cells around infected lesions. A slightly different pattern of induction is found in bean in which three classes of PAL genes are present, gPAL1, gPAL2, and gPAL3, with each class containing polymorphic forms. All classes were found to be induced by wounding but only gPAL1 and gPAL2 were induced by fungal elicitor (Cramer et. al., 1989). By using a transient expression system, a promoter fragment from the pea PAL gene, gPAL1, has been identified that is responsible for activation by fungal elicitor (Hashimoto et. al., 1992). PAL appears to be regulated by its end product, trans -cinnamic acid, since over expression of the bean PAL2 gene in tobacco plants, under the control of the CaMV 35S promoter, caused a down-regulation of phenylpropanoid biosynthesis and suppression of endogenous tobacco PAL transcripts (Elkind et. al., 1990).

1.3.2 4-Coumarate CoA Ligase

The cinnamic acid formed as a product of the reaction with PAL is the precursor of all phenylpropanoids. The second enzyme of the phenylpropanoid biosynthesis pathway, coumaric acid 4-hydroxylase (C4H), converts cinnamic acid to p-coumaric acid. Coumaric acid can then be converted sequentially through caffeic acid to ferulic acid, and to sinapic acid. Each of these products can enter several pathways but any one of the coumaric, ferulic, and sinapic acids can be converted by the third core enzyme of the phenylpropanoid biosynthesis

pathway, 4-coumarate CoA ligase (4CL), to their corresponding coenzyme A esters (reviewed by Bowles, 1990).

The expression of 4CL has been studied in parsley, where the enzyme exists in two isomeric forms encoded by single-copy genes (Douglas *et. al.*, 1987). Expression of 4CL is closely regulated with that of PAL (Lois *et. al.*, 1989), being rapidly expressed in a highly localised fashion around sites of infection by *Phytophthora megasperma* (Schmelzer *et. al.*, 1989). Like PAL, 4CL is also wound-inducible (Schmelzer *et. al.*, 1989).

Following the action of 4CL, the phenylpropanoid biosynthesis pathway branches into pathways that lead to the synthesis of the flavonoid phytoalexins and to the synthesis of the precursors of lignin.

1.3.3 Chalcone synthase

The action of chalcone synthase (CHS) on 4-coumaryl CoA to form naringenin chalcone (reviewed by Dangl *et. al.*, 1989) is the first committed step into flavonoid biosynthesis. Parsley contains two alleles of the CHS gene which differ in the possession of a transposon-like element in the promoter region of one allele (Herrmann *et. al.*, 1988). Parsley CHS is light induced, corresponding with the role of flavonoids as UV protectants, and regulatory sequences have been identified which are involved in light activation (Schulze-Lefert *et. al.*, 1989). However, the parsley CHS gene is insensitive to other environmental stimuli including wounding and pathogen invasion. In contrast, bean contains eight CHS genes which, unlike the parsley CHS gene, can be differentially activated by elicitor and wounding, correlating with the accumulation of isoflavonoid phytoalexins (Ryder *et. al.*, 1987).

The naringenin chalcone formed by the action of CHS then undergoes various reactions to give rise to a large range of phytoalexins, pigments and UV protectants.

1.3.4 The lignin pathway

An alternative phenylpropanoid biosynthesis pathway branch following the production of 4coumaryl CoA occurs through the actions of the enzymes 4-coumaroyl-CoA reductase and cinnamyl-alcohol dehydrogenase, which yield 4-coumaryl alcohol, a direct precursor of lignin. Coniferyl alcohol and sinapyl alcohol, the other precursors of lignin, are ultimately formed from coumaric acid, a product of the action of 4CL (reviewed by Lewis & Yamamoto, 1990). Lignification of cell walls requires the polymerisation of these precursors, a reaction requiring the presence of hydrogen peroxide and peroxidase enzymes. A wound-inducible peroxidase has been identified and cloned from potato (Roberts *et. al.*, 1988) and found to be induced also by abscisic acid (Roberts & Kolattukudy, 1989), a compound thought to be involved in the signalling process (discussed in section 1.8.1). Extracellular peroxidases have also been identified from barley which are induced in response to infection with the powdery mildew pathogen, *Erysiphe graminis* (Kerby & Somerville, 1992).

1.4 Defence products that modify the cell wall

Strengthening of the cell wall is one of the first responses to wounding, providing a barrier to prevent dessication and the entry and spread of pathogens (reviewed by Bostock and Stermer, 1989). Products formed in this respect include the extensins, also known as hydroxyproline-rich glycoproteins (HRGPs) and the glycine-rich proteins (GRPs).

1.4.1 The extensins (HRGPs)

The extensins are a family of rod-like, hydroxyproline-rich glycoproteins found as structural components in plant cell walls which rapidly accumulate in response to wounding or pathogen invasion (reviewed by Cassab & Varner, 1988). Localisation studies show that the transcripts of HRGP genes are expressed in the meristematic cells (Ye *et. al.*, 1991, and references therein) suggesting a possible role in the early stages of wall assembly. During cell differentiation additional HRGPs may be required depending on the function of different cell

types, for example, tobacco HRGP appears to be specifically expressed at sites of lateral root initiation (Keller & Lamb, 1989). The role of extensin in wound healing and disease resistance is postulated from the observation that extensin mRNA and protein accumulate in response to wounding and pathogen infection. In this respect it is interesting to note that sunflower plants accumulate HRGP transcripts when infected with *Sclerotinia sclerotiorum*, the agent of white mould, and also when treated with oxalic acid, the toxin produced by the fungus (Mouly *et.al.*, 1992). Not only does oxalic acid act as a toxin in this particular instance, it also serves to elicit the induction of the accumulation of HRGP transcripts.

HRGPs are characterised by the possession of a repeating pentapeptide sequence of $Ser(Hyp)_4$ where Hyp refers to hydroxyproline, formed by modification of proline residues by a prolyl hydroxylase associated with the endoplasmic reticulum (Bolwell *et. al.*, 1985). The extensins are highly glycosylated. For example, an extensin from carrot was found to contain a carbohydrate content of 50%, the majority being arabinose and the rest a smail quantity of galactose (Stuart & Varner, 1980). More recently, a histidine-rich extensin has been identified in maize which contains 33% arabinose and 31% galactose (Kieliszewski *et. al.*, 1992). The carbohydrate groups may be involved in stabilising the extensin within the cell wall where it is immobilised by peroxidase-mediated tyrosine cross linking. It has been suggested by Bradley *et. al.* (1992) that the insolubilisation of cell-wall structural proteins through oxidative cross-linking is one of the first stages of plant defence since it precedes the expression of proteins which are reliant on transcription.

Extensins are encoded by multigene families which have been studied extensively in tomato, in which three distinct classes of extensins exist. Classes I and II accumulate in a localised manner in response to wounding (Showalter *et. al.*, 1992), while class IV contains sequence characteristic of a glycine rich protein on one DNA strand while the other DNA strand contains extensin sequence, both of which accumulate on wounding (Showalter *et. al.*, 1991). Wounding-associated accumulation of extensin transcripts has also been observed in carrot (Ecker & Davis, 1987) and bean (Corbin *et. al.*, 1987).

A subset of the HRGPs are the proline-rich proteins (PRPs), examples of which have been cloned from soybean (Hong *et. al.*, 1987), wheat (Raines *et. al.*, 1991) and bean (Sheng *et.*

al., 1991). These proteins are characterised by having a high proline content (reviewed by Marcus *et. al.*, 1991). They are thought to play a structural role and are highly associated with lignified tissue, particularly the xylem vessel elements (Ye *et. al.*, 1991; Wyatt *et. al.*, 1992). Involvement of the PRPs in the defence response has been observed in bean (Sheng *et. al.*, 1991) in which transcript levels were found to increase in response to wounding, although a decrease in transcript levels was reported in response to fungal elicitor.

1.4.2 The glycine rich proteins

The glycine rich proteins (GRPs) are characterised by having a glycine content of around 60%. Genes have been cloned from bean (Keller et. al., 1988), petunia (Condit & Meagher, 1986) and tomato (Showalter et. al., 1991). All show a rapid accumulation of transcript following wounding. Isolation of the promoter for the bean GRP has enabled study of expression using the reporter gene, GUS, which showed rapid induction following wounding in areas immediately adjacent to the wound boundary (Keller et. al., 1989a). Localisation studies show that GRP mRNAs are primarily found in lignified cell walls such as xylem vessels elements (Ye et. al., 1991). Polyclonal antibodies raised against one of the bean GRPs have enabled localisation of the protein to the unlignified primary cell walls of the oldest protoxylem elements (Ryser & Keller, 1992), suggesting in this instance that the protein is produced by xyiem parenchyma cells that export the protein to the wali of xylem vessels. Ye et. al. (1991) raise the possibility that since both PRPs and GRPs are deposited in cell walls prior to lignification, the function of these proteins is to provide a tensile structure for the differentiation of these cells. Alternatively, the high tyrosine content of both sets of proteins may have a catalytic effect on the oxidative polymerisation chain reactions of lignification, effectively serving as a type of scaffold for the deposition of lignin (Keller et. al., 1989b).

An alternative function for some GRPs has been put forward by Sturm (1992). Based on the homology of a wound-inducible GRP from carrot with various RNA-binding proteins, particularly the A1 protein of the heterogeneous ribonucleoprotein complex, Sturm suggests that the carrot GRP is involved in the maturation or transport of specific mRNAs in response to wounding. However, further work, such as the localisation of the transcript and protein, is

required in order to confirm this possible function.

1.5 Defence products with deterrent properties

The phytoalexins are one group of compounds synthesised during the defence response with deterrent properties. Other compounds include endohydrolases, protease inhibitors (PI) and thionins.

1.5.1 The phytoalexins

The phytoalexins are formed as byproducts from branches of the phenylpropanoid biosynthesis pathway. They are classified as low molecular weight compounds with antimicrobial activity that accumulate rapidly around sites of infection (reviewed by Kuc & Rush, 1985). It has been reported that phytoalexins in groundnuts accumulate in response to wounding (Arora & Strange, 1991), but in general phytoalexin accumulation is very much associated with pathogen infection. For example, it has been shown that under aerobic conditions, the phytoalexins rishitin and phytuberin are produced in potato in response to infection by *Erwinia* (reviewed by Lyon, 1989). Rishitin was also shown to inhibit growth of *Erwinia*. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMGR) catalyses the rate-limiting step in isoprenoid biosynthesis, leading to accumulation of phytoalexins. HMGR is encoded by a family, isogenes of which are differentially activated by wounding or pathogen challenge (Yang *et. al.*, 1991).

1.5.2 Endohydrolases

The two most studied endohydrolases are the chitinases and the β -glucanases (reviewed by Dixon & Harrison, 1990; Bol *et. al.*, 1990; Bowles, 1990). Both enzymes are members of the group of classical pathogenesis-related (PR) proteins, initially isolated from tobacco as a consequence of induction resulting from infection by Tobacco Mosaic Virus (TMV; Legrand,

et. al., 1987; Kauffmann, et. al., 1987; reviewed by Bol et. al., 1990,). Chitinases and β -glucanases degrade fungal and bacterial cell walls respectively. The presence of chitinases and β -glucanases inhibits fungal growth (Mauch et. al., 1988) while the breakdown of fungal cell walls releases elicitors that activate other defence genes (reviewed by Darvill & Albersheim, 1984).

Genes for chitinases and β-glucanases have been isolated from many species including bean (Hedrick et. al., 1988), potato (Kombrink et. al., 1988) and tobacco (Payne et. al., 1990). Analysis of β-glucanases in tobacco revealed the presence of gene families with eight genes for acidic β -glucanase and a smaller number of genes encoding basic β -glucanase (Linthorst et. al., 1990). The acidic enzymes are secreted into the apoplast, while the basic isozymes contain a C-terminal extension that is cleaved during targeting to the vacuoles (van den Bulcke et. al., 1989; Linthorst et. al., 1990). Chitinases are also concentrated in the vacuoles (Mauch & Staehelin, 1989). It is thought that the chitinases and β-glucanases present in the vacuole provide a last line of defence against attack, being released when the host cell lyses (Mauch & Staehelin, 1989). The cell wall-localised β-glucanases are thought to be involved in recognition, the breakdown of fungal cell walls releasing elicitors to activate other defence related genes. Chitinases and β-glucanases are induced by fungal attack (Roby et. al., 1990), ethylene (Vögeli et. al., 1988) and wounding (Hedrick et. al., 1988). Induction of chitinases and β-glucanases occurs less rapidly than the induction of other defence related genes such as PAL, with expression spreading from the infection site throughout the infected leaf and also to adjacent, non-infected leaves (Schröder et. al., 1992).

1.5.3 Protease inhibitors

Protease inhibitors (PI) are thought to contribute to the defence response by making plant tissue less digestible to insects (reviewed by Ryan, 1990), being inhibitors of animal and microbial proteases but not of plant proteases. Studies of protease inhibitors have mainly concentrated on the wound-inducible inhibitors found in potato and tomato. PI genes have been cloned from potato (Cleveland *et. al.*, 1987) and tomato (Graham *et. al.*, 1985a, 1985b). Recently, a PI I gene has been cloned from tobacco though, unlike the potato and tomato

genes, the tobacco gene shows only local induction by wounding (Linthorst et. al., 1993).

Analysis of PI II expression in potato leaves showed that induction by wounding led to a systemic response in non-wounded leaves (Peña-Cortes *et. al.*, 1988; discussed further in section 1.8.3). Though tobacco contains no genes homologous to the potato PI II gene, transgenic tobacco plants containing the potato PI II gene with corresponding upstream promoter sequences showed wound-inducible expression in the same manner as that in potato (Sanchez-Serrano *et. al.*, 1987), showing that common *trans* -acting factors responsible for wound-inducible expression must be present in both tobacco and potato. Potato PI II gene expression is greatly enhanced by sucrose, suggesting some metabolic control operates besides wound-induction (Johnson & Ryan, 1990). No enhancement by sucrose can be detected in non-tuber-bearing species of potato or in tomato (Hansen & Hannapel, 1992).

1.5.4 Thionins

The thionins are small, basic proteins found in both monocots and dicots which have toxic effects on bacteria, fungi and yeast (reviewed by Bohlmann & Apel, 1991). Their role in defence is postulated by the possession of toxicity to a variety of organisms and by the fact that their synthesis can be activated by pathogens (Bohlmann *et. al.*, 1988). The thionins have been studied most extensively in barley in which they have been found to be encoded by a complex multigene family consisting of 50-100 members per haploid genome (Bohlmann *et. al.*, 1988). The leaf-specific thionin genes contain a leader sequence which localises the thionins to the cell wall.

1.6 Defence genes with unknown functions

Many genes have been identified as being induced by wounding or pathogen attack for which a function has not yet been assigned. These include the potato *win* and *wun* genes, several classical PR proteins, and a new group of intracellular PR proteins.

1.6.1 The potato win and wun genes

Both classes of *win* and *wun* genes were isolated by differential screening of a potato cDNA library constructed using poly (A)⁺ RNA isolated from wounded tissue (Logemann *et. al.*, 1988; Shirras & Northcote, 1984). A wound-induced cDNA was used as a probe to isolate a genomic clone which was found to contain two genes arranged in tandem, *win1* and *win2* (Stanford *et. al.*, 1989). Both genes are highly homologous but intron and flanking sequences show very little similarity enabling the construction of probes specific to each gene. The two genes exhibit differential organ-specific expression following wounding, with *win1* expression restricted to leaves and stems while *win2* was expressed in the entire plant. Analysis of the *win2* promoter attached to a GUS reporter gene showed the expected wound-inducible expression pattern of *win2* in potato but showed no inducible expression in tobacco (Stanford *et. al.*, 1990), an unexpected result considering that *win2* gene homologues have been detected in tobacco (Stanford *et. al.*, 1989). Though the function of the *win* genes is unknown, they share similarities with many plant proteins thought to be involved in the defence response, including chitinase and several lectins.

The *wun1* and *wun2* genes are rapidly and highly expressed in wounded potato tubers, being detectable from 30 minutes (*wun1*) to 4 hours (*wun2*) after wounding. Unlike the case with the *win2* promoter, the promoter from *wun1* imparted wound-inducible expression to transgenic tobacco plants (Logemann *et. al.*, 1989). Again, the function of the *wun* genes is unknown but 35% homology was found with an anionic peroxidase from potato, an enzyme involved in suberisation (Logemann & Schell, 1989).

1.6.2 Pathogenesis-related (PR) proteins

The classical PR proteins were identified as proteins induced in tobacco by infection with TMV (reviewed by Bol *et. al.*, 1990). Some of the proteins have now been identified as possessing chitinase (PR 3) or β -glucanase (PR 2a, PR2b) activity, as described in section 1.5.2.

The functions of other PR proteins is as yet unknown though some do share homology with

known proteins. Genes for the PR1a, b, and c family have been isolated from tobacco and share 60% homology (Cornelissen *et. al.*, 1986). A homologue has also been cloned from maize (Casacuberta *et. al.*, 1991) which showed increased expression following infection with the fungus *Fusarium moniliforme*. The PR-1 proteins are normally targeted to the extracellular spaces following induction by pathogen infection or chemical treatment. However, if the genes are expressed in specialised cells called crystal idioblasts, the proteins accumulate within the vacuole, suggesting that specialised cell sorting is occurring (Dixon *et. al.*, 1991).

The PR 4 group of proteins is less well characterised than the other groups and includes proteins of low molecular weight, for example of 13 kD and 15 kD identified by Pierpoint (1986). The PR 5a group show a 65% amino acid homology to thaumatin (Cornelissen *et. al.*, 1986; Pierpoint *et. al.*, 1987) and also a serological relationship to osmotin (Singh *et. al.*, 1987), a protein involved in salt stress in tobacco that has also been shown to be induced by wounding (LaRosa *et. al.*, 1992). A cDNA coding for a thaumatin-like protein has been isolated from maize (Frendo *et. al.*, 1992) that is induced by various stresses including UV light irradiation and wounding. The predicted nucleotide sequence shows the presence of a signal peptide, suggesting that the protein is secreted into the intercellular spaces in a similar fashion to the acidic PR proteins. Little is known about group PR 5b, for which no function has been assigned though a serological relationship between other PR 5 proteins and proteins in group PR 1 has been reported (Van Loon *et. al.*, 1987).

1.6.3 Intracellular pathogenesis-related proteins

The intracellular PR proteins are a class of proteins induced by pathogen infection. They share no homology with the tobacco PR proteins, though they have similar molecular weights to some of the classical PR proteins of around 16-17 kD. The intracelluar PR group are distinctive in not possessing signal sequences. Members of the group include the parsley PR1-1 proteins, isolated by elicitor treatment of parsley cell cultures (Somssich *et. al.*, 1988) and the bean PvPR 1 and PvPR 2 proteins, also isolated by elicitor induction of bean cell cultures (Walter *et. al.*, 1990). Analogues have been found in pea, birch and potato (Walter *et. al.*, 1990) and references therein), and recently in soybean (Crowell *et. al.*, 1992) and

asparagus (Warner et. al., 1992). No function has yet been assigned to the gene products.

1.7 Products induced as a consequence of oxidative stress

Wounding and pathogen invasion lead to the release of superoxide anions and hydrogen peroxide (Salin & Bridges, 1981; Thompson *et. al.*, 1987a; Doke & Ohashi, 1988). Superoxide anions and hydrogen peroxide react to produce the highly reactive hydroxyl radical (reviewed by Elstner, 1982). These radicals can react with organic compounds to produce organic radicals which in turn can react with further compounds, initiating a chain reaction (Rabinowitch & Fridovich, 1983). Such chain reactions are the cause of the peroxidation of polyunsaturated lipids in biological membranes, the breakdown of which is one of the results of wounding (Thompson *et. al.*, 1987a). Production of superoxide radicals is also thought to play a role in the hypersensitive death of plant cells, an incompatible reaction observed with most pathogens (Doke, 1983; Doke & Ohashi, 1988) resulting in death of the host cells with concomitant prevention of the spread and multiplication of the pathogen. The role of superoxide anions in the hypersensitive response is discussed further in section 1.9.1.

1.7.1 Formation of superoxide anions

Superoxide anions are formed from many oxidation reactions in the cell, most notably in the mitochondria with production of oxygen radicals by the electron transfer chain, the final step of which is transfer of electrons to oxygen; and in the chloroplasts where superoxides are formed by the transfer of electrons to oxygen from the electron acceptor of photosystem I and from ferredoxin (Asada & Takahashi, 1987).

1.7.2 Mechanisms involved in the deactivation of superoxide

Hydroxyl radicals react indiscriminately to cause lipid peroxidation, denaturation of proteins,

and the mutation of DNA. The plant has therefore evolved mechanisms to prevent the production of hydroxyl radicals by detoxifying superoxide anions and hydrogen peroxide as they are formed. Both enzymic and non-enzymic mechanisms are involved in protecting cells from oxidative stress (reviewed by Rabinowitch & Fridovich, 1983; Bowler *et. al.*, 1992). Nonenzymic mechanisms include the production and presence of antioxidants such as ascorbate, glutathione, α -tocopherol and carotenoids. The major enzymes involved are superoxide dismutase (SOD) which catalyses the dismutation of superoxide anions to produce hydrogen peroxide and molecular oxygen; and catalases which convert hydrogen peroxide to water and molecular oxygen (reviewed by Scandalios, 1990). The cell is therefore in a constant state of balance between superoxide anions being formed and removed. Any imbalance in this state, resulting as a consequence of environmental stress, causes an upregulation of SOD and catalase enzyme activity.

1.7.3 Types of superoxide dismutase

Three types of SOD are known to exist which are classified according to the metal cofactor present at the active site and can be identified by their sensitivities to cyanide and hydrogen peroxide (reviewed by Scandalios, 1990). Cytosolic and chloroplastic Cu/Zn SODs are sensitive to both inhibitors, Fe SOD is sensitive only to hydrogen peroxide, while Mn SOD is resistant to both. In eukaryotes, the different SODs are highly compartmentalised with the Mn SOD being found in the mitochondria, Fe SOD and chloroplastic Cu/Zn SOD in the chloroplasts, and the cytosolic Cu/Zn SOD in the cytosol (reviewed by Bowler *et. al.*, 1992). With the exception of a few plants such as mustard and water lilies, Fe SOD is only found in bacteria (reviewed by Hassan, 1989).

1.7.4 SOD gene expression

SOD genes have been cloned from a variety of plants including maize (Cannon *et. al.*, 1987), tomato (Perl-Treves *et. al.*, 1988), spinach (Sakamoto *et. al.*, 1990), Brussels sprouts (Walker *et. al.*, 1991), petunia (Tepperman *et. al.*, 1988), pea (White & Zilinskas, 1991), soybean

(Crowell & Amasino, 1991), rice (Sakamoto et. al., 1992) and Arabidopsis (Hindges & Slusarenko, 1992). The Cu/Zn SODs have been most extensively studied and ali show a very high degree of homology at the protein level of greater that 70%. SODs are induced by a range of environmental stresses that cause an increase in the production of superoxide radicals (Perl-Treves & Galun, 1991; Tsang et. al., 1991). These stresses include pathogen attack which was found to induce a Mn SOD in tobacco (Bowler et. al., 1989), and wounding, which resulted in an increase in both chloroplastic and cytosolic Cu/Zn SODs in tomato (Perl-Treves & Galun, 1991). The type of SOD that is induced depends mainly on the particular cell compartment that is undergoing oxidative stress. For example, the plant cell response to pathogen invasion involves an increase in metabolism, with the extra respiratory stress this imposes on the mitochondria resulting in an increase in the mitochondrial Mn SOD (Bowler et. al., 1989). Over-expression of Mn SOD in mitochondria was found to reduce cellular damage caused by oxidative stress induced in this compartment (Bowler et. al., 1991). The herbicide paraquat exerts its toxic effects through a free radical mechanism by transferring electrons to molecular oxygen and is active mostly in the chloroplasts and to a lesser extent in the mitochondria. Application of paraquat to maize resulted in a significant increase in the activity of chloroplastic and cytosolic SODs but only in a slight increase in the mitochondrial SOD (Matters & Scandalios, 1986), corresponding with the levels of oxygen radicals formed in these compartments. Incubation of plants in the dark causes paraquat to exert its effect mostly in the mitochondria and by using this technique Bowler et. al. (1991) were able to show that over expression of MnSOD in the mitochondria of transgenic tobacco plants gave increased resistance to paraquat. They also showed that targeting the MnSOD to the chloroplasts by replacing the mitochondrial leader sequence with a chloroplast transit peptide, gave increased resistance to paraquat during light conditions, showing that it is not the particular type of SOD itself which is important but the compartment in which it has an effect.

1.7.5 SOD gene regulation

In general, an increase in superoxide anion production in a particular cell compartment causes a corresponding increase in the respective SOD. The mechanism by which this occurs is not known but does result in the increased synthesis of protein as shown by *in vivo* labelling with
[³⁵S]-methionine (Matters & Scandalios, 1986). All the SODs have been shown to be encoded by nuclear genes (reviewed by Scandalios, 1990) so an increase in a particular SOD mRNA would require that an increase in superoxide production in the mitochondria, for example, sends a mitochondria specific signal to the nucleus to activate only the mitochondrial SOD gene. A mechanism to achieve this specific signalling has been suggested by Bowler *et. al.* (1992) who postulate that lipid-derived molecules could act as a compartment-specific signal. Oxidative events could lead to cleavage of fatty acids specific for chloroplastic, mitochondrial, or plasma membranes, leading to the release of a hydrophilic molecule that could diffuse to the nucleus. At the nucleus it could interact with specific transcription factors to activate the gene for the required SOD enzyme. Biologically active lipids exist in mammalian systems (Samuelsson *et. al.*, 1987) and lipid-derived molecules such as jasmonic acid (discussed further in section 1.8.3) have been shown to induce defence related genes.

Partial regulation of SOD activity may be due to the presence of inactive proenzymes. Evidence for this comes from the observation that during kernal development in maize, mRNA levels of the mitochondrial SOD do not mirror the increase in observed Mn SOD activity (White *et. al.*, 1990). White *et. al.* (1990) suggest that there is a posttranscriptional event involved in regulating the translational output or the half-life of the protein. When yeast are grown in anaerobic conditions, Cu/Zn SOD was found to exist as an inactive proenzyme, correlating with over expression of metallothionein (Galiazzo *et. al.*, 1991). Evidence also exists that in yeast Cu/Zn SOD and metallothionein gene expression are controlled at the level of transcription by copper (Carri *et. al.*, 1991). Copper regulation of an inactive proenzyme in plants may therefore also exist but has not yet been proven.

1.8 Elicitors of the defence response

Elicitors are compounds which, when applied to plant cells, cause an induction of genes involved in the defence response. Use of cell cultures has enabled the study of elicitors in the expectation that such studies will provide insights into signal transduction pathways operating during pathogen and wounding induction. Some elicitors induce responses that differ from those when a wounding stimulus is applied. For example, when a promoter from one of the bean chalcone synthase genes was attached to the GUS reporter gene to analyse responses to elicitors, Doerner *et. al.* (1990) found that the wounding stimulus alone caused a slow induction of the GUS gene while application of mercuric chloride to a fresh wound site greatly increased the induction rate. Many elicitors have been studied for their capacity to induce the defence response including various chemicals and elicitors derived from plant and microbial cell walls.

1.8.1 Chemical elicitors

One of the first events detected following either wounding or pathogen invasion is the production of ethylene (Smith *et. al.*, 1986). PAL, 4-coumarate-CoA ligase, CHS and HRGPs have all been found to be induced by the application of ethylene (Ecker & Davis, 1987). The response to ethylene requires the presence of calcium, with calcium-deficient plants showing a reduced pathogenesis response (Raz & Fluhr, 1992). The reduced form of glutathione also induces the defence genes (Wingate *et. al.*, 1988; Dron *et. al.*, 1988; Edwards *et. al.*, 1991). Other elicitors include xylanase (Lotan & Fluhr, 1990), abscisic acid (Peña-Cortés *et. al.*, 1989), salicylic acid (reviewed by Raskin, 1992), arachidonic acid (Bostock *et. al.*, 1982), gibberellic acid (Weiss *et. al.*, 1992), and heavy metals such as mercury (Doerner *et. al.*, 1990) and vanadate (Steffens *et. al.*, 1989).

It is not known how these elicitors function. They may mimic chemicals involved in the signal transduction pathway or may themselves be involved in signal transduction, bypassing the normal pathway functioning in plants. Ethylene is synthesised following wounding or pathogen invasion and mRNAs which may be involved in the synthesis of ethylene are known to be induced by wounding (Smith *et. al.*, 1986; Wang & Woodson, 1992). A biosynthetic pathway has also been proposed for the synthesis of salicylic acid from intermediates of the phenylpropanoid pathway (Ward *et. al.*, 1991). Reduced glutathione is involved in the removal of hydrogen peroxide through the Halliwell-Asada pathway (reviewed in Bowler *et. al.*, 1992). The role of hydrogen peroxide as a secondary messenger has been postulated (Apostol *et. al.*, 1989), correlating with an oxidative burst during elicitation of cultured plant

cells. Rogers *et. al.* (1988) showed that phytoalexin biosynthesis could be induced in bean cells by adding generators of oxygen radicals such as xanthine, suggesting that oxidative events may be involved in the initial signalling and induction of defence genes. Arachidonic acid is released from membrane phospholipids on cell stimulation (Samuelsson *et. al.*, 1987), possibly as a consequence of lipid peroxidation caused by the action of hydroxyl radicals.

1.8.2 Elicitors derived from plant and microbial cell walls

The induction of the defence response by pathogen infection can be mimicked by adding elicitors derived from the cell walls of phytopathogenic fungi, leading to the postulation that cell wall derived fragments are involved in the activation of the defence response (reviewed by Ryan & Farmer, 1991). Fragments from plant cell walls can also induce the defence response, providing a possible common elicitor of both the wounding and defence responses.

The induction of chitinases and β -glucanases (discussed in section 1.5.2) during the defence response provides enzymes which digest microbial cell walls, releasing fragments capable of activating further defence proteins in the process. Elicitor activity is associated with β -glucan fragments (Darvill & Albersheim, 1984) released during the infection process. Host cell wall fragments of oligogalacturonides are released by the activity of fungal and bacterial pectic enzymes (Bruce & West, 1982) and also by the action of hydrolases present within the plant cell. The degree of polymerisation of oligogalacturonides affects elicitor activity with trisaccharides being capable of inducing PAL activity (De Lorenzo *et. al.*, 1987) while longer oligomers are required for the induction of phytoalexin biosynthesis in soybean (Nothnagel *et. al.*, 1983).

1.8.3 Signalling and systemically acquired resistance to pests and pathogens

1.8.3.1 Systemic response to pathogens

The induction of the hypersensitive response by incompatible pathogen interactions (discussed in section 1.9) leads to the expression of defence genes in locations far removed from the initial infection site, imparting a broad range long-term resistance to a variety of pathogens. This response is termed systemically acquired resistance (SAR) and involves long range signalling by the plant (reviewed by Gianinazzi, 1984).

The phenomenon of SAR has been extensively studied in tobacco following the hypersensitive response induced by TMV infection. The traditional PR proteins isolated as a consequence of induction by TMV infection are systemically induced and this induction can be mimicked by the addition of salicylic acid (reviewed by Bol et. al., 1990). Acquired resistance in Arabidopsis has also been shown to be induced by salicylic acid (Uknes et. al., 1992), leading to the speculation that salicylic acid acts as a systemic signal. It has been shown that endogenous salicylic acid accumulates in TMV-infected tobacco (Malamy et. al., 1990) but if tobacco plants are grown at temperatures which prevent induction of defence related genes, endogenous salicylic acid levels do not increase (Malamy et. al., 1992). Coordinate regulation of the PR proteins, including chitinases and β-glucanases has been shown using exogenous application of salicylic acid (Ward et. al., 1991). A biosynthetic pathway has been proposed for the synthesis of salicylic acid from intermediates of the phenylpropanoid pathway (Ward et. al., 1991). This suggests possibilities for the coordinate regulation of the phenylpropanoid biosynthesis pathway, with its role in the hypersensitive response, and the regulation of salicylic acid production with induction of the systemic response.

The role of ethylene in inducing a systemic response has also been analysed using the potato *win2* gene (section 1.6.1). Fusions of the *win2* promoter attached to the GUS reporter gene showed that local wound-inducible expression did not require ethylene, but, if present, imparted high expression levels (Weiss & Bevan, 1991). However, systemic

expression required both the wound stimulus and ethylene, while ethylene alone did not affect win2 gene expression.

1.8.3.2 Induction of PI genes

The PI proteins (section 1.5.3) are systemically induced by wounding. However, unlike the PR proteins which only become detectable in uninfected leaves several days following the initial infection (Ward et. al., 1991), systemically induced PI proteins can be detected 20 minutes after wounding (Peña-Cortes et. al., 1988), the same time that they become detectable in the wounded leaves. Cell wall polysaccharides released during wounding have been shown to induce the PI genes (Bishop et. al., 1981) and an 18 amino acid polypeptide named systemin has been shown to be capable of inducing a systemic reaction in the PI genes of tomatoes (Pearce et. al., 1991). Abscisic acid (ABA) has also been shown to induce PI II genes in a systemic manner in potato and tomato (Peña-Cortés et. al., 1989) with ABA deficient mutants showing a greatly reduced PI response to wounding. Recent work has concentrated on the role of jasmonic acid and methyl jasmonate in cell signalling. Methyl jasmonate induces the synthesis of PI proteins (Farmer & Ryan, 1990), and also of a leaf thionin in barley (Andresen et. al., 1992). Transgenic tobacco containing the upstream and downstream untranslated regions of PI II showed methyl jasmonate induced expression of a reporter gene (Farmer et. al., 1992), and a G-box sequence required for methyl jasmonate induced activity has been identified in the promoter region of the potato PI II gene (Kim et. al., 1992).

Farmer and Ryan (1992) present a model postulating that systemin, the systemic signal, wounding, or oligouronides released as a result of pathogen attack, interact with plasma membrane receptors causing the release of linolenic acid from the membrane by the action of a lipoxygenase, an enzyme that uses molecular oxygen to produce hydroperoxides from fatty acids, then converts the linolenic acid to a precursor of jasmonic acid. Lipoxygenase activity has been observed to increase in response to the presence of methyl jasmonate (Bell & Mullet, 1991; Grimes *et. al.*, 1992), wounding (levinsh, 1992), and in . response to infection with pathogenic bacteria (Koch *et. al.*, 1992). Lipoxygenase activity

also appears to coincide with ethylene production (levinsh, 1992), a known elicitor of the defence response.

Farmer and Ryan (1992) tested various octadecanoids for their ability to activate the PI genes but found that only precursors specific for the synthesis of jasmonic acid had inducing activity, demonstrating the specificity of jasmonic acid in the intracellular signalling process. The involvement of the plasma membrane in the signalling process has previously been demonstrated by Thain *et. al.* (1990) who showed that oligosaccharide elicitors of the PI genes induced changes in the membrane potential, while Farmer *et. al.* (1991) showed that oligogalacturonides enhanced the *in vitro* phosphorylation of at least three proteins in the plasma membrane. The possibility that signals are transmitted throughout the plant as an electrical potential cannot, however, be ruled out: Wildon *et. al.* (1992) have shown that inhibiting translocation in the phloem has no effect on the systemic accumulation of PI transcripts and proteins.

1.9 Plant resistance and the gene-for-gene hypothesis

Resistance of plants to pathogens can be classified as horizontal or vertical. Horizontal or variable resistance is under polygenic control and is therefore difficult to study genetically. Vertical or specific resistance is due to the presence of a major resistance gene which is inherited in a Mendelian fashion. Pioneering studies by Flor (1942) using flax as the host plant and the flax rust, *Melampsora lini*, as a pathogen led to the conclusion that for each resistance gene in the host there is a specific and related virulence gene in the pathogen (reviewed by Sidhu, 1975). The gene-for-gene hypothesis therefore states that an incompatible or resistance reaction occurs when the product of the dominant avirulence gene product in the pathogen interacts with the dominant resistance gene in the plant. If either the plant resistance gene product or the avirulence gene product are absent then a compatible or susceptible reaction occurs. Due to variable and non-host resistance, the compatible reaction is a rare occurrence and requires that the pathogen is able to overcome the defence response of the plant or is able to detoxify products of the response such as the phytoalexins. Various models have been put forward to explain the -gene-for-gene

hypothesis including the elicitor-receptor model and the ion channel defence model (reviewed in Gabriel & Rolfe, 1990), but no particular model has yet been substantiated. The biochemical basis of resistance of maize to the fungal pathogen *Cochliobolus carbonum* race 1 has been determined and shown to be due to the possession of the dominant allele of the nuclear locus *hm* (Meeley *et. al.*, 1992). Resistant lines possess the enzyme HC-reductase which inactivates HC-toxin produced by the pathogen, a toxin that imparts specific pathogenicity to maize.

1.9.1 The hypersensitive response

An incompatible interaction is characterised by the so-called hypersensitive response which leads to localised host cell death and induction of SAR. The invading pathogen remains localised, being prevented from spreading throughout the plant by the fact that the living tissue around the site of necrosis rapidly becomes resistant (reviewed in Fritig et. al., 1987). Resistance is conferred by the induction of genes involved in the defence response. The same gene products are also induced in a compatible reaction; the differences between the compatible and incompatible reactions appear to be due to the rapidity and extent of induction of certain genes. For example, study of a compatible and incompatible reaction in French bean (Phaseolus vulgaris) showed that in the incompatible reaction PAL and CHS transcripts were detectable rapidly in tissue localised mainly around the site of infection, whereas in the compatible reaction there was no early accumulation of these transcripts, with the response being delayed and more widespread (Bell et. al., 1986). A similar study using tobacco cell suspensions indicated the presence of two distinct groups of mRNAs involved in the defence response: group I were induced 3-7 fold higher in the incompatible response when compared with the compatible response, whereas group II contained mRNAs which were induced to the same extent in both reactions (Godiard et. al., 1991). Schröder et. al. (1992) present evidence that β-glucanases and chitinases are members of group II since both protein and mRNA distribution patterns were the same in both compatible and incompatible interactions.

Involvement of superoxide radicals in the defence response has been demonstrated by the

induction of phytoalexin biosynthesis in bean cells by the addition of oxygen radical generators (Rogers *et. al.*, 1988), and by the inhibition of phytoalexin accumulation in legumes by the addition of oxyradical scavengers (Epperlein *et. al.*, 1986). A requirement for lipoxygenase has also been demonstrated by Vaughn and Lulai (1992) who showed that a potato tuber callus line lacking lipoxygenase activity did not undergo a hypersensitive response when elicitor was added, whereas rapid cell death occurred in tuber callus lines containing lipoxygenase activity.

There is evidence that superoxide, SOD and lipoxygenase are involved in determining the establishment of a compatible or incompatible interaction. Koch et. al. (1992) showed that in an incompatible interaction between tomato and pathogenic bacteria, lipoxygenase mRNA was induced within 3 hours, whereas in the compatible interaction accumulation of the mRNA was induced much later. In both the reaction of potato with Phytophthora infestans and the reaction of tobacco with TMV, there is evidence of a superoxide-generating NADPH-oxidase bound to the plant cell membrane that is stimulated immediately following invasion by an incompatible pathogen (Doke, 1983; Doke & Ohashi, 1988; reviewed in Bowler et. al., 1992). Also, in interactions of tomato roots infested with the nematode Meloidogyne incognita, SOD activity was seen to increase markedly in the compatible reaction, but no induced SOD activity was observed in the incompatible reaction which underwent a hypersensitive response (Zacheo & Bleve-Zacheo, 1988). High SOD activity has also been detected in the compatible interaction between pepper plants and Phytophthora capsici, unlike the incompatible interaction which showed relatively low SOD activity (Hwang et. al., 1991). These observations indicate that the formation of superoxide radicals may play a direct role in establishing the hypersensitive response with superoxides and related oxygen species causing the destruction of plant cells via such processes as lipid peroxidation. In a compatible interaction, the rapid induction of SOD activity detoxifies the oxygen radicals and rapid localised cell death does not occur. Alternatively, superoxide or hydrogen peroxide could act as secondary messengers with signal transduction capabilities being modified depending on the compatibility or incompatibility of the reaction.

1.10 Engineering resistance

The compatible plant-pathogen interaction is a rare occurrence when all possible plant and pathogen combinations are considered. However, modern agricultural practice has severely limited the number of plant species used as food crops which, in turn, has created problems due in part to the uniformity of crop growth. In natural populations, for example trees, the spread of disease is inhibited by the presence of a mix of trees. In uniform plantations there is no natural barrier to the spread of disease so if a virulent form of a pathogen arises (a frequent occurrence due to a high mutation rate) the effect is devastating. The aim of plant breeders is therefore to provide a constant supply of crop cultivars varying in the number and types of resistance genes they possess to outsmart the pathogen. However, there are many problems with plant breeding which are outlined below.

1.10.1 The limitations to plant breeding

Breeding to introduce one characteristic into a desirable line is a very time consuming business. Initial crosses have to be made to transfer the required quality into the new line. Several backcrosses then have to be made to the original desired line to retain all the qualities of the line while continuing to select for the new quality. Also, the introduction of a new characteristic with retention of all other advantageous qualities may not be possible if desirable genes are linked to undesirable phenotypes, for example, resistance to a particular pathogen linked to a low yield.

There are many limitations to conventional plant breeding: for example, there is only a limited gene pool available restricted by sexual incompatibility (reviewed by Lindsey, 1992). Also, genetic variation is presently decreasing because of an invasion of the habitat of natural plant varieties by cultivated crop plants. These generally have a limited genetic base due to centuries of inbreeding and selection by man resulting in the rapid replacement of a large pool of wild endogenous plants with plants possessing only a small genetic base. The narrowing of genetic diversity in plants has therefore made it desirable to pursue interspecies transfer. The ideal situation, to eliminate the element of chance in crop breeding, would be to isolate a

single gene conferring the required characteristic and transfer this directly to the desired line. It is in this situation that genetic engineering can play a role.

1.10.2 The advantages of genetic engineering

Gene transfer, using the *Agrobacterium tumefaciens* Ti plasmid as vector for introducing foreign DNA into plant cells (Hernalsteens *et. al.*, 1980; Ream & Gordon, 1982), eliminates many of the disadvantages of crop breeding. The ability to introduce individual genes bypasses the problem of linked genes and also of the necessity of backcrossing to retain the required qualities of a line. The available gene pool is extended to include all organisms, providing endless possibilities. For example, plants have been transformed with genes imparting resistance to specific herbicides (for example De Block *et. al.*, 1987). Of particular interest in this case was the fact that the resistance gene was isolated from the bacterium *Streptomyces hygroscopicus* (Thompson *et. al.*, 1987b) opening the possibility that genes for a number of characteristics could be acquired by isolating bacterial mutants.

The major disadvantage of genetic engineering with present technology is that transfer is limited to one or only a very few genes. Routine transformation of the most important crops, the cereals, has also not yet been achieved. Crop breeding will therefore continue to play an important role in these situations and also in the selection of traits encoded by many genes, such as polygenic resistance.

1.11 Compounds with potential for genetic engineering of resistance

Any compound with proven antimicrobial or anti-pest properties has the potential to be used in engineering resistance, examples of which are described below. Viral components, for example the expression of coat protein, have also been shown to increase resistance to viral infections. Ideally, the compound used should be encoded by just one gene for the purposes of genetic engineering, which eliminates compounds such as the phytoalexins which require complex biosynthetic pathways.

1.11.1 Bacilius thuringiensis (Bt) toxin

The Bt toxin is a family of proteins which together provide toxicity against a variety of insects (reviewed in Whiteley & Schnepf, 1986). Ingestion of the toxin by insects leads to its breakdown and release of active δ -endotoxins in the alkaline environment of the midgut. The toxin has been used as a biological insecticide by spraying directly onto crop plants but cloning of the gene has now enabled its expression in transgenic plants. Expression of Bt toxin in transgenic tobacco plants (Vaeck *et. al.*, 1987) and tomato plants (Fischhoff *et. al.*, 1987) has been shown to protect the plants from feeding damage caused by insect larvae.

1.11.2 Products of the plant defence response

Supplementation of the defence response may be achieved by introducing further products known to be involved in the response. Chitinases and β -glucanases are natural candidates with their known antifungal activities but most work has concentrated on the protease inhibitors known to exert deleterious effects on insects (reviewed by Ryan, 1990). Expression of the cowpea trypsin inhibitor gene in tobacco plants was shown to improve resistance to insect damage (Hilder *et. al.*, 1987), while constitutive expression of PI I and II in tobacco also increased resistance to insect damage (Johnson *et.al.*, 1989).

1.11.3 Viral components

The phenomenon of cross protection, in which inoculation of a plant with a mild strain of virus imparts protection to more virulent strains, prompted research into the mechanism of this protection. One model proposed was that coat protein produced by the first infection encapsidates the RiNA of any succeeding viral challengers and so prevents its replication. This model was tested by Abel *et. al.* (1986) who discovered that tobacco plants transformed

with the TMV coat protein gene were resistant to subsequent TMV infections. Resistance is thought to be conferred by a delay in disease development (Nelson *et. al.*, 1987). This type of protection is called coat protein mediated protection and has now been demonstrated in many cases (reviewed by Beachy *et. al.*, 1990; Gadani *et. al.*, 1990). Resistance to viruses has also been achieved by expressing viral satellite RNAs (for example Harrison *et. al.*, 1987; Gerlach *et. al.*, 1987), and an open reading frame encoding a putative viral replicase gene (Braun & Hemenway, 1992). The mechanism by which these achieve resistance is not known but is thought to be due to interference of the normal virus replication process.

1.11.4 Insect immune proteins

Bacterial infections in insects result in the induction of peptides containing antimicrobial activity which are secreted into the haemolymph (reviewed by Boman & Hultmark, 1987; Boman *et. al.*, 1991). Many families of antibacterial peptides have been identified including lysozymes, cecropins and attacins (Boman, 1986) which are all involved in the insect immune response. Though the peptides act in a synergistic fashion (Hultmark *et. al.*, 1983) the majority of the antibacterial activity is contributed by the cecropins (Boman & Hultmark, 1987).

The cecropins are small, highly basic proteins of approximately 3.5 kD containing only 35-39 amino acid residues. NMR studies indicate that cecropins have a structure consisting of two amphipathic α helixes joined by a hinge region (Boman, 1991). The cDNAs for the cecropins from the Cecropia moth, *Hyalophora cecropia*, have been isolated (van Hofsten *et. al.*, 1985; Lidholm *et. al.*, 1987) and indicate that the cecropins are made as larger precursor proteins whereas lysozyme, by comparison, is synthesised with only the addition of a signal peptide (Boman, 1991). Besides insects, cecropins have also been isolated from mammalian systems in the form of a porcine cecropin (Lee *et. al.*, 1989) which shares a degree of similarity at the amino acid level ranging from 64% to 75% with two insect cecropins, containing 12 identical amino acid residues in 36 positions with cecropin B from the Cecropia moth. The porcine cecropin differs from the insect cecropins in that it lacks a C-terminal amide thought to contribute to the broad spectrum activity of the insect cecropins (Li *et. al.*, 1988). Isolation of cecropins from a broad range of species suggests that antibacterial peptides may form a

ubiquitous means of defence against bacterial infections since an antibacterial defence based on only RNA and protein synthesis is faster and under greater control than immune systems which are dependent on cell division (Lee *et. al.*, 1989). The possession of antibacterial activity and the requirement for only a single gene to ensure their production has prompted suggestions for the use of cecropins in genetically engineering resistance to bacterial pathogens in plants (for example, Jaynes *et. al.*, 1987).

1.12 Aims of this thesis

The project aims are to explore strategies that may in the future allow the use of genetic engineering to increase resistance in plants to bacterial infections. A major objective will be to use a promoter activated at sites of wounding to drive the production of a secreted antibacterial protein.

1.12.1 The problems of soft rot

Bacterial diseases were targeted because though they may be of lower economic importance than viral or fungal diseases, continual destruction of crops in successive seasons can greatly affect the profitability of a crop. Examples of bacterial infections which cause major problems include bacterial wilt of potato and tomato caused by *Pseudomonas solanacearum*, fire blight of pear caused by *Erwinia amylovora*, and losses in rice caused by *Xanthomonas campestris* (reviewed by Billing, 1987). Bacteria such as *Erwinia carotovora* are also the cause of soft rot spoilage of many vegetables, a common storage problem particularly in potatoes (reviewed by Lund, 1983; Beukema & Zaag, 1990). The susceptibility of potatoes to soft rot during storage is of particular concern to major distributors such as the company Dalgety PLC, who kindly provided part of the funding for this project.

The bacterial soft rots are opportunistic pathogens to which potatoes are normally resistant (reviewed by Lyon, 1989). However, under appropriate conditions of high humidity in an anaerobic atmosphere, such as may occur during storage, all varieties of potato are susceptible. The problems of storage are exacerbated by the fact that *Erwinia* are soildwelling bacteria and easily gain entrance to tubers through the lenticels and through wounds caused during the harvesting process and so cannot be excluded prior to storage. A novel approach to engineering resistance to soft rot is therefore required since there are no specific resistance genes which may be transferred into desirable varieties through more traditional methods of crop breeding.

The approach to be used in this particular case would be to engineer the secretion of antibacterial substances at wound sites since these are the main entry points for *Erwinia*. Though *Erwinia* also enters tubers via the lenticels, the bruising around wound sites containing softened tissue make an especially attractive breeding ground for the bacteria. It is expected that multiplication and spread of the bacteria within tuber tissue would be prevented from the area of the wound site. The aims of this individual thesis are therefore to initially study the secretion of an antibacterial substance, and also to search for a suitable promoter to be used to activate the secretion of an antibacterial substance.

1.12.2 Secretion of an antibacterial substance

Insect immune proteins containing antibacterial activity are secreted into the haemolymph while some plant defence proteins, such as the chitinases and β -glucanases, are secreted into the intercellular fluid (discussed in section 1.5.2). This suggests that secretion forms an important component of the defence system and, for maximum impact, proteins containing toxic activity towards microbes should be secreted into the intercellular fluid.

Cecropin was chosen as the antibacterial agent to be studied in this project because it is active against both Gram positive and negative bacteria and there is evidence that it is also effective against some fungi (Destefano-Beltran *et. al.*, 1990). Cecropins act in a stoichiometric fashion and are believed to form channels in bacterial lipid bilayer membranes in a similar fashion to the defensins, antimicrobial peptides found in neutrophils and macrophages (Kagan *et. al.*, 1990). No effect of cecropins on erythrocytes has been noted with resistance thought to be due to a lack of affinity (Steiner *et. al.*, 1988): consumption by

humans and other animals is not therefore thought to be a problem. The effect of cecropin against some plant protoplasts has been investigated (Nordeen *et. al.*, 1992) and showed that the lethal concentration for the protoplasts was up to 70 times higher than that required to have a lethal effect on bacterial phytopathogens, indicating that the use of cecropins in plants is feasible. Porcine cecropin was specifically chosen in preference to the insect cecropins because it is active without the C-terminal amide thought to confer broad spectrum activity to the insect cecropins (Li *et. al.*, 1988). The C-terminal amide may be specific to insect hosts and not be processed correctly within plant cells.

Many secreted products have been identified in plants including some of the defence-related proteins and enzymes such as the amylases. Though signal sequences from various organisms vary widely in sequence, they share common structural properties of a hydrophobic domain and a signal peptidase cleavage site, enabling recognition in diverse systems (Glerasch, 1989). At the start of this project very little information concerning signal peptides in plants was available. Recently, hen egg white lysozyme has been successfully expressed in transgenic tobacco with its own 18 amino acid signal peptide (Trudei *et. al.*, 1992), though in this case no more than 10% of the lysozyme activity was found secreted into the interceilular fluid. Lund and Dunsmuir (1992) have also recently shown that the efficiency of secretion of the bacterial protein ChIA in transgenic tobacco could be greatly improved by replacing the bacterial signal sequence with a plant-derived signal from the protein PR1b. They suggest that the use of a plant signal Improves the ability of the bacterial protein to enter the plant secretory pathway.

The secretion signal chosen in this project for studying the secretion of antibacterial substances at wound sites was that from wheat α -amylase. The amylase is naturally secreted from the aleurone of wheat into the endosperm during germination. The secretion signal from wheat α -amylase has been shown to function effectively in yeast (Rothstein *et. al.*, 1984) where it is recognised both in its natural internal location and also when it is located N-terminaliy (Rothstein *et. al.*, 1987). Efficient recognition of the wheat amylase signal enabled yeast to be used as a test system to study the secretion of cecropin and the results from this study are presented in chapter 3.

1.12.3 Search for an inducible promoter

An inducible promoter is required to ensure that the antibacterial substance is only synthesised when required and so prevent problems, such as yield loss or interference of normal metabolic processes, that may result as a consequence of constitutive expression of a foreign protein. Transgenic plants containing foreign proteins of plant origin appear to shown no deleterious effect on yield when grown in controlled growth room conditions (Hilder & Gatehouse, 1991) but the introduction of an insect protein may affect plant processes. Once transgenic plants containing the foreign protein were produced, effects on yield could be assessed in field trials. Ideally, the promoter should be induced by a signal not involved in establishing the compatibility or incompatibility of a pathogen interaction. Many pathogens such as the soft rot bacteria gain entry through wound sites, requiring the initial puncturing of the outer plant tissue layers prior to infection. A wound-inducible promoter would therefore appear to be an ideal choice with inducibility not dependent on a specific pathogen but on the more universal response to wounding.

Previous work at Leicester concentrated on the use of an asparagus ceil culture system in which wounded cells were generated mechanically and which represents an enriched source of wound-induced genes (Harikrishna et. al., 1991). The validity of this approach has been confirmed by Grosset et. al. (1990) who showed that many mRNAs synthesised by tobacco protopiasts are wound-inducibie. The mechanically isolated cell system involves grinding asparagus ciadodes using a pestle and mortar and placing the isolated cells into media, where they remain viable and continue to grow and divide (Figure 1.1). The asparagus system has been used to isolate genes shown to be inducible by wounding (Warner et. al., 1992) leading to subsequent isolation of a wound-inducible promoter. Ideally, the promoter used should be activated within one to two days by the wound stimulus in preparation for pathogen attack and then activation maintained to ensure sustained resistance to pathogen invasion around the wound site. Two-dimensional gel analysis of poiy (A)+ RNA isolated from mechanically wounded asparagus cells identified a family of genes showing these required characteristics (Figure 1.2). One member of this family, named DD1, was identified as a candidate for cloning since its expression profile appeared to fit that required. An antibody was made to the protein and an N-terminal amino acid sequence obtained. Chapters 4 and 5 describe the successful

Figure 1.1

a) Asparagus fern showing cladodes.

b) Asparagus mechanically isolated cells produced by grinding ciadodes and placing into suitable cell culture medium. Viable cells contain intact chloroplasts.



Figure 1.2

Two-dimensional gel analysis of in vitro translated poly (A)⁺ RNA isolated from day 0 and day 2

following single cell isolation of asparagus cells. The DD1 group is shown.



cloning of DD1 and subsequent expression analysis.

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During the course of the work, an asparagus wound-inducible promoter became available. The characterisation of this promoter in transgenic potato plants in response to wounding and pathogen invasion is described in chapter 6.

The approach of using a wound-inducible promoter attached to a cecropin gene has been suggested and attempted (Destefano-Beltran *et. al.*, 1990) but without apparent success. The approach outlined above differs significantly in including the secretion of cecropin and in the use of a synthetic cecropin gene optimised for expression in plants.

In summary, the aims of the work presented in this thesis were: 1) to study the secretion of cecropin in yeast (chapter 3); 2) to clone a candidate gene whose expression profile indicated that it would provide a suitable promoter (chapters 4 and 5); and 3) to analyse the expression of an asparagus wound-inducible promoter in transgenic potatoes (chapter 6).

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CHAPTER 2

MATERIALS AND METHODS

This chapter describes the materials and methods used to obtain the results described in the succeeding results chapters.

2.1 Materials

2.1.1 Chemicals

All chemicals were obtained from either Sigma, BDH, Clontech or Pharmacia unless otherwise stated. Oligonucleotides used as primers in PCR reactions were obtained from the DNA synthesising facility in the Biochemistry Department, Leicester University. Deionised destilled water (QH₂O) was prepared using a Milli-Q reagent water system (Millipore).

2.1.2 Nucleotides

Nucleotides were obtained from Pharmacia. Labelled nucleotides were supplied by Amersham: [³⁵S] α -dATP at a concentration of 370 MBq/ml and with a specific activity greater than 22 TBq/mmoi; [³²P] α -dCTP at a concentration of 370 MBq/ml and a specific activity of 110 TBq/mol; and [³²P] γ -dATP at a concentration of 370 MBq/mi and a specific activity of 110 TBq/mol.

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2.1.3 Enzymes

DNA modifying enzymes were supplied by Bethesda Research Laboratories (BRL), Pharmacia, Stratagene or Boehringer Mannheim. Taq polymerase was obtained from CamBio.

2.1.4 Plant material

Asparagus officinalis cuitivar Conover's Collosai seeds were purchased from Nickersons seeds. These were germinated and grown for six weeks in John innes number 3 compost in a greenhouse at 25°C under normal daylight hours.

Potato tubers (*Solanum tuberosum* cuitivar Deslree) for transformation purposes were obtained from the Dalgety Food Technology Centre, Cambridge, and stored in the dark at 4°C. Tubers were no longer used once sprouting had occurred.

2.1.5 Bacterial host strains and plasmids

2.1.5.1 Bacterial strains

Escherichia coli XL1-blues (Builock et. al., 1987): rec A1, end A1, gyr A96, thi -1, hsd R17, sup E44, rel A1, lac, [F' pro AB, lacl^QZ Δ M15, Tn10 (tet^r)].

This strain was used as a host for the amplification of plasmids described below. *E. coli* were grown in either LB (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, deionised water to 1 litre, pH adjusted to 7.0 using 5 M NaOH prior to autoclaving) or NZY (10 g Difco Casein hydrolysate, 5 g NaCl, 5 g Difco bacto-yeast extract, 2 g MgSO₄.7H₂O, completed and autoclaved as for LB). Liquid cultures were placed in a Gallenkamp orbital incubator at 200 rpm at a temperature of 37°C for 16 hours. Solid agar plates were prepared by adding 15 g Difco bacto-agar per litre of liquid medium prior to autoclaving and growth allowed to occur at 37°C in a LEEC incubator for 16 hours. Ceils retaining the F' pilus were selected by growth in the presence of 12.5 μ g/mi tetracycline.

Agrobacterium tumefaciens pGV2260 (Debleare et. al., 1985) and LBA4404 (Hoekma et. al., 1983).

These strains were used as hosts for vectors such as pBiN19 which can be transferred into plants via *Agrobacterium* -mediated transformation. The hosts are disabled and do not cause crown-gall disease but possess the virulence factors required for transfer of DNA between the T-DNA borders into host genomic DNA. Both contain a chromosomaliy located rifampicin (Rf)-resistance marker allowing selection in the presence of 100 μ g/ml Rf. The strain pGV2260 also contains an ampiciliin (amp)-resistance marker allowing selection with the addition of 100 μ g/mi amp. The bacteria were grown in LB at a temperature of 25°C for up to 48 hours.

All bacterial stocks were stored in the form of glyceroi stocks, made by adding 1.2 ml of a 16 hour (*E. coli*) or 48 hour (*A. tumefaciens*) liquid culture to 300 μ i of glyceroi, flash freezing in liquid nitrogen and storing at -70°C.

2.1.5.2 Plasmids

pBluescript (Short et. al., 1988; Stratagene cloning systems).

This is a multi-purpose plasmid vector derived by replacing the pUC19 polylinker with a synthetic polylinker containing 21 unique restriction sites. Plasmids containing DNA inserted into the polylinker site can be selected by a blue/white colour assay. The assay is possible because the vector carries DNA derived from the *lac* operon of *E. coli*, encoding the amino-terminal fragment of β -galactosidase, surrounding the polylinker site. The synthesis of this fragment can be induced by the presence of isopropyl thio- β -D-galactoside (IPTG) and is capable of complementation with a defective form of β -galactosidase encoded by the host, such as *E. coli* XL1-blues. On exposure to iPTG both fragments of the enzyme are synthesised and blue colonies are formed when plated on media containing the chromogenic substrate 5-bromo-4-chloro-3-indoiyl- β -D-galactoside (X-gal). Insertion of DNA into the polylinker site inactivates the amino-terminal fragment of β -galactosidase, giving rise to white colonies.

Other features of pBluescript relevant to this thesis are: the possession of the f1 origin of

replication from the f1 filamentous phage allowing rescue of single-stranded DNA for sequencing upon co-infection with helper phage; an inducible *lac* promoter upstream from a *lacZ* gene allowing the production of fusion proteins which may be detected with antibody probes; and the possession of T3 and T7 bacteriophage promoters allowing *in vitro* synthesis of strand-specific RNA. The plasmid contains an amp resistance marker and is selected in the presence of 100 µg/ml amp.

Of relevance to this plasmid is the phage vector λ -ZAP (Short *et. al.*, 1988; Stratagene cloning systems). This vector allows the insertion of up to 10 kb of DNA and is used in the construction of cDNA iibraries. it contains multiple cloning sites within plasmid sequences that can be excised *in vivo* with the aid of a heiper phage and converted to the plasmid vector, pBluescript. Phage particles were stored in SM (5.8 g NaCi, 2 g MgSO₄.7H₂O, 50 ml of 1 M Tris-HCl pH 7.5, 5 ml of 2% gelatin, deionised water to 1 litre) containing 1 drop of chioroform at 4°C.

pBIN19 (Bevan, 1984).

This is a wide host range binary cloning vector used for *Agrobacterium* -mediated DNA transfer to plants. The vector contains a kanamycin (km)-resistance marker and is selected in the presence of 50 µg/mi km.

pBI101 (Jefferson et. al., 1987).

This is a general purpose vector for constructing gene fusions to the reporter gene β glucuronidase (GUS). It was made by ligating the coding region of GUS to the 5' end of the nopaline synthase polyadenylation site in the polylinker site of pBiN19.

Plasmids constructed during the course of the work are introduced in the relevant text.

2.1.6 Yeast host strains and plasmids

The yeast host strain used for the results obtained in chapter 3 was *Saccharomyces cerevisiae* JRY188: MATα; *sir* 3-8; *leu* 2-3, 112; *ura* 3-52; *his* 4; *rme*. Cells were either grown

in rich YPD medium (5 g Bacto-yeast extract, 10 g Bacto-peptone, 10 g glucose, deionised water to 500 ml) or in minimal medium (Difco Semi Defined medium prepared according to manufacturer's instructions with the addition of 2% glucose, required amino acids at 20 μ g/ml, uracil at 1 μ g/ml). In the case of JRY188 the required amino acids were trp, his and leu. Growth in liquid medium took place in a Gallenkamp orbital incubator at 200 rpm at a temperature of 30°C for periods of up to 48 hours in YPD and 72 hours in minimal medium. Lids were taped on iooseiy to allow CO₂ produced to escape. Solid agar plates were prepared by adding 15 g Difco bacto-agar per litre of liquid medium prior to autoclaving and yeast growth allowed to occur at 30°C in a LEEC incubator for periods of time as described for liquid medium.

The plasmid vector used to obtain the results in chapter 3 was pEMBLyex4 (Cesareni & Murray, 1987). This is an expression vector containing an inducible galactose promoter with a *ura* marker gene, and transformants are selected in *ura*- host strains such as JRY188 on media deficient in uracii.

2.1.7 Potato pathogens

Some of the results obtained in chapter 6 required the infection of transgenic potato plants with plant pathogens.

2.1.7.1 Phytophthora Infestans

The *P. infestans* Isolate used was number 36609, race 3,4 which was grown on rough rye agar (60 g rye soaked for 36 hours in half quantity water, bolied for 1 hour and then macerated in a blender, 15 g agar, 20 g sucrose, water to 1 litre). Growth occurred in the dark for 6 days at 16°C, after which time the sporangia were scraped off with a small volume of water. Zoospores were released by placing the sporangia at 4°C and then continuing incubation at room temperature. Zoospores are released as the temperature of the water rises. The zoospores were used to infect potato leaves.

2.1.7.2 Erwinia carotovora

E. carotovora pv *carotovora* isolate 177 was grown for 24 hours in LB in a shaking waterbath at 27°C with 200 strokes per min.

2.1.7.3 Potato Virus Y (PVY)

A common isolate of PVY at the Scottish Crop Research Institute (SCRI) was used. inocuiant was prepared by removing the leaves from an infected plant and grinding them up in water using a pestie and mortar. The resulting suspension was then frozen until required.

The majority of the methods described here are based on those found in Sambrook *et. al.* (1989) and Draper *et. al.* (1988) in which further explanations and detailed references can be found.

2.2 Extraction and quantification of macromolecules

2.2.1 Nucleic acid extractions

isolation of nucleic acids basically involves breaking open the tissue containing the required nucleic acids and then various purification steps to eliminate protein and undesirable RNA or DNA. Phenol is most commonly used to remove protein, while different alcohols containing varying sait concentrations are used to differentially separate the nucleic acids.

2.2.1.1 Plant DNA extraction

After Murray and Thompson (1980).

Genomic DNA was isolated for the purpose of Southern blotting (2.4.1). Plant material was frozen in iiquid nitrogen and stored at -80°C or used fresh. Approximately 10 g of plant tissue was placed in a pre-cooled pestle and mortar and ground under liquid nitrogen. This was then placed in a 50 mi phenoi resistant tube and incubated with 2 x CTAB (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% CTAB (w/v), 40 mM β-mercaptoethanoi) at 1 mi/g tissue for 30-60 mins at 60°C, shaking the tube occasionally. The suspension was then extracted with an equal volume of phenol: chloroform: isoamyl-alcohol (25:24:1) with the tube being mixed by inversion. The two phases were separated by centrifugation and the top aqueous phase removed to a new tube. To this was added 0.1 volumes of 10% CTAB (10% CTAB (w/v), 5.0 M NaCl) prewarmed to 60°C and the components mixed. The aqueous phase was then re-extracted with phenol: chloroform: isoamyl-alcohol. After centrifugation, nucleic acids were precipitated from the aqueous phase by addition of 2.5 volumes of cold absolute ethanol with the tube being left on ice for 10 mins. The nucleic acids were collected by centrifugation for 10 mins at 4000 pm. They were then washed twice with 0.5 ml 70 % (v/v) ethanol, left to air dry and redissolved in a suitable quantity of distilled water (usually 1-3 ml). Insoluble matter, mainly carbohydrate, was removed by centrifugation with the supernatant retained. RNA present in the nucleic acid mix was digested using RNase at a final concentration of 0.1 mg/mi of nucleic acid solution with incubation for 1 hour at 37°C. DNA was then precipitated by adding 0.1 volumes of 2 M sodium acetate pH 5.6 and 3 volumes of absolute ethanol with the peilet collected by centrifugation. The final DNA pellet was dissolved in an appropriate volume of distilled water (approximately 1 mi) and its concentration measured spectrophotometricaily.

2.2.1.2 Plant RNA extraction

After Covey and Hull (1981).

RNA was extracted for the purpose of northern blotting (2.4.2). Plant material stored at -80°C was ground in a pre-cooled pestle and mortar under liquid nitrogen until it resembled a fine powder. Grinding buffer (6% (w/v) 4-aminosalicyiate, 1% (w/v) triisopropyl naphthalene sulphonate (Kodak), 6% (v/v) phenol, 50 mM Tris-HCl pH 8.4) at 2 ml/g tissue was added and grinding under liquid nitrogen continued. While still frozen the material was transferred to a 50 ml phenol resistant tube and allowed to thaw in the presence of an equal volume of phenol: chloroform: isoamyl-alcohol with periodic mixing. Once thawed, the mixture was centrifuged and the upper aqueous phase re-extracted with phenol: chloroform: isoamyl-alcohol. The aqueous phase was removed into a new tube and nucleic acids precipitated by the addition of 0.05 volumes 4 M sodium acetate pH 6.0 and 2.5 volumes absolute ethanol with incubation on ice for 10 mins. The nucleic acids were collected by centrifugation at 10,000 rpm for 10 mins at 4°C. They were left to dry on the bench and then dissolved in a small volume of water, usually 100-300 μi . Differential precipitation of RNA was achleved by the addition of 3 volumes of 4 M sodium acetate pH 6.0 and leaving at -20°C for 20 mins. The RNA was then collected by centrifuging in a microfuge, washed in 200 µi 70% (v/v) ethanol and air dried. After dissolving in distilled water the concentration was measured spectrophotometricaliy, the RNA dijuted to a final concentration of 5 mg/ml and stored at -80°C.

2.2.2 Quantification of nucleic acids

The concentration of nucleic acids was measured spectrophotometrically using a dual beam spectrophotometer (Perkin-Eimer Lambda 5 UV/VIS). DNA and RNA concentrations were determined at OD_{260} and OD_{280} respectively and a scan obtained from 200 to 300 nm to obtain some indication of sample purity.

2.2.3 Extraction of protein

Fresh or pre-frozen tissue samples were ground with acid-washed sand under liquid nitrogen using a pestie and mortar. Protein extraction buffer (100 mM Tris-HCl pH 8.0, 5 mM DTT, 5 mM EDTA pH 8.0) was added at 0.5 ml/g tissue fresh weight and 0.02 volumes of 200 mM PMSF and grinding continued until a consistency of fine powder was reached. Frozen tissue was then transferred to 1.5 mi microfuge tubes and centrifuged for 10 min at 4°C. Protein-containing supernatant was removed, concentration measured and samples stored at -70°C.

2.2.4 Quantification of protein

Protein concentrations were calculated using the Bradford assay (Bradford, 1976) in a microtitre plate. Wells were filled with 200 μ l of Bradford's solution (600 mg/i Coomassie brilliant blue G-250, 2% (v/v) perchloric acid, settled overnight and filtered through Whatman number 1 paper) to which were added 10 μ i of protein sample. Standards were prepared using Bovine serum albumin and the microtitre plate read by a Dynatech MR5000 microtitre plate reader. The reader was programmed to calculate the concentration of protein samples directly.

2.3 Electrophoresis

This is a method of separating molecules under the application of an electric field based on differences in molecular weight or in folded structure. DNA fragments were separated for sequencing (2.10), Southern blotting (2.4.1), and gel electrophoresis was also used in various methods in the manipulation and modification of DNA (2.9). FINA fragments were run on gels for the purpose of northern blotting (2.4.2). Protein samples were applied to gels for western blotting (2.5) and for the purpose of performing *in gel* enzyme assays (2.14.3). In all cases gel electrophoresis was also used as a method of assessing the quality of samples.

2.3.1 DNA agarose gel electrophoresis

Gels of 0.7 to 1.2% (w/v) agarose were prepared in 1 x TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA, 0.5 µg/ml ethidium bromide) depending on the size of the DNA fragments to be separated. Prior to loading and running the DNA samples, 0.2 volumes of loading buffer were added (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in water). Electrophoresis was carried out at 1-10 V/cm in 1 x TAE buffer. Marker DNAs of known size were run alongside samples to enable an estimate of fragment size to be made when the gel was viewed on a UV trans-illuminator (UVP inc.). The gel was photographed using a video camera (UVP Inc.) and video processor (Mitsublshl). If the gel were to be Southern blotted it was photographed alongside a ruler to enable estimation of DNA fragment sizes following hybridisation.

2.3.2 RNA formaldehyde gel electrophoresis

Gels of 1.2% (w/v) were prepared by melting an appropriate amount of agarose in water. After cooling to 60°C, 10 x MOPS buffer (200 mM MOPS pH 7.0, 50 mM sodium acetate pH 7.0, 10 mM EDTA pH 8.0) was added to give a final concentration of 1 x, and formaldehyde added to give a 2.2 M final concentration. RNA samples were prepared by mixing up to 30 μ g of RNA in sterile water in a total volume of 6 μ l with 12.5 μ i of delonised formamide, 2.5 μ i 10 x MOPS buffer, and 4 μ l 37% (v/v) formaldehyde in a sterile microfuge tube. The samples were then incubated at 65°C for 5 mlns, 2.5 μ l tracking dye (50% (v/v) glyceroi, 0.1 mg/ml bromophenol blue) added, and run in 1 x MOPS buffer at 3-6 V/cm. Rlbosomal RNAs were used as molecular mass markers and were vlsualised using UV light after staining with ethidium bromide (0.5 μ g/mi in 0.1 M ammonium acetate) for 45 mins. Gels were photographed alongside a transparent ruler to enable transcript sizes to be estimated foliowing northern biotting.

2.3.3 DNA sequencing gels

A BioRad seqencing gel kit was used with giass plates of 20 cm x 40 cm x 0.5 mm. The top plate was siliconised with Sigmacote and the base sealed according to manufacturer's instructions. The gel mix was made by combining 80 mi of 6% (w/v) gel solution, prepared by mixing 34.11 g DNA sequencing grade acrylamide, 1.8 g N,N'-methylenebisacrylamide, 252 g urea, 60 ml 10 x TBE buffer (108 g Tris base, 5.8 g EDTA, 55 g boric acid, deionised water to 1 litre) and deionised water to 600 mi, with 480 µl AMPS and 64 µl TEMED and poured between the plates using a 50 mi syringe. A "shark's tooth" comb was Inserted and the gel allowed to polymerise. The gel was pre-run ln 1 x TBE buffer at 2700 V until a gel temperature of 50°C was reached before samples were loaded. Electrophoresis was then continued at 2300 V to maintain the gel temperature.

Following electrophoresis, the plates were dismantled and the gel transferred to 3MM Whatman paper. This was covered with Saran wrap and the gel dried down onto the paper using a Biorad gel slab vacuum drier. Autoradiography was usually carried out for 1-3 days depending on the number of radioactive counts detectable.

2.3.4 Polyacrylamide gel electrophoresis (PAGE) of proteins

A BioRad protein minigei kit was used in electrophoresis of proteins for both *in situ* gei assays and western blotting with the kit being assembled according to manufacturer's directions.

Denaturing and native gels were prepared in exactly the same manner with the SDS being omitted from the native gel mix and from the running buffer.

The following table gives amounts of constituents used in preparing the bottom resolving gels varying in the percentage of bis-acrylamide:

Gel percentage	7.5%	10%	15%	20%
Distilled water	4.9 ml	4.1 ml	2.4 ml	0.8 ml
1 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Bls-acrylamide *	2.49 ml	3.325 ml	4.99 ml	6.65 ml
10% SDS	100 μl	100 µl	100 μl	100 μl

*Bls-acrylamide in the proportions 0.8%:30%.

The top stacker gel was the same regardless of the percentage of the resolving gel and was prepared by mixing the following:

3.25 ml
1.25 ml
0.5 ml
50 μl

Constituents for the resolving gel were mixed and de-gassed before adding 37 μ I 10% (w/v) AMPS and 3 μ I TEMED. The gel was then poured and overlaid with 700 μ I 1:1 water: propan-2-ol and allowed to set for 15 mln. Once the gel had set the overlay was removed and the stacker gel mixed. After de-gassing, 15 μ I of 10% (w/v) AMPS and 5 μ I of TEMED were added and the stacker poured on the bottom gel, the gel comb set in place and allowed to set for 30 min. Gels were run at 200 V for 45-60 min in glycine running buffer (14.4 g glycine, 3 g Tris base, 1 g SDS, deionised water to 1 litre) until the blue dye present in the loading buffer reached the bottom of the gel.

Protein samples to be run on denaturing gels were prepared by adding 15-100 μ g of protein In a volume of 30 μ i distilled water to 0.25 volumes of 4 x cracking buffer (2% (w/v) SDS, 20% (v/v) β -mercaptoethanol, 40% (v/v) glycerol, 0.25 M Tris-HCI pH 6.8, 0.01% (w/v) bromophenol blue). Samples were boiled for 2 mln to denature the protein and immediately piaced on ice until loaded onto the gel. Samples to be run on native gels were added to the same cracking buffer containing no SDS and were loaded Immediately.

2.4 Nucleic acid blotting

2.4.1 Southern blotting

Modified after Southern (1975).

This technique enables the transfer of DNA fragments from agarose gels (2.3.1) to a nylon membrane enabling the detection of homoiogous bound sequences by probing with a known segment of DNA. DNA gels run as described in 2.3.1 were prepared for Southern blotting by initially placing the gel in 2-3 volumes of depurinating solution (0.25 M HCi). After 10 mins the gel was transferred to 2-3 volumes of denaturing solution (0.5 M NaOH, 1.5 M NaCi) for 30 mins to 2 hours and then finally to neutralizing solution (3.0 M NaCi, 0.5 M Tris-HCl pH 7.4) for 40 mins to 2 hours. The gel was then rinsed in distilled water and transfer carried out as described for northern blotting (section 2.4.2).

2.4.2 Northern blotting

Modified after Lehrach et. al. (1977).

This procedure enables the transfer of RNA to a nyion membrane, enabling the detection of transcribed sequences using a known fragment of DNA as probe. Following electrophoresis as described in 2.3.2, RNA geis were rinsed in distilied water and biotted without further treatment.

Both DNA and RNA geis were biotted in the same manner as described below with the only exception being that in the case of RNA geis care was taken to ensure that equipment was free from RNases by cleaning all equipment with DECON.

Gels were blotted in a tray containing 20 x SSC (3.0 M NaCi, 0.3 M Na citrate, pH 7.0) into which was placed a heavy duty sponge. Two pieces of 3MM Whatman paper cut to size were

placed on the sponge and allowed to soak up $20 \times SSC$. The gel was placed on the paper and a piece of Hybond-N (Amersham) carefully iaid on the gel making sure that no bubbles were trapped. Cling film was then placed over the areas of the tray not covered by the filter to ensure that movement of the $20 \times SSC$ occurred through the Hybond-N. Two pieces of 3MM soaked in $20 \times SSC$ were then placed on top of the filter followed by four pieces of dry paper. On top of this was placed a stack of absorbant paper towels and a water-filied bottle (approximately 500g) and the nucleic acids allowed to transfer by capillary action to the filter overnight. The filters were then rinsed in $3 \times SSC$ and dried prior to fixing.

2.4.3 Bacterial colony blotting

This method is a modification of Southern biotting, allowing the detection of hybridising sequences contained within plasmlds present in bacteria without having to first isolate the plasmid DNA and electrophorese it on an agarose gei. It was used to detect cioned fragments of DNA following ligation to vector DNA (2.9.4) and transformation into a bacterial host (2.9.5). Bacterial colonies were grown overnight on NB agar plates (9 cm diameter) containing appropriate antiblotic. A Hybond-N filter of 82 mm diameter was placed on the colonies for 1 min and the filter orientated using holes made with a needle. After this time the filter was removed and placed colony-side up on 3MM Whatman paper soaked in denaturing solution for 5 min. It was then transferred to 3MM paper soaked in neutralising solution for 5 mln. The filter was finally washed in 2 x SSC prior to drying and fixing.

2.4.4 Fixing blots

Nucleic acids were fixed onto Hybond-N filters by placing the filter, nucleic acid side down, on a UV transiiluminator for 2 mins.

2.5 Western blotting

This method provided a means of transferring proteins to cellulose membranes which could then be reacted with antibodles to detect specific proteins. PAGE minl gels were run as described (2.3.4). The apparatus used was disassembled, the stacker gel removed and the resolving gel placed on a piece of Hybond-C nitrocellulose filter cut to size. The gel was then electroblotted using a Milliblot SDE system (Millipore) accoding to manufacturer's instructions. After blotting, the filter was put in a smail sandwich box containing Ponceaus stain for 5 min and then rinsed with distilled water. Ponceaus stains protein bands pink and is a method of checking that protein transfer was successful. Stain was removed by washing in TBS (50 mM Tris-HCl pH 7.4, 200 mM NaCl) containing 0.1% (v/v) Tween 20. The filter was then placed in blocker (TBS with 0.1% (v/v) Tween 20 and 3% (w/v) dried milk - Marvei) and left overnight at 4°C. The following morning the filter was transferred to primary antibody buffer (TBS with 10% (v/v) glycerol, 10% (w/v) Marvei), the appropriate dijution of antibody added and left on a rocking platform at room temperature for 1.5 hours. The filter was washed 3 times for 15 min each in TBS containing 0.1% (v/v) Tween 20 on a rocking platform. It was then placed in secondary antibody buffer (TBS with 1% (w/v) Marvei), the appropriate dilution of secondary antibody added and the filter left on a rocking platform for 40 min. The filter was then washed 3 times for 10 min each in the same wash solution as described above and finally rinsed in 100 mM Tris-HCI pH 8.0. Treatment of the filter from this point was dependent upon the conjugate attached to the secondary antibody.

Since the primary antibodies used were invariably made in rabbits the secondary antibodies were goat/anti-rabbit with varying conjugates (obtained from Sigma). If the conjugate was alkaline phosphatase a choice of two substrates were routinely used. The first substrate was Fast Red (Sigma) which involved transferring the filter to 20 ml 100 mM Tris-HCl pH 8.0 containing 2 mg Naphthol-AS phosphate (Sigma), previously dissolved in DMF, and 20 mg of Fast Red. The filter was then placed, covered with foil, on a rocking platform for 10-30 min until the colour had developed. Filters were rinsed in distilled water and stored in 1% (v/v) ethanoic acid. The second substrate involved using BCIP and NBT. The filter was rinsed as before and then placed in 20 mi of 100 mM Tris-HCi pH 9.5, 100 mM NaCl, 5 mM MgCl₂. To this was added 330 µl of BCiP (made up as a stock of 30 mg/ml in DMF) and 660 µl of NBT
(made up as a stock of 50 mg/ml in 70% (v/v) DMF). The purple colour develops almost immediately. The filter was rinsed twice in distilled water and dried by placing between 3 MM filter paper. Filters were stored dry.

If the conjugate attached to the secondary antibody was biotin, filters were rinsed and placed in secondary antibody buffer containing the appropriate dilution of Extravidin-alkaline phosphatase conjugate (Sigma). They were agitated for 1 hour at room temperature and washed 3 times in wash solution described above. Filters were blotted dry and then treated in the same manner as for secondary antibody/aikaline phosphatase conjugate. The biotin conjugate was used to impart greater sensitivity since 2 moiecules of extravidin bind to 1 molecule of biotin giving 2 reactive molecules for every antibody bound to the filter.

2.6 Radio-labelling of probes

2.6.1 Radio-labelling of double-stranded DNA probes

After Feinberg and Vogelstein (1984).

Double-stranded DNA probes were used routinely in Southern and northern biotting and for colony and plaque hybridisations. Probe stock was prepared by suitable restriction digests (2.9.1) and separation of DNA on agarose geis (2.3.1). The gei slice containing the required DNA was removed from the gel and the DNA purified by Genecieaning (2.9.2).

Oligo iabelling buffer (OLB) was prepared by mixing 2 volumes of solution A (625 μ I 2 M Tris-HCI pH 8.0, 25 μ I 5 M MgCl₂, 18 μ I β -mercaptoethanol, 5 μ I 0.1 M dATP, 5 μ I 0.1 M dTTP, 5 μ I 0.1 M dGTP, 350 μ I distilled water) with 5 volumes of solution B (2 M HEPES pH 6.6 titrated with NaOH) and 3 volumes of solution C (hexadeoxyribonucleotides suspended eveniy In 3 mM Tris-HCI pH 7.0, 0.2 mM EDTA pH 7.0 at 90 OD₂₆₀ units/mi).

Probes were labelied by placing 3.0 μ i OLB, 0.6 μ l BSA (DNase free), 1.5 μ I [³²P] α -dCTP and 0.6 μ I of DNA polymerase I Klenow fragment in a 1.5 mi screw-capped tube. To this was

added 10-20 ng of probe stock in 9.3 μ l distilled water, preboiled to denature the DNA, and the mixture incubated at 37°C for 1 hour. After this time, 85 μ l of distilled water were added. Incorporation of label into the probe was measured by removing 1 μ i of probe into a tube containing 0.5 ml herring sperm carrier DNA (0.5 mg/ml in 20 mM EDTA). DNA was precipitated by adding 125 μ l of 50% (w/v) TCA with unincorporated counts removed by filtering through a Whatman GF/C disk in a filter tower and washing twice with 10% (w/v) TCA and once with 100% ethanol. This filter provided a measure of incorporated counts. A 1 μ i allquot of the probe was pipetted onto a second GF/C disk to provide a measure of unincorporated counts. Counts were measured by liquid scintiliation counting. If incorporation proved to be acceptable (approximately 70%), the probe was bolied for 3 mins and added to hybridisation solution.

2.6.2 End-labelling of oligonucleotides

Labelied oligomers were used in library screening and were prepared by combining 1.0 μ i of oligomer (50 pmoie of ends) with 2.0 μ I 10 x T4 polynucleotide kinase buffer (500 mM Trls-HCi pH 7.6, 100 mM MgCl₂, 50 mM DTT, 1 mM spermidine, 1 mM EDTA pH 8.0, to volume with distilled water), 10.0 μ i [³²P] γ -dATP, 6.0 μ i distilled water and 1.0 μ I (10 units) T4 polynucleotide kinase (Pharmacia) in a screw-capped 1.5 mI tube. After mixing, the tube was incubated at 37°C for 1 hour. To eliminate unincorporated label, the probe was precipitated by adding 40 μ i distilled water, 240 μ I 5 M ammonium acetate, and 750 μ I cold absolute ethanol. After leaving on ice for 30 min, the tube was centrifuged in a microfuge for 10 min, the probe washed with 70% (v/v) ethanoi and air drled. It was then dissolved in 100 μ i of TE (10 mM Tris-HCi pH 7.5, 1 mM EDTA pH 7.5) and added to hybridisation buffer. incorporation was measured approximately using a Geiger counter.

2.7 Pre-hybridisation and hybridisation of filters

Both hybridisation and pre-hybridisation of filters were carried out in the same solutions and consisted of 6 x SSPE (from a 20 x stock: 3.0 M NaCi, 0.2 M NaH₂PO₄.2H₂O, 0.02 M EDTA,

to pH 7.7 with 1.0 M NaOH), 5 x Denhardt's reagent (from a 50 x stock: 2% (w/v) each of BSA, Ficoll 400, PVP), 0.5% (w/v)SDS and 100 μ g/ml sheared herring sperm DNA. Hybridisation solution for Southern biotting (2.4.1) was made up in distilied water while hybridisation solution for northern blotting (2.4.2) contained 50% (v/v)de-ionised formamide.

The purpose of pre-hybridisation is to block sites on the membrane to which the probe may bind non-specifically and was carried out by placing filters in an appropriate chamber, adding hybridisation solution to a depth of 2-3 mm and leaving for 2-5 hours. DNA blots were left at 60-65°C and RNA blots at 42°C. Labelled probe was then added and hybridisation continued at the same temperature for 4-5 hours for high copy number target sequences or overnight for iow copy number sequences.

2.7.1 Washing stringency conditions

Filters were washed at 60°C initially in wash A (3 x SSC, 0.1% (w/v) SDS) with several changes of wash solution. If the counts present on the filters were still high, the filters were transferred to a higher stringency wash, wash B ($0.5 \times SSC$, 0.1% (w/v) SDS) and washing continued until the counts on the filter dropped or until no further counts could be detected in the wash itself. Filters were then dried between sheets of Whatman 3 MM paper and wrapped in Saran wrap.

2.7.2 Autoradiography

Autoradiography was carried out using X-ray film (Amersham Hyperfilm-MP) in a cassette fitted with an intensifying screen. Cassettes were left at -70°C for periods ranging from several hours to 3 weeks depending on the radioactivity detectable on the filter.

2.7.3 Stripping of filters

On some occasions it was prudent to strip filters of any attached labeiled probe and re-use the

filter in a fresh hybridisation reaction. This enabled the direct comparison of labelled bands on different autoradiographs.

DNA filters were stripped by placing at 45°C for 30 min in 0.4 M NaOH, and then at 45°C for 15 min in 0.1 x SSC, 0.1% (w/v) SDS, 0.2 M Tris-HCl pH 7.5.

RNA filters were stripped by placing at 65°C for 1-2 hours in 5 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.1 x Denhardt's reagent.

Filters were judged to be successfully stripped when radioactive counts were no longer detectable.

2.8 Library screening

Screening a cDNA library enables the identification of phage plaques containing a desired sequence. The sequence can be identified by homology to a known fragment of DNA used as a probe in a similar fashion to Southern blotting (2.4.1), or, if an antibody probe is available, plaques can be identified in a manner similar to western blotting (2.5) by inducing them to express proteins transcribed and translated from the target DNA. The library used for screening whose results are described in chapters 4 and 5 was obtained from Dr. Simon Warner (Botany, Leicester University). It was constructed in the phage vector λ -ZAP (see 2.1.5.2), using poly (A)⁺ RNA isolated from a mixture of 1-3 day old cultured asparagus cells.

2.8.1 Plating out phage librarles

Before the library could be plated out, phage-competent *E. coli* cells were prepared. This involved growing an overnight culture from a single colony of XL1-blue cells in LB containing 12.5 μ g/ml tetracycline. The following morning, 1 ml of the overnight culture was added to 50 ml LB containing 0.2% (w/v) filter-sterilised maltose, 10 mM MgSO₄ and 12.5 μ g/ml

tetracycline and grown to an OD_{600} of 0.5. The cells were then pelleted by centrifuging at 4,000 rpm for 10 min at 4°C and resuspended in a half volume of cold 10 mM MgSO₄. Phage-competent cells were stored at 4°C and were used for up to 2 weeks.

Library phage stock was diluted to the required titre (stated in the relevant text) using SM, mixed with 300 µl of phage-competent cells and left at room temperature for 5 min. To this was added 10 ml of molten top agar (NZY broth, 0.7% (w/v) agar) at 50°C and the mix poured onto a 140 mm Petri dish containing NZY agar. The plate was gently swirled to mix and allowed to harden before incubating overnight at 37°C. Plaques were visible as clear zones on a turbid lawn of bacteria.

2.8.2 Plaque lifts for hybridisation with DNA probes

After removing from the incubator, plates containing phage were placed at 4°C for 30 min to allow the agar to harden. Circles of Hybond-N, 132 mm in diameter, were placed onto the plates for 1 min. Duplicate filters were left on the same plates for 2 min. The filters were orientated by piercing with a needle while the plates were marked at corresponding points with a felt tipped pen. The filter was then peeled off the plate and placed phage-side up on a tray containing Whatman 3 MM paper soaked in denaturing solution. After 5 min the filter was transferred to paper soaked in neutralising solution for 5 min. It was then rinsed in 2 x SSC, dried and fixed using UV light.

2.8.3 Screening using double-stranded DNA probes

Probes were labelled (see 2.6.1) and pre-hybridisation, hybridisation, washing and autoradiography carried out in the same manner as for Southern blotting (2.4.1).

2.8.4 Screening using end-labelled oligonucleotides

Filters were prepared as described in 2.8.2 and placed in prehybridisation solution at 60°C for 3 hours. The filters were then removed to oligo hybridisation solution which consisted of the following: 3.0 M tetramethylammonium chloride, 0.01 M Na₂PO₄ pH 6.8, 1 mM EDTA pH 7.6, 2.5 x Denhardt's reagent, 0.5% (w/v) SDS and 100 μ g/ml sheared herring sperm DNA. The probe, labelled as described in 2.6.2, was boiled and added to the hybridisation solution and the filters left overnight at a temperature 5-10°C below that of the calculated Ti. Filters were first washed in 6 x SSC, 0.1% (w/v) SDS at room temperature and then in wash A also at room temperature before being placed on film.

The addition of tetramethylammonium chloride (TMACi) enables the hybridisation temperature to be increased since hybridisation becomes independent of the GC content of the oligonucleotide and dependent solely upon the length of the oligomer used (Melchior & Hippel, 1973; Jacobs *et. al.*, 1988). This is important when degenerate oligomers are being used since an increase in hybridisation temperature greatly increases the stringency of the reaction. Ti is melting temperature measured in the presence of TMACI and is calculated by applying the equation contained in Jacobs *et. al.* (1988).

2.8.5 Screening using antibodies as probes

The phage library (constructed in lambda ZAP) was plated out (see 2.8.1) at a density of 2000 pfu per 140 mm Petri dish and incubated at 42°C for 3.5 hours or until cleared zones the size of pinpricks had appeared. The plates were overlaid with Hybond-C nitrocellulose filters that had previously been soaked in 10 mM IPTG (to induce protein expression) and dried and then the plates incubated overnight at 37°C. The following morning the plates were removed from the incubator and the filters marked for orientation purposes. The plates were then placed at -70°C for 5 min to allow the agar to harden and facilitate removal of the nitrocellulose filters. Filters were carefully peeled off the agar and placed in TNT (10 mM Tris-HCi pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20) and rinsed before placing in a fresh batch of TNT and leaving on a rotating shaker at room temperature for 30 min. Filters were transferred to blocking buffer

(TNT containing 5% (w/v) dried milk - Marvel) and left on a rotating shaker overnight. Filters were then placed into blocking buffer containing the appropriate dilution of primary antibody and agitated for 2 hours at room temperature. Excess antibody was removed by washing the filters firstly in wash 1 (TNT with 0.15% (w/v) Marvel), then wash 2 (as wash 1 plus 0.1% (v/v) Nonidet P40) and lastly in wash 3 (as wash 1). The filters were then transferred to secondary antibody in blocking buffer and left on a rotating shaker for 1.5 hours. After this time washes 1 to 3 were repeated to remove excess antibody. Treatment of filters from this point was dependent upon the conjugate attached to the secondary antibody and is described in the section on western blotting (2.5).

2.8.6 Removal and storage of positive plaques

Positive plaques were removed by placing the narrow end of a Pasteur pipette over the plaque and using a teat to suck the plug of agar into the pipette. The piug was then expelled into a 1.5 ml microcentrifuge tube containing 1 ml of SM (5.8 g NaCl, 2.0 g MgSO₄.7H₂O, 50 mM Tris-HCl pH 7.5, 2% (v/v) gelatin solution, distilled water to 1 litre) and 2 drops of chloroform and stored at 4°C. Titre was measued by plating out dilutions made in SM.

2.8.7 Excision of pBluescript from λ -ZAP

Once a positive phage stock had been identified, the Bluescript plasmid was rescued by mixing 50 μ l of phage stock in SM with 200 μ i phage-competent cells and 10 μ l R408 helper phage (10⁷ pfu) in a 1.5 ml tube. After incubating for 15 min at 37°C, 5 mi of NZY broth was added and the tube placed in a shaker at 37°C for 3-6 hours. The tube was then placed in a water bath at 70°C for 20 min to kill the cells and the phage while allowing the rescued phagemid to survive. The dead cells were removed by centrifuging for 5 min in a microcentrifuge with the phagemid-containing supernatant being retained. Plasmid was recovered by mixing 20-200 μ l of phagemid stock with 100 μ l of phage competent cells, spreading on NZY agar plates containing ampicillin at 100 μ g/ml and incubating overnight at 37°C. Colonies that grew contained double-stranded Bluescript plasmid and could be

amplified and purified as described in 2.9.6 and 2.9.7.

2.9 Manipulation and modification of DNA

DNA can be manipulated using a variety of enzymes enabling stretches of DNA to be cloned and amplified. Restriction enzymes recognise 4 or 6 bp palindromic sequences and cut the DNA in a very specific fashion, leaving cut ends which can be joined, using DNA ligase, to other pieces of DNA cut using compatible restriction enzymes. Following ligation, the DNA can be transferred into *E. coli* cells to allow amplification.

2.9.1 Restriction digestion of DNA

DNA was digested to provide fragments to be cloned and also to provide vectors cut with compatible restriction enzymes into which required fragments could be inserted. Digests were carried out according to manufacturer's instructions (BRL). A typical digest contained 1.0 μ g DNA, 1 μ i 10 x restriction enzyme buffer (supplied by manufacturer), 0.5 μ l (5 units) restriction enzyme and made up to a volume of 10 μ l with distilled water. If the DNA preparation used contained large amounts of contaminating RNA, 1 μ l of RNase at 0.5 mg/ml was added. Reactions were left at the required temperature for periods ranging from 1 to 3 hours.

Vector DNA was prepared for ligation following digestion by adding an equal volume of phenol: chloroform: isoamyl-alcohol, mixing the contents and centrifuging for 5 min in a microfuge. DNA was precipitated by adding 3 volumes of absolute ethanol, leaving at -70°C for 10 minutes, centrifuging for 10 min in a microfuge, washing with 70% (v/v) ethanol, and then drying and redissolving the pellet of DNA in distilled water. This procedure effectively removed the restriction enzyme from the digested DNA. A total of 1 μ g of digested DNA was dissolved in a final volume of 20 μ l of distilled water and 1 μ l of this (approximately 50 ng) used in a ligation reaction.

2.9.2 Purification of DNA fragments from agarose gels

Digested DNA was frequently run on agarose gels to separate fragments. The required fragment was then purified from the gel using solutions provided in the Geneclean kit (BIO 101 Inc.). The gel slice was placed in a 1.5 ml microcentrifuge tube, 2-3 volumes of 6 M Nal added, and the tube placed at 50°C for 5 mins or until the gel slice had melted. The glassmilk was vortexed, 5 μ I added to the melted gel slice and left on ice for 5 mins to allow the DNA to bind to the glassmilk. The bound DNA was then pelleted by centrifuging in a microfuge at 13,000 rpm for 5 secs and the supernatant discarded. The pellet was washed 3 times by resuspending in 200 μ I of NEW wash, centrifuging for 5 secs and removing the supernatant. After all traces of NEW wash had been removed, the pellet was resuspended in 10 μ I of distilled water and the tube incubated at 50°C for 3 mins to elute the DNA from the glassmilk. The tube was then centrifuged for 30 secs and the DNA-containing supernatant removed to a new tube and stored at -20°C.

2.9.3 Dephosphorylation of DNA

If vector DNA was digested with only one enzyme, reclrcularisation of the vector during ligation was prevented by dephosphorylating the 5' ends. This disables the ligase from joining both ends and ligation will only occur with a fragment of DNA that contains an intact phosphate group at the 5' end. Following digestion of 2 µg vector with the relevant restriction enzyme, the DNA was extracted with phenol: chloroform: isoamyl-alcohol and precipitated with absolute ethanol. After drying, the DNA was dissolved in water containing 0.1 volumes of CIP buffer (100 mM Tris-HCI pH 8.5, 10 mM MgCl₂ and 10 mM ZnCl₂), 1 unit of Calf Intestinal Alkaline Phosphatase added and the reaction allowed to proceed at 37°C for 30 min. The dephosphorylated vector was then re-extracted with phenol: chloroform, ethanol precipitated, dried and dissolved in distilled water to give a final concentration of 50 ng/µl.

2.9.4 Ligation of DNA fragments

T4 DNA ligase joins DNA fragments together by catalysing the formation of a covalent phosphodiester bond from a 5'-phosphoryl group and an adjacent 3'-hydroxyl group. A typical ligation reaction was placed in a 1.5 ml microfuge tube and contained 10-100 ng digested vector DNA, 100-250 ng purified insert DNA, ligase buffer containing ATP (supplied by manufacturer), 1-10 Weiss units T4 DNA ligase with the volume made up to 20 μ l with distilled water. The contents of the tube were mixed and left either for 1-2 hours at room temperature (approximately 20°C) or at 15°C overnight prior to transforming competent *E*. *coli* cells (2.9.5).

2.9.5 Transformation of E. coli using calcium chloride competent cells

Modified after Cohen et. al. (1972).

Following ligation, DNA was transformed into *E. coli* to enable the selection of successful ligation events and also to provide a means by which the ligated DNA may be amplified. Transformation requires competent cells which were prepared by using 2 ml of an overnight culture of *E. coli* XL1-blues to inoculate 100 ml of LB. Cells were grown to an OD₆₀₀ of 0.6, centrifuged at 4000 rpm for 10 min at 4°C in 50 ml tubes and resuspended in 50 ml of ice cold 50 mM CaCl₂. The tubes were incubated on ice for 1 hour, re-centrifuged and the pelleted cells resuspended in 5 ml of 50 mM CaCl₂, 20% (v/v) glycerol. Cells were then divided into 100 μ l aliquots, flash-frozen in liquid nitrogen and stored at -70°C until required.

Transformation was accomplished by combining ligated DNA with 10 µl of 10 x TCM (100 mM Tris-HCi pH 7.5,100 mM CaCl₂, 100 mM MgCl₂) made up to 100 µl total volume with distilled water. A 100 µl aliquot of competent cells was thawed on ice and added to the mix which was left on ice for 30 min. The tube was placed in a water bath at 37°C for exactly 2 min to heat shock the cells, 1 ml of LB added and the tube placed in a shaker at 37°C for 1 hour to allow expression of antibiotic resistance genes. The cells were then pelleted by centrifuging in a microfuge for 3 min before being resuspended in 100 µl of fresh LB. Dilutions of the

transformed cells were plated out on agar containing the appropriate antibiotic and incubated at 37°C overnight. If blue/white colour selection of recombinants was possible, 80 µg/ml X-gal and 20 mM IPTG were added to the plates before spreading the cells. Due to disruption of the LacZ gene, recombinant plasmids would appear white rather than the non-recombinant blue.

2.9.6 Small scale plasmid DNA preparations from E. coli

After Birnboim and Doly (1979).

Once transformants were obtained, it was necessary to check that correct inserts had been retained. This was achieved by preparing small quantities of DNA from a bacterial culture originating from a single transformation and then performing a series of diagnostic restriction enzyme digests. A bacterial culture harbouring the required plasmid was grown overnight in LB containing appropriate antibiotic in a shaker at 37°C. A total of 1.5 ml of this culture was spun down in a microfuge for 3 mins. Supernatant was removed and cells resuspended in 100 µl lysis buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 500 mM glucose) prior to adding 200 µl of 200 mM NaOH, 1% (w/v) SDS. Contents were gently mixed by inversion, 150 µl of 3 M potassium acetate pH 5.2 added and the tube shaken. The white precipitate so formed (containing genomic DNA and proteins) was removed by centrifugation and the aqueous supernatant extracted using phenol: chloroform: isoamyl-alcohol. The crude plasmid DNA was then precipitated by adding 2.5 volumes of absolute ethanol and placing the tube at -70°C for 10 mins. After centrifuging, the pellet of DNA was washed in 70% (v/v) ethanol, air dried and resuspended in 20-30 μ I of distilled water. An aliquot of 2 μ I was then used in restriction digests. Since this method does not remove RNA, digests were routinely carried out with the addition of 0.1 volumes 0.5 mg/ml RNase.

2.9.7 Large scale plasmid DNA preparations from E.coli

Large quantities of DNA were required for cloning procedures and as stocks for the

preparation of DNA fragments to be used as probes in library screening (2.8), northern (2.4.2) and Southern (2.4.1) blotting. Cells from 500 ml of an overnight culture grown in LB containing appropriate antibiotics, were harvested by centrifuging at 10,000 rpm at room temperature in a Sorval RC-5B centrifuge using GSA polypropyiene bottles. The pellet was resuspended in 18 ml of lysis buffer and the bacteria lysed by addition of 40 ml of 0.2 M NaOH, 1% (w/v) SDS. Genomic DNA and protein were precipitated by the addition of 20 mi 3 M potassium acetate pH 5.2 and the bottle shaken to mix the contents. The precipitate was removed by centrifugation at 10,000 rpm for 5 mins and the supernatant collected by filtering through polyallomer wool into clean bottles. Nucleic acids were precipitated by adding an equal volume of propan-2-ol and collected by centrifuging at 10,000 rpm for 10 mins. The nucleic acid pellet was dissolved in 3 ml distilled water, transferred to a siliconised corex tube and 3 ml of 8 M LiCl added. After vortexing, the precipitated large RNAs were removed by centrifugation at 10,000 rpm at 4°C for 10 min. The supernatant was retained and DNA precipitated by adding an equal volume of propan-2-ol, mixing and then centrifuging at 10,000 rpm for 10 min. After redissolving the pellet in 500 μi of distilled water, the solution was transferred to a 1.5 ml microfuge tube and 20 µl of 10 mg/ml RNase added. The tube was incubated at 37°C for 30 mins. A 500 µl aliquot of 1.6 M NaCl containing 13% (w/v) PEG 6000 was mixed into the DNA solution and the pellet of DNA collected by centrifugation at 4°C for 10 mins. After discarding the supernatant, the pellet was resuspended in 300 μ i of distilled water, extracted twice with phenol: chloroform: isoamyl-alcohol, and then precipitated by the addition of 100 µl 10 M ammonium acetate and 2 volumes of absolute ethanol. DNA was recovered by centrifugation for 5 mins, washed in 70% (v/v) ethanol, air dried and redissolved in 500 µl distilled water. Following concentration measurements (2.2.2), the DNA was redissolved at a concentration of 1 mg/ml and stored at -20°C.

2.9.8 Amplification of DNA using the polymerase chain reaction (PCR)

Modified after Mulls and Faloona (1987).

PCR provides a means of amplifying (vector) inserts, for example, to be used for probe stocks

if primers to the vector containing the insert are available. The reaction can also be used to amplify specific DNA from a mixture providing there is some knowledge of DNA or protein sequence to which PCR primers can be designed. The basic protocol followed was that contained in the Perkin-Elmer Cetus manual with a typical reaction mix containing 0.1 volumes $10 \times PCR$ buffer (100 mM Tris-HCI pH 8.3, 500 mM KCI, 15 mM MgCl₂, 0.1% (w/v) gelatin), 0.1 volumes $10 \times dNTPs$ (2 mM of each, Pharmacia Ultrapure), 250 ng of each primer, 1 µl of phage stock in SM or DNA template (10^3 - 10^6 copies), 0.5 µl Taq polymerase and sterile water to a volume of 50 µl. The reaction mix was then overlaid with 2 drops of paraffin oil to prevent evaporation during the heating cycles and placed in a PCR machine (a Perkin-Elmer Cetus Thermocycler). The first cycle consisted of denaturation at 95°C for 5 min, annealing at 65°C for 1 min and extension at 72°C for 1 min followed by 29 cycles of 1 min denaturation, 1 min annealing and 1 min extension. The final extension was allowed to run for 8 min. For some reactions the number of cycles and the annealing temperature were changed to reflect the template used and the melting temperature of the primers. The oil was then extracted using 100 µl of chloroform and 5 µl of the PCR product anaylsed by running on an agarose gei.

2.10 Sequencing of DNA

Once clones had been isolated by library screening (2.8), they were sequenced to enable comparison with known DNA sequences. Dideoxynucleotide sequencing (Sanger *et. al.*, 1977) was carried out using the Sequenase 2.0 kit supplied by USB Inc. All solutions required were provided in the kit and sequencing carried out according to manufacturer's directions.

2.10.1 Preparation of single stranded template

DNA fragments inserted into the polyiinker of pBluescript, which possesses the f1 origin of replication from the f1 filamentous phage, allows the rescue of single-stranded DNA for sequencing upon co-infection with heiper phage. An overnight culture of the clone to be sequenced was grown in 100 μ g/ml ampicillin and 12.5 μ g/ml tetracycline (ensures selection

of male sex pill of strain through which helper phage infects) and used to inoculate 10 ml of NZY medium containing the same antibiotics. The culture was grown to an OD_{650} of 0.5 and 1 ml of this infected with 10^9 pfu of the helper phage M13K07. This was left in a shaker at 37° C for 1 hour. After this time, 9 ml of NZY were added containing 100 µg/ml ampicillin and kanamycin at 70 µg/ml and the culture left overnight in a shaker at 37° C.

The following morning the cells were centrifuged at 4,000 rpm for 10 min and the phage containing supernatant removed to a new tube. Phage was precipitated by the addition of 0.2 volumes 20% (w/v) PEG 6,000/1 M NaCl and incubating on ice for 15 min. Phage particles were pelleted by centrifuging at 10,000 rpm in a Sorval RC-5B centrifuge and the pellet resuspended in 400 μ l of distilled water. The solution was extracted twice with phenol: chloroform and DNA precipitated by adding 0.1 volumes 2 M sodium acetate pH 5.6 and 3 volumes absolute ethanol and leaving at -70°C for 10 min. After centrifuging at full speed in a microfuge the DNA pellet was washed with 70% (v/v) ethanol, dried and redissolved in 20 μ i of water. A 2 μ l aliquot was run on an agarose gel to assess quantity and quality.

Annealing of template DNA with suitable sequencing primers was carried out by mixing 1 μ g of the template in a volume of 7 μ l with 1 μ l (10 ng) of a suitable primer. A total of 0.2 volumes of 5 x sequencing buffer was added and the tube placed in a beaker containing water at 65°C and allowed to cool to room temperature before continuing with the sequencing reactions.

2.10.2 Preparation of double stranded templates for sequencing

Sometimes single stranded DNA preparations were not possible so double stranded DNA of a quality suitable for sequencing was prepared. Crude plasmid DNA was first prepared as described for small scale preparation of plasmid DNA (2.9.6) with the DNA dissolved In a final volume of 50 μ I distilled water. Large RNAs were precipitated by adding 75 μ I 8 M lithium chloride and leaving on ice for 5 min. After centrifuging for 5 min in a microfuge the supernatant containing DNA was removed to a new tube. DNA was precipitated by adding 3 volumes of absolute ethanol and leaving for 5 min. The tube was centrifuged, the pellet washed in 70% (v/v) ethanol, dried and redissolved in 50 μ i distilled water. Any remaining

small RNAs were removed by adding 1 μ I of RNase at 10 mg/mI and incubating at room temperature for 5 min. The solution was phenol: chloroform extracted twice to remove the RNase and the DNA re-precipitated by adding 0.1 volumes 2 M sodium acetate pH 5.6, 3 volumes absolute ethanol. After centrifuging, washing, and drying the DNA pellet it was dissolved in 20 μ i distilled water and a 2 μ I aliquot run on an agarose gel to assess quality and quantity.

Template DNA was denatured by mixing 10 μ I DNA (5-10 μ g) with 2 μ I 2 M NaOH and incubating at room temperature for 5 min. The solution was neutralised by adding 5 μ i 5 M ammonium acetate pH 4.8 and DNA precipitated by adding 3 volumes absolute ethanol. Annealing was achieved by adding the denatured DNA in 6 μ I of water to 2 μ i of a suitable primer (10 ng) and 0.2 volumes 5 x sequencing buffer and continuing as for single stranded template.

2.10.3 Analysis of DNA sequences

Computer aided sequence analysis and database searches were carried out using the University of Wisconsin programmes on a VAX (Devereux *et. al.*, 1983).

2.11 In vitro transcription and translation

This technique was used to enable the comparison of a known DNA sequence with a particular band of protein that had previously been identified by using an *in gel* enzyme assay (2.14.3; chapter 5). The transcription reaction was carried out using a kit from Stratagene which provided ail solutions. The reaction mix was combined in a 1.5 ml microfuge tube and contained 0.5-1.0 µg linearised template DNA, 0.2 volumes 5 x transcription buffer, 1 µi each of 10 ml rATP, rCTP, rGTP and rUTP, 30 ml DTT, 1 unit of RNase block II, 10 units of T7 RNA polymerase, and DEPC treated water to a total volume of 25 µl. After mixing, the reaction was incubated at 37°C for 30 min and then phenol: chloroform extracted. Nucleic acid was precipitated by adding 0.1 volumes 2 M sodium acetate pH 5.6, 3 volumes absolute ethanol,

leaving at -70°C for 10 min, and then centrifuging in a microfuge for 10 min. The nucleic acid pellet was then washed with 70% (v/v) ethanol, dried, and dissolved in 20 μ l of DEPC-treated water. A 5 μ l aliquot was run on an agarose gei to check the quantity and quality of RNA produced.

The translation reaction was set up with solutions provided in a kit from Promega in a 1.5 ml microfuge tube and contained 17.5 μ l nuclease-treated rabbit reticulocyte lysate, 40 units RNase inhibitor, 5.0 μ i of RNA template from the transcription reaction, 0.5 μ l of an amino acid mix without methionine, 1.0 μ l [³⁵S]-Methionine, and DEPC treated water to a final volume of 25 μ i. The reaction was incubated at 30°C for 60 min after which time half the mix was run initially on a 15% denaturing protein gel to ensure that the reaction had worked. The second half of the mix was later run on a native gel alongside previously identified protein bands. The gel was then placed on Whatman 3 MM paper, covered with cling film and dried in a BioRad gel drier on cycle 1 at 60°C for 1.5 hours. The dried gel was placed on film for 2 nights and bands on the autoradiograph compared with protein bands present on the dried gel.

2.12 Yeast transformation

Yeast were used as a host to study the secretion of cecropin (chapter 3). This involved transforming the cecropin gene inserted into a plasmid carrying a *ura* marker gene into yeast under the control of a galactose inducible promoter. The production and secretion of active cecropin was then assessed using the *E. coli* overlay test.

2.12.1 Transformation of yeast using the lithium acetate method

Modified after Ito et. al. (1983).

An overnight culture of the required yeast strain was subcultured into fresh YPD media at 10⁶ cells/ml in a total volume of 10 ml and placed in a shaker at 30°C until cell density reached

 10^7 /ml. Cells were collected by centrifuging for 7 min at 3,000 rpm, washed in 2 ml TE and resuspended in 1 ml LA buffer (0.1 M lithium acetate in TE). The tube containing the cells was left in a shaker at 30°C for 1 hour. Plasmid DNA (0.1-10 µg) in 10 µl of TE was added to 300 µl of the yeast cells and mixed with 700 µi of 50% (w/v) PEG 4,000. This was placed in a water bath at 30°C for 1 hour and then heat shocked for 5 min at 42°C. After centrifuging at 4,000 rpm for 5 min the cells were resuspended in 400 µi sterile distilled water. Selective plates, lacking in uracil, were then spread with 200 µi of transformed cells and incubated at 30°C for 2 to 4 nights until colonies of approximately 1-2 mm in diameter were visible. This method generally yielded 5-800 colonies per transformation.

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2.12.2 Small scale isolation of plasmid DNA from yeast

From Leicester Biocentre manual.

Following transformation of yeast, plasmid was isolated to confirm that they were transformants and not revertants. A total of 4.5 ml of an overnight culture of the yeast transformant in SD minimal media was spun down in a 1.5 ml microfuge tube by first spinning down 1.5 ml, discarding the supernatant and repeating the process. The cells were resuspended in BME buffer (0.9 M sorbitol, 0.05 M Na_2PO_4 pH 7.5, 1 $\mu i/ml$ $\beta\text{-}$ mercaptoethanol) and 25 µl of yeast lytic enzyme (Sigma) at 10 mg/ml added. The tube was incubated at 37°C for 1-1.5 hours until sphaeroplasts formed which was confirmed by observing under a microscope. Cells were centrifuged for 30 secs and resuspended in 100 µl of 1 M sorbitol before adding 800 µi of lysis buffer (100 mM Tris-HCi pH 9.7, 50 mM EDTA pH 8.5, 0.5% (w/v) SDS). Contents were mixed and then incubated at 70°C for 20 min. Protein and genomic DNA were precipitated by adding 200 µl of 5 M potassium acetate and leaving on ice for 45 min. The tube was then centrifuged and supernatant removed to a new tube. To the plasmid- containing supernatant was added 0.55 ml propan-2-ol, the contents mixed and left at room temperature for 5 min. Plasmid DNA was then pelleted by centrifuging, washed in 70% (v/v) ethanol, dried and dissolved in 20 µl TE. Half of this was then used to transform calcium chloride competent E. coll cells (2.9.5) in order to recover enough amplified DNA to perform restriction analysis of the plasmid.

2.12.3 Galactose promoter induction

Yeast cells were grown for 16 hours in either YPD or for 48 hours in selective media. They were then centrifuged for 7 min at 3,000 rpm in a Heraeus centrifuge and the cell pellet washed twice with YPgal medium before resuspending in YPgal at a density of 4-5 x 10⁸ ceils per ml. Cells were then placed in a shaking incubator at 30°C for a minimum of 2 hours before assaying the medium for the desired protein.

2.12.4 E. coll overlay test for the presence of active cecropin

A 200 μ I aliquot of an overnight culture of *E. coli* XL1-blues grown in LB was added to 5 ml of top agar and spread on a Petri dish containing solid LB medium. Once the top agar had set, wells were made in the agar using the narrow end of a Pasteur pipette into which were placed 3 μ I of the medium to be tested. The plates were then incubated overnight at 37°C. Antibacterial activity was indicated by the presence of a clear halo around the wells in an otherwise turbid lawn of bacteria.

2.12.5 Yeast RNA extraction

The following method is based on that contained in the Leicester Biocentre manual.

Confirmation that transcripts were produced from plasmids transformed into host yeast cells was obtained by isolating RNA and performing northern blots. Yeast cells were grown for 16 hours in appropriate media and then centrifuged at 5,000 rpm for 10 min at 4°C in a Sorval RC-5B centrifuge using a GSA rotor. After discarding the spent media, the cells were resuspended in 25 ml of sterile, distilled water, transferred to a 30 ml plastic Nunc tube and repelieted in a Heraeus centrifuge at 5,000 rpm for 10 min at 4°C. The pellet was then resuspended in Kirby mix (6% (w/v) 4-amino salicylate, 1% (w/v) triisopropyl naphthalene sulphonate, 6% (v/v) phenol, 50 ml Tris-HCl pH 8.4) using 1 ml per 100 ml of yeast culture. The mix was transferred to a 15 ml corex tube, acid-washed sterile glass beads added until

they were just covered by the liquid and the tube vortexed in bursts of 30 secs, keeping the tube on ice for 10 min between each burst. Cell breakage was determined by examining cells under the microscope. When the majority of the cells had burst an equal volume of phenol: chloroform was added and mixed thoroughly by shaking the tube. The phases were separated by centrifuging for 10 min at 5,000 rpm in a Heraeus centrifuge and the top aqueous phase re-extracted a further two times with phenol: chloroform. Nucleic acid was then precipitated by adding lithium acetate to 0.2 M, 2.5 volumes of absolute ethanol and leaving at -70°C for 30 min. After this time the nucleic acid was collected by centrifuging at 10,000 rpm for 15 min. The pellet was drained and redissolved in 400 μ i of sterile distilled water. RNA was precipitated by adding an equal volume of 6 M lithium acetate and incubating on ice for 1 hour. RNA was then pelleted by centrifuging for 10 min at 10,000 rpm. The pellet was washed twice in 3 M lithium acetate and then redissolved in 400 μ l sterile water. After transferring to a 1.5 ml microfuge tube, RNA was re-precipitated by adding 15 μ l of 6 M lithium acetate and 2.5 volumes of absolute ethanol. After pelleting, the RNA was dissolved in water and its concentration measured (2.2.2) prior to storing at -70°C.

2.13 Potato transformation

Potato tubers were transformed with a *gus* A reporter gene under the control of a woundinducible promoter in order to assess the promoter activity in response to pathogen activity (chapter 6). This was achieved by infecting transformed potato with various pathogens and measuring the activity of the reporter gene both quantitatively by fluorimetric assays and qualitatively using histochemical localisation studies.

2.13.1 Agrobacterium-mediated transformation of potato

After Sheerman and Bevan (1988).

Transformation involves transferring the desired gene into potato linked to a marker gene to enable the identification of successfully transformed cells. In this case, the marker gene was

neomycin phosphotransferase-II which confers resistance to kanamycin. Tubers were peeled, cut in half and sterilised by placing in 10% (v/v) chloros containing 6 drops of Tween-20 and left for 20 min, stirring occasionally. Using a number 10 sterile cork borer, cores were removed from the sterilised tubers and sliced into discs 1-2 mm thick. The discs were placed into 20 ml Steriiin tubes containing Agrobacterium stocks harbouring the plasmid to be transferred into the potato (grown for 2 nights in LB containing appropriate antibiotics, then centrifuged at 4000 rpm for 5 min and resuspended in half a volume of MS30 liquid media) and left for 20 min with occasional shaking. The discs were then removed, excess liquid discarded by draining on sterile filter paper, and placed on plates containing shoot regeneration medium (MS salts (table 2.1), R3 vitamins (1 µg/ml thiamine HCl, 0.5 µg/ml nicotinic acid, 0.5 µg/ml pyridoxine HCi), 3% (w/v) sucrose, 5 µM zeatin riboside, 3 µM iAA aspartic acid, pH 5.9, solidified with 0.8% Difco agar). Plates were placed in a growth cabinet at 20°C with 16 hours of illumination followed by 8 hours of darkness. After 48 hours, after which time the Agrobacterium would have infected the potato discs, the discs were transferred to shoot regeneration plates containing 50 µg/ml kanamycin to select transformed potato shoots and 200 µg/ml cefotaxime to kill the bacteria. Once green shoots appeared, which took 8-12 weeks, these were transferred to rooting medium (MS medium containing R3 vitamins, 3% (w/v) sucrose, 50 µg/ml kanamycin, 200 µg/ml cefotaxime) in 20 ml Sterilin tubes. Any white shoots which grew were considered to be untransformed "escapes" having been bleached by the presence of kanamycin.

Once plantlets had been obtained, they were removed from the Sterilin tubes under sterile conditions and cut into sections containing internodes which were replaced into Sterilin tubes containing fresh rooting media. This provided a way of bulking up and maintaining stocks prior to planting out in soil. Before placing in soil, sections containing internodes were placed in Petri dishes and left for a week in a growth room until shoots and roots had developed. After this time, the plantlets were placed into pots containing a mixture (half and half) of peat and vermiculite and allowed to grow in a Fison's growth cabinet at 20°C with 16 hours of light alternating with 8 hours of darkness. For the first few days of growth, the pots were placed into plastic bags to retain a high humidity. The humidity was gradually decreased until the plantlets had grown a thick cuticle. Tubers were visible after 4-5 weeks of growth in pots, with the tuber size being dependent upon the size of pot used to grow the potato plants.

2.13.2 Microbial infections of potato plants

All work Involving piant pathogens was carried out at the Scottish Crop Research Institute (SCRI) under the supervision and guidance of Drs. Gary Lyon and Hugh Barker, SOAFD licence number GM/22/1992. Potato plants used were transformed lines obtained at Leicester University, transported to the SCRI as piantlets in Petri dishes, and grown for 5 weeks in a greenhouse at 20°C under natural light conditions. Plants were sprayed with nicotine to prevent aphid infestation.

2.13.2.1 Infection with Phytophthora Infestans

Leaves were removed from the potato piants and placed upsidedown into square Petri dishes containing a sponge presoaked in water to maintain a high humidity. The leaves were then infected by placing 20 µi dropiets containing 100, 1000, or 5000 zoospores in water onto flat sections of the leaf, with a total of 4 droplets on each leaf. Leaves were then incubated at 16°C in a growth cabinet with a cycle of 16 hours of illumination followed by 8 hours of darkness until symptoms consisting of regions of necrosis appeared (3-5 days). Leaves were then tested histochemically for GUS activity (section 2.13.3.2).

2.13.2.2 Infection with Erwinia carotovora

A 24 hour culture (2.1.7.2) was harvested by centrifuging at 10,000 rpm at room temperature in a Sorvai RC-5B centrifuge using GSA polypropylene bottles, and then resupended in an equal volume of water which gave a density of approximately 5×10^9 cells per ml. Potato tubers were then submerged in the bacterial suspension and a vacuum applied for 10-15 min to infiltrate the bacteria into the tubers via the lenticels. The tubers were then placed in both aerobic and anaerobic chambers at 20°C under conditions of high humidity, achieved by placing the tubers in plastic bags which were loosely tied. Uninfected tubers were also placed under the same conditions to provide controls. Symptoms consisting of soft areas in the tubers appeared after 5-7 days. Tuber slices through the soft areas were then assessed histochemically for GUS activity (2.13.3.2).

2.13.2.3 Infection with Potato Virus Y (PVY)

Potato leaves were infected on the plant by shaking carborundum, a mild abrasive, over the leaves to be infected. Inoculant, prepared as described in 2.1.7.3, was then rubbed gently onto the leaves, with the abrasive causing breaks in the plant epidermis which allows the virus particles to enter the plant. Controls were set up in the same manner with water used Instead of the virus-containing inoculant. Primary symptoms consisting of smail regions of necrosis appeared on the inoculated leaves after 10-12 days. Secondary symptoms of regions of chlorosis appeared on the uninoculated leaves after a further 3-4 days. Samples of leaves of inoculated and uninoculated leaves before and after the appearance of symptoms were tested histochemically for GUS activity (2.13.3.2).

2.13.3 β-glucuronidase (GUS) assays

2.13.3.1 Fluorimetric assays of GUS activity

Protein was isolated by grinding tissue of approximately 0.5 cm² using a small pestle and mortar in 400 μ I of GUS extraction buffer (50 mM Na₂PO₄ pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (v/v) Sarkosyl, 10 mM β -mercaptoethanol). The material was then placed in a microfuge tube and centrifuged for 10 min. Supernatant was removed to a new tube and used immediately in GUS assays.

Assays were performed by placing 20 μ I of the isolated protein in a 1.5 ml microfuge tube containing 180 μ I of GUS fluorimetric assay buffer (GUS extraction buffer with 1 mM methyl umbelliferyl glucuronIde) pre-warmed to 37°C. The tube was then incubated at 37°C. Every 20 min, including at time zero, a sample of 20 μ I was removed from the tube and placed in a well of an opaque microtitre plate containing 180 μ I of stop solution (200 mM Na₂CO₃). When all samples had been taken, fluorescence was measured in a Perkin-Elmer fluorimeter. A

calibration curve (Figure 2.1) was obtained by preparing dilutions of the reaction product 4methyl umbelliferone (4-MU), adding them to stop solution and plotting fluorescence units against nmoles 4-MU. Protein concentrations were measured using the Bradford assay (2.2.4) to enable calculation of GUS activity per mg of total protein.

2.13.3.2 Histochemical localisation of GUS activity

Tissue sections to be stained were placed in X-GLUC solution (50 mM Na_2PO_4 pH 7.0, 2 mM X-GLUC made up as 200 x stock in DMF and stored at -20°C) and incubated overnight at 37°C. Sections were then washed in several changes of 70% (v/v) ethanol to remove chlorophyll and allow observation of the stained tissue.

2.14 Preparation of asparagus for experimental work

Asparagus was used as a model system for the identification of wound-induced genes (Harikrishna *et. al.*, 1991). The system involves uniformly grinding asparagus cladodes to isolate single cells which are then cultured in suitable medium. The activity of genes isolated using this system were studied both in isolated single cells and in dark-grown seedlings wounded by slicing, to ensure that the genes were wound-inducible and not upregulated as a consequence of either the isolation process or by growth in cell culture medium.

2.14.1 Mechanical isolation of asparagus cells

A totai of 10 g of ciadodes were stripped off 6 week old seedlings and surface sterilised by placing in a solution of 10% (v/v) bleach for 20 min before washing in several changes of sterile tap water. They were then placed in a mortar together with 10 ml of sterile tap water and gently ground using a pestle. Waste cladode material was removed by filtering the cell suspension through a sterile 64 μ m mesh filter and the cells collected by centrifuging at 800 rpm in a Sorval RT6000B centrifuge for 2 min. The supernatant was pipetted off and a known

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Figure 2.1

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Calibration curve of 4-MU. A stock solution of 10 μ m 4-MU was diluted in water to a number of known concentrations. The number of nmoles was then calculated and this value plotted against the fluorescence reading obtained for each concentration using an excitation energy of 365 nm and reading the emission spectra at 455 nm.



Calibration curve for 4-MU

volume (usually 80 ml) of fresh sterile tap water added. An aliquot was removed and placed on a haemocytometer slide to count the number of cells. After centrifuging again, the cells were resuspended to a concentration of 4×10^5 cells/ml in asparagus medium (table 2.2). The cells were then either divided into aliquots of 10 ml and placed in 90 mm Petri dishes sealed with Nescofilm, or divided into 50 ml aliquots and placed in 250 ml sterile flasks sealed with a cotton bung and foil. In both cases cells were incubated in the dark at 25°C on a rotating platform set to 40 rpm. Before harvesting the cells were checked for infection by observation under a microscope. They were then centrifuged at 1,000 rpm for 10 min and the cells either used directly or flash frozen in liquid nitrogen and stored at -70°C.

2.14.2 Growth of seedlings for wounding studies

Asparagus seeds were surface sterilised in 100% iMS for 3 min, washed in sterile tap water and left to imbibe sterile tap water for a period of 3 hours. The seeds were then scattered on sterile vermiculite soaked in water contained in a foil covered sterile washing up bowl. Growth occurred in the dark at 25°C. Once the hypocotyls had grown to a height of 50 mm (approximately 2 weeks post-germination) the seedlings were harvested. They were cut into sections of the required size and placed in 90 mm Petri dishes containing filter paper soaked in water. Dishes were sealed using Nescofilm and left in the dark at 25°C for the duration of the wounding experiment. Sections were then harvested, flash frozen in liquid nitrogen and stored at -70°C.

2.14.3 In gel superoxide dismutase (SOD) assay

After Beauchamp and Fridovich, 1971.

One of the genes isolated from the asparagus model system showed very high homology to the enzyme superoxide dismutase (chapter 5) so SOD assays were undertaken to compare gene activity with enzyme activity. SOD assays were performed *in gel* which enabled a direct comparison of the activity of different SODs in the same protein sample. A total of 20 μ g of

protein isolated as described in 2.2.3 were run on a 10% native PAGE mini-gel (2.3.4) for 2.5 hours at 100 V in a cold room at 4°C. The gel was then placed in 100 ml SOD assay buffer (50 mM Tris-HCl pH 8.4, 0.25 mM EDTA pH 8.0, 3 mg riboflavin, 10 mg NBT made up as 30 mg/ml stock in 70% (v/v) DMF) and incubated in the dark at room temperature. The gel was then exposed to bright light by placing on a light box and left until white bands showing SOD activity appeared on a purple coloured background which took approximately 20 min. Cyanide insensitive SODs were detected by adding 1 mM KCN to the SOD assay buffer prior to placing the gel in the dark. Gels were stored in 50% (v/v) glycerol solution in the dark.

2.14.4 Testing the effects of cell culture medium on SOD activity

Apart from the wounding stimulus, isolated cells in the asparagus cell culture system could be affected directly by constituents of the asparagus cell culture medium, or by growth in the dark. The following experiment was performed to determine whether light or an increase in respiration caused by the presence of certain medium constituents affected SOD activity.

Six week old asparagus seedlings were cut at the stem above soil level and approximately 2 inches of the cut stem immersed in either water, 1% (w/v) sucrose, 3% (w/v) mannitol, or both sucrose and mannitol in a glass jar, leaving the rest of the seedling protruding above the top of the jar. A total of 10 seedlings were placed in each jar. Sucrose and mannitol concentrations are the same as that found in asparagus cell culture medium (table 2.2). Duplicates of each condition were set up and each left at 25°C in either the dark or under light conditions. After 4 days, protein was isolated from the cladodes as described in 2.2.3 and a SOD assay conducted as described in 2.14.3.

Table 2.1 MS salts

After Murashige and Skoog, (1962).

Constituent	Weight per litre
CaCl ₂ .2H ₂ O	440 mg
NH4NO3	1.65 g
KNO3	1.9 g
КІ	0.83 mg
CoCl ₂ .6H ₂ O	0.025 mg
KH ₂ PO ₄	170 mg
H ₃ BO ₃	6.2 mg
Na ₂ MoO ₄ .2H ₂ O	0.25 mg
MgSO ₄ .7H ₂ O	370 mg
MnSO ₄ .4H ₂ O	22.3 mg
CuSO ₄ .5H ₂ O	0.025 mg
ZnSO ₄ .4H ₂ O	8.6 mg
FeSO ₄	27.85 mg
Na ₂ EDTA	37.25 mg
Glycine	2.0 mg
Inositol	100 mg
Nicotinic acid	0.5 mg
Pyridoxine HCI	0.5 mg
Thiamine HCI	0.1 mg

The pH was adjusted to 5.8 with 0.1 M HCl. All the above components are supplied in preweighed packets by Flow Laboratories Ltd.

Table 2.2 Asparagus medium

Modified from Nagata and Takebe (1971).

Constituent	Weight per litre
NH4NO3	825 mg
KNO3	925 mg
CaCl ₂ .2H ₂ O	220 mg
MgSO ₄ .H ₂ O	1.233 g
КН ₂ РО ₄	680 mg
Na ₂ EDTA	37.3 mg
FeSO ₄ .7H ₂ O	27.8 mg
H ₃ BO ₃	6.2 mg
MnSO ₄ .4H ₂ O	22.3 mg
ZnSO ₄ .7H ₂ O	10.58 mg
КІ	0.83 mg
NaMoO ₄ .2H ₂ O	0.25 mg
CuSO ₄ .5H ₂ O	0.03 mg
Mannitoi	30 g
Sucrose	10 g
Myo Inositol	100 mg
Thiamine HCI	1.0 mg
NAA	1.0 mg
6-BAP	0.3 mg

pH was adjusted to 5.8 with 1.0 M KOH. Prior to use 3.4 ml of filter sterilised glutamine at 23.5 mg/ml was added per 80 mi of medium.

CHAPTER 3

ANALYSIS OF YEAST TRANSFORMANTS CONTAINING CECROPIN CONSTRUCTS

3.1 Introduction and background to previous work

One of the important components in the proposed route for the genetic engineering of plants for bacterial resistance is the secretion of an antimicrobial polypeptide.

Cecropin was chosen as the antibacterial substance because it is active against a range of bacterial pathogens (Destefano-Beltran *et. al.*, 1990) while not being toxic to the plant host (Nordeen *et. al.*, 1992). The reasons for choosing porcine cecropin are elaborated further in section 1.12.2. A synthetic cecropin gene was designed and constructed using synthetic oligomers by Simon Firek (Botany Department, Leicester University) based on the amino acid sequence of the mature porcine cecropin (Lee *et. al.*, 1989). The codon usage was biased towards that of plants by studying the most commonly used codons taken from the sequence data of small subunit rubisco from several plants and designing the synthetic gene to incorporate mainly these codons. The synthetic cecropin gene was combined with the wheat α -amyiase secretion signal which was also synthesised using oligomers. The efficiency of cecropin production and secretion was initially examined in tobacco under the control of the CaMV 35S promoter (Nagy *et. al.*, 1985). Transgenic plants were obtained and analysed for the presence of the cecropin transcript and also for the production of protein. Though in some lines a transcript was detected on northern blots (S. Firek, personal communication) no active cecropin could be detected either intracelluiarly or in the intercellular spaces.

Further constructs were then prepared containing a viral translation enhancer (VTE) element. Much research has been carried out concerning the ability of 5' leader sequences from viral mRNA to greatly enhance translation both *in vitro* and *in vivo* (Gaille *et. al.*, 1987a, 1987b, 1988). These sequences include the omega sequence obtained from Tobacco Mosaic Virus. Research conducted by Dr. Gary Foster (Leicester University) has resulted in the isolation of a VTE element from Potato Virus S (PVS). Initial results indicated that this element greatly enhanced translation *in vitro*. The element was therefore incorporated into a construct containing the wheat α -amylase secretion signal and the synthetic cecropin gene in the expectation that this would enhance translation *in vivo* and enable the detection of a product.

In vitro transcriptions and translations of the secretion signal and cecropin gene constructs with and without the VTE element were carried out with the results shown in Figure 3.1. The addition of microsomes indicated that with the control RNA correct processing was achieved. in the absence of the VTE element no product was detected whereas with the element a product was highly visible, though there were no detectable differences between the samples with and without microsomes. The lack of product without the VTE element appeared to indicate that either very little product was being formed, or that any product formed was being rapidly degraded. A degraded product would explain the smears present in lanes 3 and 4. The presence of visible product with the addition of the VTE element indicates that it may be highly advantageous to use the element in constructs containing the cecropin gene.

Constructs containing the VTE element attached to the wheat α -amylase secretion signal and cecropin gene again under the control of the CaMV 35S promoter were transferred into tobacco plants. Northern analysis showed that detectable transcript was present in some lines but again no active cecropin product could be found.

It was therefore decided to study the expression and secretion of cecropin in the yeast *Saccharomyces cerevisiae*. The yeast would be used as a test system to determine whether viable product could be obtained from the constructs used in tobacco. Yeast has two major advantages over continuing the study of secretion of cecropin in tobacco: transformants can be obtained within a week rather than the minimum of two months required to generate transgenic tobacco; and also with yeast every cell would have the potential to express and secrete product, eliminating the dilution effect caused by non-expressing cells in tobacco. It is possible that the CaMV 35S promoter is not constitutive for every tissue or does not direct

Figure 3.1

In vitro transcription and translation of synthetic cecropin.

Lane 1. Translaton of control RNA.

Lane 2. Translation of control RNA in the presence of microsomes.

Lane 3. Translation of cecropin RNA with attached wheat α -amylase secretion signal.

Lane 4. Translation of cecropin RNA and wheat signal in the presence of microsomes.

Lane 5. Translation of cecropin RNA with wheat signal and attached VTE element.

Lane 6. Translation of cecropin RNA with wheat signal and attached VTE element in the presence of microsomes.

(Reproduced with permission of Dr. Simon Firek, Botany Department, University of Leicester.)



high expression in every cell. Were this to be the case, the lack of detection of an active product in tobacco may have been due to dilution of any cecropin produced by cells which were not expressing the gene or not expressing to high levels.

Yeast was chosen as an expression vector since as a eukaryote it should not be sensitive to cecropin (Steiner *et. al.*, 1988). Yeast have also been used previously to successfully express many heterologous animal and plant proteins including the expression and secretion of human interferon (Hitzeman *et. al.*, 1983), successful synthesis and processing of thaumatin (Overbeeke, 1989), and the expression and secretion of α -amyiases from both rice (Kumagai *et. al.*, 1990) and bariey (Søgaard & Svensson, 1990). *S.cerevisiae* can be easily manipulated genetically and has the advantage that it secretes very few proteins into the growth medium, greatly simplifying the purification of secreted foreign proteins.

3.2 Testing the effect of cecropin on yeast and bacterial growth

Though cecropin was not expected to affect yeast adversely, some reports suggest that cecropin has a toxic effect on certain fungi (Destefano-Beltran *et. al.*, 1990). Because yeast and filamentous fungi are closely related it was decided to test the effect of cecropin on yeast, and also assess the sensitivity of *E. coli* to cecropin since this is the test bacterium that would be used to determine whether active cecropin was being produced from the transformed yeast.

Figure 3.2a contains the results of a bacterial overlay test, conducted as described in 2.12.4, in which wells were filled with 3 μ l of varying dilutions of cecropin. It can be seen that 600 ng (a concentration of 3 μ g/ml) of cecropin diffused throughout the depth of the plate gives a clear indication of antibacterial activity.

The effect of cecropin on yeast was tested by growing the host strain, JRY188, in YPD media containing commercially synthesised porcine cecropin (Peninsula laboratories, inc., California) at a concentration of 10 μ g/ml. After overnight growth, the number of yeast cells per ml of media were counted and compared with equivalent counts of yeast grown in the same media

Figure 3.2

Effect of cecropin on growth of E. coli and the yeast S. cerevisiae.

a) Effect on *E.coli* present in top agar in the form of an overlay. Wells were made in the agar using the narrow end of a Pasteur pipette and varying quantities of cecropln (amounts as shown) placed into the wells. Plates were left overnight at 37°C. Ciear zones indicate inhibition of bacterial growth. The dilutions refer to dilutions of the cecropin stock solution at 2 mg/ml used to give the amounts of cecropin shown in the wells.

b) Table showing the number of cells of *S.cerevisiae* present in media following overnight growth in YPD with and without cecropin present at 10 μ g/ml. Three replicates were set up to obtain the standard error shown.



b)

no. yeast cells x 10 ⁷	
no cecropin	with cecropin
51 +/-9.7	52.5 +/-8.3
without cecropin. From the table In Figure 3.2b it can be seen that the counts of yeast in media with and without cecropin are virtually the same which leads to the conclusion that externally supplied cecropin has no effect on yeast growth at the concentration used.

3.3 Transformation of yeast with cecropin constructs

The plasmid pSKCEC4, containing the synthetic cecropin gene attached to the wheat α amylase secretion signal in pBluescript, was obtained from Dr. Simon Firek. The wheat signal and cecropin fragment were removed from the plasmid using the restriction enzymes *Xba* I and *Hin* d IIi and ligated into similarly restricted pEMBLyex4 vector, a shuttle vector containing origins of repiication for both *E. coli* and *S. cerevisiae* (obtained from Graham Plastow, Dalgety Food Technology Centre, Cambridge). This resulted in the plasmid pYWCE1 (Figure 3.3a). The shuttle vector contains an inducible galactose promoter enabling the expression of foreign proteins to be controlled in cases where constitutive expression may prove to be toxic. The promoter is repressed by the presence of glucose in the external medium. The vector also contains a yeast terminator which is required for high level expression in yeast (Zaret & Sherman, 1982).

Similar manipulations using the piasmid pSKVTE9, containing the VTE element attached to the synthetic cecropin gene and wheat α -amylase secretion signal in pBluescript (also obtained from Dr. Simon Firek), resulted in the construction of pYWCE2 (Figure 3.3b).

The plasmids so constructed were used to transform yeast. The vector pEMBLyex4 was also transformed into yeast to provide a non-cecropin producing control. Transformed yeast were selected and confirmation that the transformation was successful was obtained by isolating yeast plasmid DNA. The DNA was used to transform *E. coli*, the plasmid amplified and confirmed to be correct by restriction enzyme digestion.

High copy number transformants were selected by plating the yeast on minimal medium plates lacking both uracil and leucine. The pEMBLyex4 plasmid vector used contains two selection markers: *ura3* (Rose *et. al.*, 1984) which is used to select initial transformants and enables the

Figure 3.3

Constructs used in assessing the viability of secretion of cecropin in yeast.

Both the cecropin gene and wheat a-amylase secretion signal were synthesised using complementary oligomers ligated together by Dr. Simon Firek (Leicester University). The VTE element was obtained from Dr. Gary Foster (Leicester University). Sequencing confirmed that both constructs formed in frame translational fusions, which then formed transcriptional fusions when transferred to the yeast vector.

a) Diagram of pYWCE1 construct showing the sequence of the wheat α -amylase secretion signal attached to synthetic cecropin gene.

b) Diagram of pYWCE2 construct showing the sequence of the viral translation enhancer element attached to the wheat α -amyiase secretion signal and the synthetic cecropin gene.

Key: ▼ indicates the signal peptidase cleavage site

- → approximate transcription start site
- B Bam Hi
- H3 Hind III
- P Pst I
- RI Eco RI
- X Xbal

The yeast vector used was pEMBLyex4, a shuttle vector containing origins of replication for both yeast and *E. coli*. The promoter present in the vector is a hybrid of the upstream activation sequence from the intergenic region between the *GAL1* and *GAL10* genes, and the 5' non-translated leader of the *CYC1* gene which contains the signals required for initiation of transcription (Cesareni & Murray, 1987).





Eco GAA1	RI FTC <i>I</i>	АТСТ 10	CAI	ATA	CAA 2	M TGO 0	A SCG <i>P</i>	N AACZ	к АААС 30	H CAC'I	L FTG1	s cccc 40	L CTCI)	S CGC	L TCI	F TTCO 50	L CTCC	V GTC(L CTC 60
	\sim mature cecropin																		
L CTT(G GGCC	L CTGT 70	S °CGG	A SCCA	S GCI 8	L TGG 0	A SCCI	s CCC	G GGAI 90	S CCI	W rgg1	L TGI 100	S CCCA	K AAGA	T ACCO 11	A GCCA	K AAG <i>I</i>	K AG:	L FTG 120
E GAGA	N AACI	s rccg 130	H CCA	K AGA	K AGC 14	R SGTA 0	I ATCI	S ICCO	E GAGO 150	G GA <i>l</i>	I ATCO	A SCCA 160	I ATCO)	A GCC <i>A</i>	I ATCO 17	Q CAGO 70	G GGAC	G GGA(Р ССА 180
R CGT	* FAA1	* FAAC	CTA	١G															

															М	P	Ρ	ĸ	P
TTA	GGT	FCAC	CAG	STA P	AGAC	TTC	CGAA	AGA	AACT	rgto	CCA	ACAC	GAGA	AAA	ATGO	CCG	CCG	AAA	CCG
		7()		6	30			90			100	C		1:	LO			120
•																			
G	S	L	0	E	F	I	S	Y	т	М	А	Ν	ĸ	н	L	S	L	S	L
GGA	rcco	CTG	CAG	GAAI	TCF	ATC:	FCA	TAT	ACAA	ATGO	SCGP	ACI	AAA	CACI	TG:	rcco	CTC	rcg	CTC
130					140				150			160				70		180	
F	L	V	L	L	G	L	S	А	S	\mathbf{L}	А	s	G	S	W	L	s	ĸ	т
TTC	CTC	GTC	CTC	CTTC	GCC	CTG	rcgo	GCC	AGC'	ГТGC	SCCI	ICC(GGAT	rcci	rGG.	LLLC.	FCC)	AAG	ACC
	190				200			210			220				230				240
А	ĸ	ĸ	L	E	N	S	А	K	ĸ	R	Ι	S	E	G	I	А	Ι	А	Ι
GCC.	AAG	AAG	FTG	GAGA	AC1	rcco	GCCA	٩AG	AAG	CGTI	ATCI	rcco	GAG	GGA	ATCO	GCC2	ATC	GCC	ATC
		250	0		26	50			270			280	0		29	90			300
Q	G	G	Ρ	R	*	*													
CAGGGAGGACCACGTTAATAACCTAG																			
		310	0		32	20													

TCTAGAGCTCACAAGAGATTTGGTGGAAGCCGTAGCAACATTGGGGCCGTTGAAGCACCT 10 20 30 40 50 60

wheat signal sequence, cecropin gene and VTE in the vector pEMBLyex4

Xba I



deficient yeast host strain to grow on media lacking supplemental uracil; and *leu2-d* (Beggs, 1978) which has a truncated promoter and is poorly expressed, enabling yeast to grow on media deficient in leucine only if it is present in high copy number. Though yeast containing high copy numbers of the plasmids pEMBLyex4 and pYWCE1 were obtained, no high copy number yeast transformants containing the construct with the VTE element, pYWCE2, could be isolated. This suggests that the yeast is unable to tolerate high multiple copies of the VTE element present in the construct, possibly as a result of greatly enhanced production of toxic protein.

3.4 Assessing yeast transformants for the production of cecropin

Initial tests for cecropin production involved concentrating the growth medium after induction of the galactose promoter and testing for the presence of active cecropin using the well test as described in 3.2. Further tests involved laying a lawn of susceptible bacteria over induced yeast colonies and looking for the presence of a zone of inhibition of bacterial growth around the colonies.

3.4.1 Testing media for the presence of active cecropin

Following galactose induction of the transformed yeast, the cells were removed by centrifugation and the remaining media tested for cecropin activity using the well test. No antibacterial activity was found, possibly due to dilution of the product by the media. The media was therefore concentrated for use in further tests by using Amicon microconcentrators. These contain filters of varying sizes which retain proteins above a certain size while allowing smaller proteins through. To concentrate cecropin a combination of two sizes of filter were used: a 10 K filter which retains proteins above 10 kD and a 3 K filter which retains proteins above 3 kD. Since cecropin is 3.5 kD in weight it was expected to be retained by the 3 K filter. However, if proteins are compactly folded, it is possible that they will pass through a filter that should in theory retain them so the filters were tested for their ability to concentrate cecropin. Filters were used according to manufacturer's instructions.

Cecropin of known concentration was initially passed through the 10 K microconcentrator. Both the filtrate (liquid passing through the filter) and retentate (liquid retained by the filter) were kept with the filtrate being passed through a 3 K filter. Both filtrates and retentates and the filters themselves were then tested for their antibacterial activity using the bacterial well plate test as described previously (3.2). Approximately half the cecropin was found in the retentate from the 3 K filter while the other half was found on the 3 K filter itself. However, no antibacterial activity was detected in the filtrate from the 3 K filter enabling its satisfactory use in concentrating cecropin. In all experiments conducted using the microconcentrators both the retentate from the 3 K filter and the filter itself were tested for antibacterial activity.

The microconcentrators are only capable of concentrating 2 ml of liquid at one time. From the above experiment it was determined that after concentration through both filters, 100 μ l of retentate remained, giving a 20-fold concentration.

Yeast transformants were grown in minimal selective media overnight until they reached 1.5- 2.0×10^7 cells per ml and then induced by resuspending the cells in YPgal medium. The rich medium was used since it has been noted that secretion is much more efficient in rich medium (Rothstein *et. al.*, 1984). Initially, media from a two hour induction of the galactose promoter was concentrated. However, no zones of inhibition were seen in the bacterial well test so the induction time was extended to a maximum of 16 hours. Despite the increase in induction time, no antibacterial activity was detected.

3.4.2 Bacterial overlay tests

A second approach for detecting secreted cecropin was attempted which involved directly overlaying yeast colonies grown on solid minimal media selective plates with a bacterial lawn and looking for inhibition of bacterial growth around the yeast themselves. It was expected that this approach would increase the sensitivity of the test used due to the direct contact between the secreted foreign gene product and the test bacterium, without the diluting effects of growth in liquid media.

Transformed yeast were grown on minimal selective plates for up to 72 hours until a colony of approximately 2 mm in diameter was reached. The whole colony was then transferred to minimal plates containing galactose to induce the promoter and left for 16 hours before overlaying the plate with a bacterial lawn and continuing incubation for a further 16 hours at 30°C (it had previously been established that growth at 30°C did not affect bacterial growth in any way). The plates were then studied for any signs of antibacterial activity.

The plates appeared to show zones of inhibition of bacterial growth around some of the yeast colonies (Figure 3.4), particularly visible in the central colony containing the pYWCE2 plasmid with the VTE, wheat α -amylase secretion signal and cecropin gene.

In order to maximise the size of the zones, very thin solid minimal selective medium plates of just 2 mm depth were poured and a minimum amount of bacteria used in the overlay to still give a confluent lawn. After 72 hour growth of the yeast colonies, they were transferred to duplicate plates, one plate containing minimal media with glucose to act as a non-induced control and one plate containing minimal media with glactose. The plates were overlaid with a bacterial lawn and growth continued for 16 hours. The resulting plates showed the presence of zones of inhibition of bacterial growth (Figure 3.5). However, zones are visible on the control yeast containing minimal media with glucose. This result suggested that yeast growth itself in some way affected bacterial growth and that growth inhibition was not due to the production of cecropin.

It was thought that the inhibition of bacterial growth could be due to a pH effect with yeast growth leading to a decrease in pH to the extent that growth of *E. coli* was inhibited. This hypothesis was tested by measuring the pH of various media following overnight yeast growth. The pH of YPgal was 5.0 while that of minimal media containing galactose was 3.5. The resulting pH of the minimal media may therefore be enough to inhibit bacterial growth. The differences in zone sizes seen could be due to differences in the total numbers of yeast cells present and in the exact thickness of the plates.

The failure to detect active cecropin in concentrated medium following induction of yeast

Figure 3.4

Yeast overlay tests for the presence of secreted cecropin. Yeast were grown on minimal selective plates for up to 72 hours at 30°C and then patched onto inducing media containing galactose. After 16 hours, the plates were overlaid with 200 μ l of an overnight culture of *E*. *coli* in 5 ml of top agar and incubation continued at 37°C for a further 16 hours. Plates were then studied for signs of inhibition of bacterial growth adjacent to the yeast colonies.

a) Yeast transformed with the control vector, pEMBLyex4.

b) Yeast transformed with the cecropin containing vector, pYWCE1.

c) Yeast transformed with the vector pYWCE2 containing both the cecropin gene and the VTE element.



Figure 3.5

Plates showing maximised zones of inhibition of bacterial growth. Yeast were grown on plates containing minimal agar only 2 mm thick and overlaid with a bacterial lawn as described for figure 3.4. Plates on the left contain non-inducing minimal agar while plates on the right contain inducing minimal agar with galactose.

a) Yeast transformed with pEMBLyex4.

b) Yeast transformed with pYWCE1.

c) Yeast transformed with pYWCE2.



could be due to a variety of factors. These include non-production of cecropin by the yeast; production but poor or no secretion of the product; synthesis of an active product which is rapidly degraded; or internal production of cecropin leading to a toxic effect on the yeast cells.

This final possibility is supported by results seen with induced yeast containing pYWCE2 in which it is possible that the possession of the VTE element is exaggerating effects that cannot be seen with yeast containing pYWCE1. For example, in section 3.3 it was noted that though high copy number transformants with pYWCE1 were obtained, no corresponding yeast containing pYWCE2 could be selected. During the course of the experiments involving the growth of yeast in selective medium, it had also been noted that yeast containing pYWCE1.

Further investigations were therefore conducted to see if cecropin transcripts were produced by the yeast and and to see if transcript levels changed over time following promoter induction.

3.5 Northern analysis of transformed yeast

3.5.1 Detection of cecropin transcripts

Yeast was grown in selective medium and induced by the removal of glucose and the addition of galactose for 2 hours (see section 2.12.3). Total RNA was isolated from samples taken at time zero, 1 and 2 hours. No RNA was obtained from yeast containing pYWCE2 due to the very low density of yeast growth in selective media. The RNA was run on a gel to ensure it was evenly loaded (Figure 3.6a) and the gel blotted overnight. Control RNA was provided by Simon Firek and originated from *in vitro* transcription of pSKCEC4, giving a transcript containing the wheat α -amylase secretion signal and the cecropin gene. The filter was probed with the wheat signal/cecropin fragment obtained from restriction enzyme digestion of pSKCEC4. The resulting autoradiograph is shown in Figure 3.6b. There is no evidence of a detectable cecropin transcript in the yeast containing the pYWCE1 plasmid. The bands that are visible are those from the control with the upper band being the DNA template used in the

Figure 3.6

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Northern analysis of yeast transformants following galactose induction. Lane numbers refer to the number of hours following promoter induction by galactose. The central lane contains control RNA originating from *in vitro* transcription of pSKCEC4, giving a transcript containing the wheat secretion signal and the cecropin gene.

a) Agarose gel showing RNA isolated from yeast transformants containing either pEMBLyex4 (EMC) or pYWCE1 (C106). A total of 6 μ g of RNA were loaded in each lane.

b) Northern blot of the gel shown in a) probed with the 200 bp wheat α -amylase signal sequence/cecropin gene fragment.

c) Northern blot in b) stripped and reprobed with the ura gene.

Both northern blots were exposed for 96 hours.



b) EMC C106 C106 C106 0 1 2 0 1 2 0 1 2 0 1 2



in vitro transcription and the lower band being the transcription product.

3.5.2 Detection of ura transcripts

One possibility for the non-detection of a cecropin transcript is that the pYWCE1 plasmid has been lost from the yeast. To test this possibility, the filter used in the above experiment was stripped and reprobed with the *ura* gene which is present only on the plasmid. Figure 3.6c shows the result. Both the yeast containing the control plasmid pEMBLyex4 and yeast containing pYWCE1 were found to retain the plasmid as evidenced by the presence of *ura* transcript. It can be seen that successful selection of high copy number plasmids was achieved using pYWCE1 with the lane at time zero containing far more *ura* transcript than the corresponding lane with pEMBLyex4. However, with the yeast containing the cecropin construct, the amount of *ura* transcript was found to decrease noticeably following promoter induction. Decrease of the *ura* transcript suggests either that the plasmid was lost from the yeast as the time following promoter induction increased, or that total yeast cell numbers were decreasing.

3.6 Plasmid stability tests

The northern blot performed in 3.5.2 showing a decrease in *ura* transcript following promoter induction indicated that the plasmid number rapidly decreased. This decrease could either be due to individual cells losing excess copies of the plasmid or due to a decrease in the total number of yeast cells. Changes in cell number after induction were therefore followed to determine which of these two possibilities caused the decrease in plasmid number.

3.6.1 Changes in yeast cell number following induction

Yeast were initially grown in minimal selective medium containing glucose and then transferred to selective medium containing galactose. Galactose induction was continued for

2 hours with samples removed at 15 min intervals. Dilutions of these samples were spread on selective glucose plates which lacked uracil to enable a count to be made of the number of cells retaining the *ura* plasmid marker. The results are presented graphically in Figure 3.7. During the 2 hour time course there is not a significant change in the overall numbers of cells retaining the ability to grow on *ura* deficient media. After 2 hours the numbers of yeast containing pEMBLyex4 and pYWCE1 were slightly lower than before induction while yeast numbers containing pYWCE2 were almost the same as at time zero. Within the 2 hour time course of the experiment yeast cell number was not expected to increase since cell division time in minimal medium is greater than 2 hours (C. Hadfield, personal communication; own observations). This result therefore suggests that the decrease in *ura* transcript seen in Figure 3.6c was due to loss of excess copies of the plasmid and not to a decrease in total cell number.

The graph shown in Figure 3.7 also reinforces a previous observation, namely that yeast containing pYWCE2 grow to a far lower density than yeast containing either pEMBLyex4 or pYWCE1.

3.6.2 Long term plasmid stability tests

Since the above experiment in 3.6.1 showed that cell numbers remained approximately the same after a 2 hour induction in selective media, it was decided to investigate long term plasmid stability following 12-56 hours growth in various media. Stability was studied after 12 hours growth in YPD and YPgal, and after 56 hours growth in selective glucose media and selective galactose media. The results would show whether the plasmid was retained when no selection was applied and also when long term induction was allowed to occur.

Plasmid stability was assessed by removing samples and spreading dilutions on YPD plates to obtain the total number of viable cells present, and also on selective glucose plates to determine the cell numbers retaining the *ura* plasmid marker. These data were then used to calculate the percentage of cells that retained the *ura* marker after growth in the various media. The results are shown graphically in Figure 3.8.

Figure 3.7

Graph showing a time course of the number of yeast transformants retaining the *ura* plasmid marker following galactose induction of the promoter. Samples of yeast cells were removed every 15 minutes for a total of 2 hours, dilutions spread on minimal selective plates, and colonies counted after 72 hour growth. The experiment was repeated three times to obtain the standard error shown as bars in the graph.

EMC - yeast transformants containing the control plasmid pEMBLyex4 C106 - yeast transformants containing the plasmid pYWCE1 V101 - yeast transformants containing the plasmid pYWCE2 Time course showing the number of yeast cells retaining the ability to grow on uracil deficient media following galactose induction





Figure 3.8

Graph showing the percentage of cells retaining the ability to grow on media deficient in uracil after initial growth in non-selective media (YPD), non-selective inducing media (YP gal), selective media (min D), and selective inducing media (min gal). The experiment was repeated three times to obtain the standard error shown as bars in the graph.

EMC - yeast transformants containing the control plasmid pEMBLyex4

C106 - yeast transformants containing the plasmid pYWCE1

V101 - yeast transformants containing the plasmid pYWCE2



percentage of cells retaining the ura3 plasmid marker After growth in YPD, approximately 70% of the cells containing pEMBLyex4 and pYWCE1 retained the *ura* plasmid marker while less than 1% of the cells containing pYWCE2 retained the marker (Figure 3.8). A similar result is seen after growth in YPgal except that approximately 50% of the cells with pEMBLyex4 and pYWCE1 retained the marker. Again, none of the cells containing pYWCE2 retained the marker plasmid.

After growth in both selective glucose and selective galactose media a higher proportion, approximately 80-90%, of the cells containing pEMBLyex4 and pYWCE1 were found to retain the *ura* marker, while 2-9% of cells containing pYWCE2 retained the marker.

The above results indicated that the pEMBLyex4 and pYWCE1 plasmids were relatively stable. Some loss of plasmid did occur for both transformants after growth in inducing media but since the effect was the same for both it could not be attributed to cecropin production. Growth in selective media led to greater retention of the plasmid but at 80-90% compared with the 70% retention seen with growth in non-selective media suggested that the plasmid was intrinsically stable.

The results seen with pYWCE2, the plasmid containing the VTE as well as the wheat α amylase signal sequence and the cecropin gene, differed markedly. After growth in nonselective media the cells almost entirely lost the plasmid with only cells lacking the marker growing. Selective media only maintained a very small proportion of the cells with the marker plasmid. These results suggest that the addition of the VTE had some disadvantageous effect on the cells.

3.7 Summary and conclusions

Expression and secretion of a synthetic cecropin gene under the control of the CaMV 35S promoter had been studied in tobacco but though transcript was detected, no active cecropin was found. Constructs were therefore transferred to yeast under the control of a galactose inducible promoter to determine whether viable products could be obtained. The advantages of yeast were the rapidity with which transformants could be obtained and the fact that every

cell would have the potential to express and secrete cecropin.

Constructs containing the synthetic cecropin gene and wheat α -amylase secretion signal (pYWCE1) were transferred into yeast together with constructs containing an extra VTE element (pYWCE2) known to enhance translation *in vitro*. Yeast containing the vector only, pEMBLyex4, were used as non-cecropin producing controls. Following promoter induction, media was concentrated and assessed for the presence of active cecropin, achieved by looking for antibacterial activity with *E. coli* as the test organism. No antibacterial activity was found in either concentrated or unconcentrated media.

When induced yeast colonies were overlaid directly with a lawn of *E. coll* there appeared to be zones of inhibition of bacterial growth directly around the yeast colonies. However, subsequent work demonstrated that the zones were due to acidification of the media by the yeast leading to inhibition of bacterial growth.

Northern blots of RNA isolated from pEMBLyex4 and pYWCE1 following promoter induction showed that the *ura* plasmid marker rapidly decreased up to 2 hours following induction in pYWCE1. This decrease was shown to be due to a decrease in plasmid copy number since total cell number remained the same over the time course.

Addition of the VTE element to the cecropin construct appeared to have a deleterious effect on yeast. No high copy number transformants containing pYWCE2 could be isolated, neither could RINA in sufficient quantities for a northern blot be obtained due to the low density to which the cells grew in selective media. Also, when the transformed yeast were grown in nonselective media none of the cells retained the plasmid. Some deleterious effect of the cecropin gene construct was noted with pYWCE1 which suffered a rapid loss of excess plasmid copies upon promoter induction. These phenomena suggest that the possession of the synthetic cecropin gene, leading to synthesis of cecropin, may have exerted some toxic effect on yeast cells which was exaggerated by the presence of the VTE element. Though the pYWCE2 plasmid appeared unstable even when not induced, slight leakage of the promoter or derepression of the galactose inducible promoter due to exhaustion of glucose in the growth medium may have been enough, with the enhancing effects of the VTE element, to exert a toxic effect. it therefore appears that cecropin may have been produced by the yeast but lack of, or poor secretion, may have led to a cytotoxic effect. When yeast containing pYWCE1 were induced, this toxicity had the effect of inducing a rapid decrease in plasmid copy number, presumably with cells retaining only enough copies of the plasmid to survive on media lacking uracil.

Several possibilities exist for the lack of or poor secretion of cecropin (discussed further in chapter 7) but they all lead to the conclusion that the constructs used would have to be altered in some way to improve secretion and were not suitable in their present form. This conclusion may also apply to the situation in plants in which again no active cecropin product could be detected.

An important property of the construct for engineering resistance in plants to bacterial infections was the wound-inducibility of the secreted antibacterial substance. The isolation of a wound-inducible promoter was therefore important. A candidate gene showing the required expression profile, named DD1, was available and attempts to clone this gene are described in the following chapter.

CHAPTER 4

SCREENING AN ASPARAGUS cDNA LIBRARY FOR DD1

4.1 Introduction and background to previous work

Previous work at Leicester has concentrated on the use of an asparagus cell culture system as an enriched source of wound-induced genes (Harikrishna *et. al.*, 1991). The system involves generating wounded cells by grinding asparagus cladodes in a pestle and mortar and placing the isolated cells into tissue culture media. The study of the asparagus system was initiated originally to provide a large source of uniformly and synchronously wounded viable cells in order to yield information concerning the control of cell development, particularly in the process of redifferentiation, that occurs at wound sites (Paul *et. al.*, 1989). It was hoped that this information would give insights into the wounding process and also, since the plant response to wounding and pathogen invasion is very similar, into the defence response. This may then reveal approaches for the engineering of resistance.

Mechanically isolated asparagus cells have previously been used in studies of photosynthesis (Hills, 1986). Cell cultures can also be established and maintained (Jullien & Guern, 1979; Paul *et. al.*, 1989) enabling the study of long term changes within the cells.

Profiles of the proteins present before and in the succeeding days following mechanical cell isolation were obtained by running isolated protein samples on two-dimensional SDS-PAGE gels. These gels showed that while some proteins remained the same after the isolation process, some decreased in abundance while others increased and novel proteins also appeared. Two-dimensional PAGE analysis of protein translated *in vitro* using poly (A)⁺ RNA isolated from asparagus cells at varying times after the isolation process also showed major

qualitative differences in the message population, suggesting that the majority of the change in protein profile was controlled at the transcriptional level (Harikrishna *et. al.*, 1991).

The two-dimensional analysis of translated poly $(A)^+$ RNA enabled the identification of several novel proteins upregulated by the mechanical isolation/wounding process. Analysis of samples taken at 2 and 4 months following cell isolation showed that some induced genes remained upregulated in cell culture (Fioroni, 1989; Harikrishna, 1989).

it has previously been noted in section 1.12.3 that the proposed promoter to be used in the engineering of resistance by secreting an antibacterial substance in plants should be one that is rapidly activated by wounding and exhibits strong expression for several days to ensure continued resistance to pathogen invasion around wound sites. Study of the twodimensional gels of *in vitro* translated poly (A)⁺ RNA identified a candidate gene based initially on its product abundance. This protein was one of a group of novel proteins present at approximately 16 kD with differing isoelectric points that were rapidly activated by the wounding process, and activation sustained for long periods (Figure 4.1). One member of this group, named DD1, was activated within 3-6 hours and on two-dimensional gels of extracted protein a spot corresponding to that o f DD1 could still be detected in protein samples isolated from 6-month old cell cultures (Fioroni, 1989). The gene encoding DD1 was therefore identified as a potential target based on its expression profile for the eventual isolation of its promoter to be used in the engineering of plants for resistance against bacterial infections.

To aid in the cloning of DD1, protein corresponding to DD1 was isolated from two-dimensional SDS-PAGE gels and used to prepare an antibody and also to obtain N-terminal amino acid sequence data (Fioroni, 1989). At the time, the amino acid sequence obtained showed no homology to other known proteins but it did enable the design of a degenerate 17 nt oligomer which was used to screen an asparagus cDNA library. The library was constructed in a plasmid vector using poly (A)⁺ RNA isolated at day 3 following the cell isolation process. A clone, DD1-34 was isolated and western and northern analysis appeared to indicate that the protein and message were coordinately regulated following cell isolation (Fioroni, 1989).

Figure 4.1

Two-dimensional analysis of *in vitro* translated poly $(A)^+$ RNA isolated from asparagus cell cultures at varying times following single cell isolation. Detail of the DD1 group is shown in cladode and at 3 hours, 1 day, and 2 days following single cell isolation. The member of the group identified to be cloned is marked with an arrow.



However, subsequent sequence analysis of the DD1-34 clone by Simon Warner (of this department) showed that it contained no homology to the DD1 N-terminal protein sequence and was eventually found to contain part of a wound-induced gene termed AoPR1 (Warner *et. al.*, 1992).

The expression profile shown by DD1 still made it desirable to clone in order to obtain the promoter. The antibody could be used to screen an expression library for cross-reacting fusion proteins, while the possession of N-terminal amino acid sequence data for DD1 would enable the design of different oligomers for use in screening.

4.2 Assessing the DD1 antibody for library screening

The first approach made to clone DD1 was to use the antibody in library screening. A second asparagus cDNA library (Warner, 1992; see 2.8) had been constructed in the phage vector λ -ZAP which contains within it the Bluescript plasmid pSK⁻. Since the plasmid contains the lac-Z promoter it can be used as an inducible expression vector to produce proteins from recombinant DNA inserts, a requirement for antibody screening. The library was construced using poly (A)⁺ RNA isolated from 1-3 day old cultured asparagus cells. Since this time period corresponded with that for the expression of DD1, the library was expected to contain abundant DD1 clones.

An antibody should be capable of detecting less than 10 ng of protein (Stanley & Luzio, 1984) and preferably picogram amounts for antibody screening to be successful. Initial experiments therefore concentrated on determining the sensitivity of the DD1 antibody and its specificity in detecting a protein at approximately 16 kDa on a Western blot. Fioroni (1989) and Harikrishna (1989) calculated that from 2-D gel analysis of protein extracts from asparagus cultures the DD1 protein constituted up to 5% of total protein. This figure was used to determine the sensitivity of the antibody to DD1 since the amount of total protein was known.

Varying dilutions of protein extracted from three day old cell cultures, shown to contain a high

proportion of DD1 protein, were run on 15% denaturing protein gels and western blotted. Different combinations of secondary antibody conjugates and substrates were then used to maximise sensitivity in detecting bound primary antibody. The combinations of antibody conjugates and substrates used in each case is depicted in Figure 4.2. The DD1 antibody had been produced in rabbit so the secondary antibody was always goat/anti-rabbit antibody. In the first case the secondary antibody used was conjugated with alkaline phosphatase with the substrate being naphthol-AS-phosphate and Fast Red. This combination showed bands at 16 kDa present in lanes containing at least 1 μ g of DD1 protein (Figure 4.3a). it was therefore unsuitable for library screening but did show that the antibody was specific for a protein at 16 kDa with slight non-specific binding to large protein bands presumed to be those of large subunit rubisco.

The second antibody cocktail was the same as in the first case but the substrate used was BCIP and NBT. The result is shown in Figure 4.3b. It can be seen that this substrate imparts roughly 4 times more sensitivity to the antibody than using Fast Red with a band faintly visible in the lane containing only 250 ng of DD1. However, it does contribute far more background staining than the previous substrate with non-specific binding to large proteins being highly visible. The sensitivity was still not good enough for library screening. Figure 4.3c shows a western blot in which a bridging secondary antibody, goat/anti-rabbit conjugated with blotin, has been used. A tertiary compound, extravidin conjugated with alkaline phosphatase, was then added. Since two molecules of the tertiary compound bind to every biotin molecule it was expected that this combination would double the sensitivity. However, only 250 ng of DD1 protein could be detected as was the case without using the bridging secondary antibody.

Though the antibody was found not to be of a high enough sensitivity for antibody screening it was still used in several library screening attempts. An effort was made to increase the amount of fusion protein produced by inducing plaques for periods of up to 16 hours. However, as expected, no positive clones were obtained with any seemingly positive spots not corresponding to the position of plaques or were due to yeast contamination found on the plates.

Figure 4.2

Combinations of conjugates and substrates used in maximising the sensitivity of the DD1 antibody. The DD1 antibody was produced in rabbit.

a) Goat/anti-rabbit antibody conjugated to alkaline phosphatase used in conjunction with naphthol-AS-MX-phosphate and Fast red as substrates.

b) Goat/anti-rabbit antibody conjugated to alkaline phosphatase used in conjunction with BCIP and NBT as substrates.

c) Goat/anti-rabbit antibody conjugated to biotin used in conjunction with an extravidin/alkaline phosphatase conjugate with BCIP and NBT as substrates.









+ BCIP and NBT

Key:





Figure 4.3

Western blots of varying amounts of asparagus protein isolated 3 days following single cell isolation. The amout of DD1 protein was calculated based on 2-D gel analysis which showed the DD1 protein to constitute up to 5% of total protein in asparagus cultures. The combinations of antibody conjugates and substrates used are depicted in detail in Figure 4.2.

a) Alkaline phosphatase conjugate with naphthol-AS-MX-phosphate and Fast Red as substrates.

b) Alkaline phosphatase conjugate with BCIP and NBT as substrates.

c) Biotin conjugate used in conjunction with an extravidin/alkaling phosphatase conjugate with BCIP and NBT as substrates.



Antibody titre can be improved by affinity binding techniques in which the antibody that binds to a western blot is eluted from the filter and is effectively concentrated. However, it was felt that the initial antibody titre was so low that even using affinity binding the titre could not be improved sufficiently for library screening. The availability of the N-termInal amino acid sequence, however, offered an alternative approach to the cloning of DD1 by enabling the design of oligonucleotides to screen the library.

4.3 Screening the library using oligonucleotides

The use of synthetic oligonucleotides to screen the library was thought to be far more sensitive than using the antibody since even if the antibody had proved to be sensitive enough for use in library screening, it could still only detect clones that were expressing the DD1 protein. Only approximately one in six of the clones containing DD1 sequence would be in the right frame and correct orientation to allow translation of the protein. Use of an oligomer would enable all clones containing sequence homologous to the N-terminal sequence to be detected.

4.3.1 Screening using the 17 nt oligomer as probe

Fioroni (1989) had previously designed an oligomer to a region of the N-terminal sequence to incorporate the least degeneracy. The oligomer consisted of a length of 17 nt and had a 128 fold degeneracy (Figure 4.4a). The melting temperature (T_m) of the oligomer in its most A/T rich form was calculated to be 38°C by using the equation that an A/T base pair imparts 2° and a G/C pair 4° to the T_m (Itakura *et. al.*, 1984). During the library screening procedure a hybridisation temperature 10-15°C below that of the calculated T_m is used which would involve hybridising at 28°C with the 17 nt oligomer. Fioroni used the oligomer in an attempt to clone the gene for DD1, but the attempt was unsuccessful with the positive clone DD1-34 later found to have no homology to the N-terminal amino acid sequence (Warner, 1992). The failure was most likely due to the low hybridisation temperature used since this would encourage the detection of false positives containing only minimum homology to the

Figure 4.4

DD1 N-terminal amino acid sequence showing regions (boxed areas) to which the 17 and 26 nucleotide oligomers were designed.

a) Sequence of the 17 nucleotide oligomer. T_m was calculated to be 38°C. b) Sequence of the 26 nucleotide oligomer. T_m was calculated to be 76°C.

(See text for equations used to calculate ${\sf T}_{m}{\sf .})$

DD1 N-terminal sequence (28 amino acids)

N-VKAVAVVAGDVVIKGQVFF\$QEGDGPTTV -C 26 mer 17 mer

a) 17 nucleotide oligomer:

5'-AAAGGACAAGTATTTTT-3' G C G C C G G T T

128 fold degeneracy

b) 26 nucleotide oligomer

5'-GTIAAAGCIGTIGCIGTIGCIGG-3'

G

2 fold degeneracy

7 inosines
oligonucleotide.

It was therefore decided to use the 17 nt oligomer screen the library, but using a different approach with the chemical tetramethylammonium chloride (TMACI) added to the hybridisation buffer. In a solution containing TMACI the T_m of oligonucleotide duplexes becomes independent of nucleotide sequence and hence of GC content (Jacobs *et. al.*, 1988). Jacobs *et. al.* (1988) provide the following equation for calculating the T_m of oligonucleotides in a solution containing 3 M TMACI:

 $T_i = -682 \times (L^{-1}) + 97$ where $T_i = T_m$ in solution containing 3 M TMACI L = number of bases in the oligonucleotide

Applying this calculation to the 17 nt oligomer yields a T_i of 57°C, enabling a hybridisation temperature of 42-47°C to be used in library screening.

A total of 5,000 plaque forming units were plated out on a 140 mm Petri dish. Plaques were transferred to a Hybond-N filter and hybridised at 42°C with the labelled 17 nt oligomer as probe. The developed autoradiograph showed approximately 40 spots. *In vitro* translation studies (Fioroni, 1989) indicated that DD1 message constituted up to 1% of total message. A figure of 1% would give an expected 50 positive clones from the screening of 5,000 plaques. The positive plaques were removed and taken through a second round of screening. However, none of the plaques in the second screen hybridised with the probe. The library screening was attempted a second time but again no positives were obtained that passed through a second round of screening. The plaques lighting up on the primary screen must therefore have been false positives. Further screening using the 17 nt oligomer as a probe was not attempted.

The inability of the 17 nt oligomer to detect positive clones was deemed to be due to its 128fold degeneracy and its high A/T content. Though the hybridisation was carried out in a solution containing TMACI to counteract these problems, washes were performed using normal wash solutions at room temperature (approximately 20°C). Despite the low wash temperature it is possible that the binding of the 17 nt oligomer in a solution lacking TMACI was so weak that it was removed in the wash solution. It was therefore decided to design a new oligomer to the N-terminal DD1 sequence to maximise T_m in both the hybridisation step and in subsequent washes.

4.3.2 Screening using a 26 nt oligomer as probe

Degeneracy in the new oligo was minimised by incorporating deoxyinosine at positions where all four DNA bases were possibilities. Deoxyinosine binds equally to all four DNA bases (Ohtsuka *et. al.*, 1985) so some specificity is lost but it was hoped that by designing a longer oligonucleotide of 26 bases that this would impart the specificity required. Figure 4.4b shows the sequence of the new oligonucleotide and compares it with the 17 nt oligomer.

For oligonucleotides longer than 17 bases the following equation is used to determine T_m (Bolton & McCarthy, 1962; Sambrook *et. al.*, 1989):

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%G + C) - (600/N)$$

where N = chain length

If an oligonucleotide contains deoxyinosine, S is substituted for N, where S equals the total number of nucleotides minus the number of deoxyinosines. The %G + C also becomes that of S. Using this equation with a sodium ion concentration of 0.9 M (the concentration found In hybridisation solution) the T_m of the 26 nt oligomer is calculated to be 76°C. A T_m of 76°C would enable a maximum hybridisation temperature of 61-66°C to be used, facilitating far more stringent conditions to be used than with the 17 nt oligomer. Binding of the oligonucleotide was initially maximised by using a hybridisation temperature of 55°C with washes conducted up to 60°C.

An asparagus genomic Southern blot was probed with the labelled 26 nt oligomer to ensure that specific binding could be achieved at the proposed hybridisation temperature. A

genomic blot would also give some indication of the organisation of the DD1 gene within the genome. The resulting autoradiograph is presented in Figure 4.5 and shows that with a *Hin* d ill digest one strongly hybridising band Is present at approximately 1.5 kb with a very faint band also present at 3.9 kb. in the lane containing DNA digested with *Eco* FI, two hybridising bands are also present at approximately 1.8 kb and 3.7 kb. The presence of two bands suggests that there are two related genes. In the lane digested with *BgI* II only one hybridising band can easily be discerned at approximately 8.5 kb. If only one band is present this may indicate that two related genes are organised in tandem array. The fact that discrete bands hybridised in the genomic Southern suggests that the 26 nt oligomer is capable of binding specifically. However, the smearing present In the lanes suggested that some non-specific binding was occurring. Therefore, in the library screeing using the 26 nt oligomer, the hybridsation temperature was increased to 60° C from 55°C and washes were conducted up to 65° C in order to eliminate any non-specific binding.

A total of 3,500 plaque forming units were plated out on 100 mm square Petri dishes at a density of 350 plaques per plate. Duplicate plaque lifts were made and the filters probed with the labelled 26 nt oligomer. A total of 5 positive plaques were picked which contained matching signals on both duplicate filters. The clones were named pDD1-1 to pDD1-5.

4.3.3 Restriction analysis of positive clones

The Bluescript plasmid was rescued from the positive clones and amplified. *Eco* RI digests of the clones revealed the presence of either multiple inserts or the presence of Internal *Eco* RI sites (Figure 4.6a). The gel was Southern blotted and probed with the 26 nt oligomer to determine which *Eco* RI fragment contained homology to the oligomer (Figure 4.6b). From the Southern blot it can be seen that out of the five clones, pDD1-1 has not hybridised to the oligomer and must therefore have been a false positive. pDD1-2 and pDD1-3 have both hybridised to bands of approximately 1 kb, pDD1-4 to a band of 800 bp and pDD1-5 to a band of 500 bp. The hybridising *Eco* RI fragments were subcloned for further restriction analysis.

Sequencing of the N-termInal regions of each clone was needed in order to determine

Figure 4.5

Genomic Southern blot of Asparagus DNA probed with the labelled 26 nucleotide synthetic oligomer. Arrows show the presence of faint bands.

A total of 10 μ g of digested DNA was loaded per lane (lanes are labelled according to the restriction enzyme used) and the products run on a 0.8% agarose gel. The hybridised filter was exposed to film for 16 hours.



Figure 4.6

Analysis of positive clones identified by a primary screen of an Asparagus cDNA library with the 26 nucleotide oligomer designed to the DD1 N-terminal amino acid sequence.

a) Restriction digests of positive clones. A total of 1-2 μ g of DNA were digested with *Eco* RI and products run on a 1% agarose gel. The lane numbers 1-5 refer to the clones pDD1-1 to pDD1-5. The presence of multiple *Eco* RI inserts can be seen in lanes 2,3 and 4. The band at approximately 3 kb corresponds to the linearised Bluescript plasmid.

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b) A Southern blot of the gel shown in a). The blot was probed with the labelled 26 nucleotide oligomer to determine which of the *Eco* RI Inserts contained sequence homologous with the oligomer. The hybridised blot was exposed for 4 hours.



b)

4.0 3.0 _____ 2.0 _____ 1.6 _____ 1.0 -0.5 _____

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2 3 4 5

a)

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whether the clones were actually those coding for DD1. The amount of sequencing required was minimised by finding the smallest fragment from each clone that hybridised with the 26 nt oligomer and only sequencing this fragment. The clones were digested with suitable restriction enzymes (Figure 4.7a) and the products run on a gel which was then Southern blotted. The blot was probed with the 26 nt oligomer and gave the resulting autoradiograph shown in Figure 4.7b. Immediately apparent from the autoradiograph is that pDD1-2 and pDD1-3 gave the same pattern of hybridising bands suggesting that they are the same. pDD1-2 was therefore abandoned and further analysis concentrated on pDD1-3. Restriction maps of the remaining clones were drawn to determine which fragment contained the region hybridising with the 26 nt oligomer (Figure 4.8).

4.3.4 Sequencing of regions containing homology to the 26 nt oligomer

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b

The restriction fragments in the positive clones containing the regions of homology to the 26 nt oligomer were subcloned and sequenced. The regions giving the greatest match with the oligomer were found and compared using computer analysis. Sequence comparisons can be seen in Figure 4.9. Full sequences obtained from the DD1 clones showing regions of homology to the 26 nt oligomer can be found in the appendix at the end of this chapter.

From the sequence comparisons it is apparent that none of the DD1 clones had a 100% match with the 26 nt oligomer. The clone showing the greatest homology, pDD1-5, had a 100% match with the oligomer only over a continuous 18 nt length. None of the clones therefore contained sequence coding for DD1. Database searches with the sequences obtained from the clones showed that pDD1-3 and pDD1-4 had no significant homology to any recorded sequence full sequence data obtained from these clones can be found in the appendix). However, at the nucleotide level pDD1-5 showed 59% identity with a bean glycine-rich cell wall protein (Keller *et. al.*, 1988) and 58% identity with a petunia glycine rich protein (Condit & Meagher, 1986; Figure 4.10). Glycine rich proteins are involved in the strengthening of the cell wall and some have been shown to rapidly accumulate in response to wounding (see section 1.4).

Figure 4.7

Further analysis of positive clones pDD1-1 to pDD1-5 to determine the smallest restriction fragment in each clone that hybridised to the 26 nucleotide oligomer. The restriction enzymes used were those known to contain sites within the clones.

a) Restriction digests of the positive clones. 1-2 μg of DNA were used In each digest and products run on a 1% agarose gel. Lane 1: pDD1-2 digested with Eco RI. Lane 2: pDD1-2 digested with Bam HI. Lane 3: pDD1-2 digested with Sal I. Lane 4: pDD1-2 digested with Xho I. Lane 5: pDD1-2 digested with Eco RV. Lane 6: pDD1-2 digested with Sac I. Lane 7: pDD1-4 digested with Eco RI. Lane 8: pDD1-4 digested with Eco RV. Lane 9: pDD1-4 digested with Eco RV and Pst I. Lane 10: pDD1-3 digested with Eco RI. Lane 11: pDD1-3 digested with Bam HI. Lane 12: pDD1-3 digested with Sal I. Lane 13: pDD1-3 digested with Xho I. Lane 14: pDD1-3 digested with Eco RV. Lane 15: pDD1-3 digested with Sac I. Lane 16: pDD1-5 digested with Eco RI. Lane 17: pDD1-5 digested with Pst I. Lane 18: pDD1-5 digested with Eco RV and Pst I.

b) Southern blot of the gel shown in a). The gel was probed with the labelled 26 nucleotide oligomer and the hybridised filter exposed to film for 16 hours.

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Figure 4.8

Restriction maps of putative DD1 positive clones shown as inserts present in the pBluescript vector pSK⁻. The approximate size of the insert is shown as calculated by comparison with marker size DNAs run on agarose gels. Regions having homology with the DD1 26 nucleotide oligomer, as deduced from figure 4.7, are shown. Areas sequenced to confirm homology to the DD1 N-terminal amino acid sequence are shown by arrows.

Sequence comparisons with the putative positive clones and the 26 nucleotide oligomer are shown in figure 4.9 while the full sequence data obtained (number of base pairs as shown) can be found in the appendix to chapter 4.

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pDD1-4







KEY

Restriction enzymes:

scale:	100 bp		
	·	X = Xho I	B = Bam HI
UD	region showing homology	S = Sal I	Sc = Sac I
	with DD1 26 nt oligomer	RV = Eco RV	P = Pst I
_	areas sequenced	RI = Eco RI	

Figure 4.9

Sequence comparisons between putative DD1 positive clones and the 26 nucleotide oligomer designed to DD1 N-terminal amino acid sequence. Regions of the positive clones showing homology to the 26 nucleotide oligomer were sequenced and computer searches performed to find sequences showing greatest homology with oligomer sequence. Results of this search are shown. : indicates a match either with the same base pair or with inosine.

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a) Comparison of 26 oligomer sequence with pDD1-3.

b) Comparison of 26 oligomer sequence with pDD1-4.

c) Comparison of 26 oligomer sequence with pDD1-5.

c) pDD1-5

b) pDD1-4

GTGGAGGCGGCGGCGGAGCTGGTGGT ::: :::::: ::::: : ::::: oligo GTIAAGGCIGTIGCIGTIGCIGG A

a) pDD1-3

Figure 4.10

Nucleotide homologies between a) pDD1-5 and bean glycine-rich cell wall protein (Keller, *et. al.*, 1988), and b) pDD1-5 and petunia glycine-rich protein (Condit & Meagher, 1986).

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a) pDD1-5 homology with bean glycine-rich cell wall protein: 59.2% identity in 223 bp overlap.

 ${\tt GTGGTGGCACTGCTGGCGGTAGCGGTGGTTGGGCTGG--ACCGTGGTGGTTCTGGAGGAG$ ******** * ******* * * ** * * * *** *** *** ::

b) pDD1-5 homology with petunia glycine-rich protein: 58.1% identity in 253 bp overlap.

--GACCGTGGTGGTTCTGGAGGAGG-TCGAGATGAGTCTGAGTCTGGCTTTGGAGGCGCT GTGTAGGTGGTGGTGTTGGAGGAGGAGGAGCTGCAGGAGGAGGTG-GTGGAGGAGGAGGAGGCGGT

 ${\tt GGGTGGGTTGGTGGTGGTGGTGGTGGTGGTGGTTGTTAAGGGAC---CGGTTGCTCAGGCTGA}$:: : : :: :::::: ::: :: :::: GGTGGCGGAGGAGGTGGTGGTGGTCTTGGCGGAGGATCAGGACATGGTGGTGGATTTGG-TGC 143 -

AGGGGGT--AATGGGTGGTCCGGAGGCGGCGGCAGTGGTTGGTAACAGCGATTAGTAGCG : : : : AGGTGGTGGAGTTGGAGGTGGAGCTGCTGGAGGAGTAGGAGGTGGAGGAGGAGGATTCGGAGG

0 290

TCTGCAGCCCGGG

4.4 An identity for the DD1 protein

Although previous database searches did not reveal any matches with the DD1 N-terminal amino acid sequence data, a further database search at this juncture showed the sequence to have 82% identity at the amino acid level with maize superoxide dismutase 2 (SOD2; Cannon *et. al.*, 1987).

4.5 Conclusions

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Attempts were made to clone the gene corresponding to the protein referred to as DD1. Initially the antibody to DD1 was assessed to determine if it was sensitive enough for library screening. By altering the combinations of secondary antibodies and substrates used the sensitivity of the antibody was enhanced but even at its most sensitive was only capable of detecting a minimum of 250 ng of DD1 protein. Library screening attempts with the antibody were therefore unsuccessful.

Further library screening attempts concentrated on the use of oligonucleotides designed to the DD1 N-terminal sequence. The first oligonucleotide used was 17 bases in length and had been designed by Fioroni (1989). Despite incorporating TMACI into the hybridisation buffer when probing with this oligonucleotide, no positive clones were detected. This was considered to be due to a combination of its high degeneracy, low T_m , and possibly the use of washes that were too stringent.

A second oligonucleotide was designed in an attempt to increase T_m . Deoxyinosines were used to minimise the degeneracy of the oligonucleotide while the length was of 26 bases to improve specificity. Library screening with the new oligonucleotide appeared to give positive clones but sequencing of regions binding to the 26 nt oligomer showed that the clones were all false positives. The oligonucleotide appears to have bound to regions containing a high GC content and in this respect it is interesting that sequence data from one of the clones analysed, pDD1-5, showed high homologies to two glycine-rich proteins which have high GC

contents.

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Genomic Southern analysis with the 26 nt oligomer as probe indicated that there may be two related genes present in the asparagus genome. The analysis also showed that the related genes were possibly arranged in tandem.

A homology of 82% with maize superoxide dismutase 2 (Cannon *et. al.*, 1987), identified the DD1 N-terminal sequence as being that belonging to an asparagus superoxide dismutase. This identification provided more targeted approaches to the cloning of this gene than continuing further with the 26 nt oligomer, and the successful cloning of the asparagus SOD is detailed in the succeeding chapter.

Appendix

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1) Sequence from pDD1-3 containing region of homology to 26 nt oligomer designed to DD1 Nterminal protein sequence. The sequence is 5'-3' with respect to Figure 4.8. The dashed lines (---) show the region of homology to the 26 nt oligomer.

CCCTCCGCC	ATCAATTO	CCCGCCACCA	CCAGCTCCGC	CGCCGCCTCC	ACCGCCGACG	ATGA
1	0	20	30	40	50	60
CCTCTACTC	CGTCCTC	AAATCCCTCC	AGCGCGAGAT	CGAGTTCATC	GACATCCAGG	AGGA
7	0	80	90	100	110	120
GTACGTCAA	GGACGAG	CTCAAGAACC	rcaagcgcga	CGATCTCGCGG	CCCAGGAGGAG	GGTC
13	0	140	150	160	170	180
	Bam	HI site				
AAGCGGGAT	CCAGTCG	GTTCCTCTCG	rcatcggcca	GTTCATGGAG	ATGGTCGATC	AGAA
19	0	200	210	220	230	240
CAACGGAAT	CTCG					
25	0					
2) Sequence f	rom pDD1-	4. The sequen	ce is 5'-3' with r	espect to Figure	e 4.8. The dash	ned lines

show the region of homology to the 26 nt oligomer.

CTTTTCACAGA	AGTAGCGTCCA	GCAGTATGT	GGAGATATGT	CGCATGCTTC	ГСАА
10	20	30	40	50 [.]	60
CAGCTATATAC	GTCAACCAGTO	GACATATCAC'	TTACATTAAC	ATCATCGAAA	GTCC
70	80	90	100	110	120
TGAAGAGCTTA	ACGTCGAGT	GCCGTCGTCT	CAACTGCCGC	CGCCGCCGCC	ACCG
130	140	150	160	170	180
			Eco RI		
GAGATGAGAG	ATCTGAGCGGC	CCGCGAATTC	site		
190	200	210			
	CTTTTCACAGA 10 CAGCTATATAC 70 TGAAGAGCTTA 130 GAGATGAGAGA 190	CTTTTCACAGAGTAGCGTCCA 10 20 CAGCTATATAGTCAACCAGTC 70 80 TGAAGAGCTTAACGTCGAGTC 130 140 GAGATGAGAGATCTGAGCGGC 190 200	CTTTTCACAGAGTAGCGTCCAGCAGTATGT 10 20 30 CAGCTATATAGTCAACCAGTGACATATCAC 70 80 90 TGAAGAGCTTAACGTCGAGTGCCGTCGTCT 130 140 150 GAGATGAGAGATCTGAGCGGCCGCGAATTC 190 200 210	CTTTTCACAGAGTAGCGTCCAGCAGTATGTGGAGATATGTG 10 20 30 40 CAGCTATATAGTCAACCAGTGACATATCACTTACATTAACA 70 80 90 100 TGAAGAGCTTAACGTCGAGTGCCGTCGTCTCAACTGCCGCG 130 140 150 160 Eco RI GAGATGAGAGATCTGAGCGGCCGCGAATTC site 190 200 210	CTTTTCACAGAGTAGCGTCCAGCAGTATGTGGAGATATGTCGCATGCTTC 10 20 30 40 50 CAGCTATATAGTCAACCAGTGACATATCACTTACATTAACATCATCGAAAA 70 80 90 100 110 TGAAGAGCTTAACGTCGAGTGCCGTCGTCTCAACTGCCGCCGCCGCCGCCG 130 140 150 160 170 Eco RI GAGATGAGAGATCTGAGCGGCCGCCGCAATTC site 190 200 210

3) Sequence from pDD1-5. The sequence is 5'-3' with respect to figure 4.8. The dashed lines show the region of homology to the 26 nt oligomer.

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ACGGTA	TCGATAAGCT	GATATCGAAT	IGCCGGCCGC	GTGGCAGTGG	TGGCTG	GGGT	GAG
	10	20	30	40	50		60
GTGGTT	CTGGAGGTGG	TGGCACTGCT	GGCGGTAGCG	GTGGTTGGGC	TGGACG	rggto	GTT
	70	80	90	100	110		120
CTGGAG	GAGGTCGAGA	TGAGTCTGAG	TCTGGCTTTG	GAGGCGCTGG	GTGGGT	rggto	GGTG
	130	140	150	160	170		180
GTGGTG	GTGGTGGTTC	TAAGGGACCG	GTTGCTCAGG	CTGAAGGGGG	TAATGG	GTGGI	rccg
	190	200	210	220	230		240
						Pst	I
GAGGCG	GCGGCAGTGG	TTGGTAACAG	CGATTAGTAG	CGTCTGCAGC	CCGGG	site	Э
	250	260	270	280	290		

CHAPTER 5

CLONING OF ASPARAGUS SUPEROXIDE DISMUTASE (SOD) AND ANALYSIS OF EXPRESSION

5.1 Introduction

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SODs are developmentally regulated and respond to various environmental stresses including pathogen attack (Bowler *et. al.*, 1989; Williamson & Scandalios, 1992), light (Tsang *et. al.*, 1991), and wounding (Perl-Treves & Galun, 1991). The upregulation of a cytosolic Cu/Zn-SOD by wounding in tomato (Perl-Treves & Galun, 1991) may indicate that the asparagus SOD corresponding to the DD1 N-terminal sequence is wound-inducible. It appears that any disturbance in the normal balance between the amount of superoxide anions formed within cells and those removed by SODs and non-enzymic antioxidants (see section 1.7.2) leads to induction of SOD activity (reviewed by Bowler *et. al.*, 1992).

The stimulation of cultured plant cells with elicitors of the defence response leads to a rapid production of hydrogen peroxide (Apostol *et. al.*, 1989; Legendre *et. al.*, 1993), one of the products of the reaction of superoxide anions with SOD. Cultured plant cells are therefore capable of exhibiting some of the same responses as intact plants to similar stresses. It is therefore possible that the upregulation of the asparagus SOD in cell culture may mirror a response to wounding in an intact plant.

MnSOD has been shown to be highly abundant in tobacco cell suspension cultures (Bauw *et. al.*, 1987) but in this instance the high abundance was thought to be due to high levels of respiration caused by the presence of sugar in the culture medium (Bowler, *et. al.*, 1989).

The activities of enzymes investigated in a cell culture system must therefore be studied in whole plants before statements can be made concerning their upregulation in response to various stresses.

The discovery that the N-terminal sequence of the DD1 protein had 82% identity at the amino acid level with maize SOD2 (Figure 5.1), a cytosolic Cu/Zn SOD (Cannon *et. al.*, 1987), provided more targeted approaches to the cloning of the gene for DD1. Cu/Zn SODs share a very high degree of homology at the amino acid level and this information has been used by many researchers to design synthetic oligonucleotides for use as probes in library screening and has led to the successful isolation of genes for several SODs (Cannon *et. al.*, 1987; Perl-Treves *et. al.*, 1988; White & Zilinskas, 1991). Primers have also been designed for use in PCR to aid in the cloning of a bovine Cu/Zn SOD (Gibbs & Shaffer, 1990) and this approach was adopted initially to clone DD1.

Once the gene for DD1 was obtained it could be used as a probe in northern analysis to study gene expression in response to wounding. Expression as a consequence of the cell culture system could also be studied to assess how expression correlated with the protein identified as DD1 on the two-dimensional gel analysis of *in vitro* translated poly $(A)^+$ RNA isolated from cell cultures (section 4.1, Figure 4.1). Knowing that the DD1 protein apparently corresponded to the SOD enzyme also stimulated the study of SOD activity in cell cultures and in wounded seedling tissue.

5.2 Study of SOD activity in cell culture and in wounded tissue

SOD activity was studied using *in situ* gel activity assays which enables the study of changes in the activity of individual SODs (Beauchamp & Fridovich, 1971). Following separation of protein in a native gel by polyacrylamide gel electrophoresis, the gel is immersed in a buffer containing riboflavin and nitro-blue tetrazolium (NBT) and incubated in the dark for up to one hour. Subsequent exposure to light causes photochemical reduction of the riboflavin, generating superoxide radicals which reduce the NBT to produce blue formazan. The gels

Figure 5.1

Diagram showing how the maize SOD2 amino acid sequence (Cannon *et. al.*, 1987) compares with the DD1 N-terminal amino acid sequence.

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COMPARISON OF MAIZE SOD2 AND DD1 N-TERMINAL AMINO ACID SEQUENCE

maize SOD2	>	м	∢	>	۲	>	ц. •	₫	77	-	6.	×. ••	· · ·	E	н・	Гч ••	Гч ••	v	α	띠	ლ	0	۲ŋ ۰۰	0	E		×
DD1 N-terminal amino acid sequence	\geq	Х	A	\triangleright	۲	>	>	₫.	ריז ניז	0	~	к Ж	0	0	\triangleright	Гц	Гц.	S	Ø	ы	U	0	(1)	с- 0.	н 5	r.	~

: identical amino acids . conserved amino acids stain uniformly blue except at positions containing SOD where the superoxide radicals are scavenged. Bands of white SOD activity are therefore clearly visible against a blue background.

A gel showing SOD activity in cladodes and in successive days in cell culture is shown in Figure 5.2a. It can be seen that in cladode 6 activity bands are visible with 4 bands (SOD-1, 4, 5 and 6) showing strong activity and 2 bands showing low activity (SOD-2 and 3). SOD-1 increases slightly in activity following single cell isolation, while SOD-2, 3, and 4 increase significantly in activity. SOD-5 and 6 are seen to decrease in activity with the activity of SOD-5 almost disappearing entirely by day 8 in culture. After 6/8 weeks in culture the activity assay pattern resembles that seen in day 8. Using the cyanide sensitivity test (see section 1.7.3), SOD-1 was determined to be a Mn-containing SOD. No evidence was found for an Fecontaining SOD. The other 5 SODs are therefore Cu/Zn-containing SODs though no differentiation has yet been made between chloroplastic and cytosolic SODs.

To see if the changes observed in SOD activity levels could be due to wounding stresses acting on the system, an *in gel* assay was performed using protein from wounded seedling tissue. Dark grown, etiolated seedlings were sliced into 5 mm segments and left on moist filter paper for a varying number of days in the dark. Protein was isolated and assayed with the resulting gel shown in figure 5.2b. Immediately apparent is the fact that the SOD activity profile in dark grown seedlings differs from that of cladode and very closely resembles that of day 8 cell culture protein. Wounding does not appear to change the activity of bands present in the unwounded seedling but gives rise to extra bands of SOD activity. These extra bands (SOD-4a and 4b) could be wound-induced SODs present in asparagus though it can not be discounted that the extra bands are a result of infection since it was very difficult to keep the seedlings sterile in the procedure used. However, the pattern observed is very much that expected of an induced enzyme with SOD-4a appearing day 1 following wounding and increasing in activity to day 4, and SOD-4b appearing from day 3. SOD activity in root protein corresponds with that seen in unwounded etiolated seedling tissue.

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Figure 5.2

In situ gel assays of SOD activity in asparagus.

a) SOD activity in cladode tissue and in cell culture. D1 to D8 refer to the number of days in cell culture following the single cell isolation procedure. Activity was also observed in six week old cell cultures and this lane also shows how the asparagus SODs were named, as referred to in chapter 5.

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b) SOD activity in chopped up seedlings and root tissue. Following germination and growth for two weeks in the dark, asparagus seedlings were removed, chopped into 5 mm sections to simulate wounding, and placed in the dark on moist filter paper in Petri dishes for varying numbers of days prior to isolating protein. D0 to D4 refer to the number of days following the wounding stimulus.

Root protein was obtained from the roots of six week old asparagus seedlings grown in the light.

The Mn-SOD lane shows the effect of adding cyanide to the buffer solution used in staining, to which only SOD containing Mn as a metal cofactor is resistant.

All activity assays shown in this figure were conducted using 20 µg of protein loaded per lane.







a)

5.3 Cloning of asparagus SOD (DD1)

5.3.1 PCR to obtain asparagus SOD probe

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The high homology that exists between SODs isolated from different plants prompted the use of PCR as a technique to obtain a fragment of DNA homologous to the asparagus SOD that could then be used to screen a cDNA Library and obtain a fuil-length gene. All plant cytosolic Cu/Zn SODs so far sequenced contain a highly conserved region of 9 amino acids present at the C-termInal end (White & Zilinskas, 1991). A 28 nt oligomer was therefore designed to this region to provide a PCR primer. The greatest variation in the amino acid sequence of different SODs occurs at the N-terminal end (Kanematsu & Asada, 1990). However, since N-terminal sequence data was already available for the asparagus SOD, this presented no problem and the 26 nt oligomer designed for use in library screening (section 4.3.2, Figure 4.4b) was used as the second PCR primer. Both oligomers to be used in PCR were determined to have similar Tms of 76°C and 80°C. Figure 5.3 shows the design of the oligonucleotides used in PCR and a description of the methodology behind the cloning of the asparagus SOD.

Conditions for the PCR reaction were optimised by using the maize SOD2 gene (Cannon *et. al.*, 1987) in the plasmid pSOD2.1c (obtained from John Scandalios, North Carolina State University, USA) as a template control, since analysis of the maize clone revealed that the oligonucleotides designed for the cloning of the asparagus SOD could also be used to amplify, by PCR, the maize gene. This enabled the maximum annealing temperature to be determined allowing amplification of the SOD gene while eliminating nonspecific amplification. It was determined that an annealing temperature of 70°C could be used and still give the expected band size of 450 bp.

Reactions were set up containing both synthetic oligomers and an aliquot of the asparagus cDNA library constructed by Simon Warner (1992) in the vector λ -ZAP, containing 500,000 plaque forming units. A control tube containing wild type λ -ZAP was also set up so that any PCR bands formed as a result of homology between the oligonucleotides and λ -ZAP could be eliminated. Following 30 cycles of amplification, PCR products were extracted and run on

Figure 5.3

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Figure showing the design of primers used in amplifying the Asparagus SOD from a cDNA library via PCR technology. A description of the methodology used to obtain 5' and 3' untranslated regions is also shown.

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CLONING OF ASPARAGUS SOD

Primers designed for PCR:

1) known N-terminal sequence

N	-	v	к	А	v	А	v	v	А	G	-	С	sense oligo
.		0.00				FOO		TOR	T 00	таа		.	26mer, 7 inosines,
э.	-	GTI	LAAA (AGC. 3	LGT.	LGC.	LG.L.	IGT	TGC	TGG	-	3.	2-tola degenerate

2) highly conserved sequence at C-terminal end of all plant Cu/Zn SODs

N - C G I I G L Q G - C antisense oligo 28mer, 2inosines, 3'- ACACCATAATAACCIAAIGTTCCATGGC - 5' 8-fold degenerate G G G C - -----

Kpn I site

Primers used to PCR SOD from cDNA library in lambda ZAP. Library constructed from poly (A)+ RNA isolated from 1-3 days following single cell isolation.

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PCR fragment obtained confirmed to be SOD by strong hybridisation to maize SOD2.

Fragment used to rescreen library to obtain clones containing 5' and 3' regulatory regions. an agarose gel (Figure 5.4a). Though extremely faint, a product band of approximately 450 bp was visible from the reaction with the asparagus library. The size corresponded with that obtained with PCR from the maize clone indicating a high probability that the 450 bp band seen was the required product.

To test whether the PCR product was homologous to the maize SOD, and hence confirm its identity, a duplicate gel was run, Southern blotted and the filter hybridised with the maize SOD2 gene as probe. The result is shown in Figure 5.4b. The asparagus PCR product clearly hybridised to the maize SOD, providing further evidence that the PCR product obtained was the correct one. The Southern blot also appears to indicate the presence of more than one hybridising band suggesting that the PCR reaction has given rise to more than one band with homology to SOD. This is not surprising considering the high degree of homology between SODs it is possible that more than one SOD can be obtained with the same primer combination.

The 450 bp PCR fragment from the asparagus library was removed from the original gel and purified using "Gene Clean" (see 2.9.2). The product was used as template in a further PCR reaction to amplify the amount of DNA. The resulting product was then run on a high percentage agarose gel (2.5%), to see if more than one band could be resolved, a possibility indicated by the Southern blot. The resulting gel can be seen in Figure 5.4c in which up to 4 bands are visible. The band most closely corresponding with the maize SOD band was removed from the gel and purified and attempts were made to clone the fragment in preparation for sequencing. However, despite incorporating a Kpn I site into the 28 nt oligomer design, the 450 bp product did not prove possible to clone by conventional methods. This is possibly due to the loss of or incorporation of incorrect nucleotides at positions which contribute to the Kpn I site. It was therefore decided to use the PCR product as a probe to screen the asparagus library. This has two distinct advantages over cloning the PCR product directly: 1) ciones obtained from the ilbrary would contain 5' and 3' untranslated regions missing from the PCR product, and 2) mistakes in DNA sequence introduced by PCR (Keohavong & Thilly, 1989) would not be present.

Figure 5.4

a) Agarose gel showing results obtained from the PCR of an asparagus cDNA library, present in the vector λ -ZAP, using primers specific for SOD. The PCR reaction consisted of 30 cycles of 1 min denaturing at 95°C, 1 min annealing at 70°C, and 1 min extension at 72°C. The template DNA used was a 1 µl aliquot of the asparagus cDNA library containing approximately 500,000 plaque forming units.

lane M - 1kb ladder as marker DNA

lane1 - PCR with the maize SOD2 gene as template

lane 2 - asparagus cDNA library as template in which a faint band at approximately 450 bp can be seen

lane 3 - wild type λ -ZAP (ie, containing no insert DNA) as template

b) Southern blot of the gel described in a). The maize SOD2 gene was used as a probe with hybridisation carried out at 50°C. The filter was exposed to film for 2 hours.

c) The 450 bp product visible in lane 2 of a) was removed and used as template in a further PCR reaction with the resulting products run on a 2% agarose gel to aid differentiation of bands.

lane M - 1 kb ladder as DNA marker

lane 1 - PCR with the maize SOD2 gene as template

lane 2 - 450 bp product as template in which up to 4 product bands are visible

In all gels showing PCR products, the reaction was set up in a total volume of 50 μ l. Following extraction of the DNA products with chloroform, 5 μ l was then run on the gel.

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5.3.2 Library screening with PCR product as probe

A total of 20,000 plaque forming units were screened using plates of 140 mm diameter with a density of 4,000 plaque forming units per plate. Duplicate filters were hybridised using the PCR 450 bp fragment as a probe, and from the initial screen 10 hybridising plaques were identified.

Positive plaques were confirmed prior to further rounds of purification by using PCR with commercial primers available to the Bluescript plasmid to remove the inserts from the λ -ZAP phage vector. Products were run on an agarose gel, Southern blotted and probed initially with the 450 bp PCR product. The filter was then stripped and reprobed with the maize SOD gene to confirm that any positives were also homologous to the maize SOD. Of the 10 initial positives picked, only 4 hybridised a second time with the 450 bp probe (Figure 5.5a) and out of these only 3 also hybridised with the maize SOD2 gene probe (Figure 5.5b). Of these 3 potential positives, number 6 gave the strongest hybridising signal and therefore seemed the likeliest candidate for the asparagus SOD. The three clones were taken through two further rounds of screening to obtain plaque pure samples (Figure 5.6). Clone 7 did not hybridise in further rounds, while the recombinant plasmids in numbers 4 and 6 were rescued and retained for further analysis. The positive plasmids were named pSOD4 and pSOD6.

5.4 Subcloning and sequencing of pSOD6

Restriction analysis of pSOD6 revealed the presence of an internal *Eco* RI site which was utilised for subcloning. A restriction map of the clone together with subcloning and sequencing strategy is shown in Figure 5.7. Sequencing was carried out using the dideoxy chain termination method (Sanger *et. al.*, 1977) with the result presented in Figure 5.8. The clone pSOD6 was found to contain a total of 719 bases. Computational analysis of the sequence revealed a predicted open reading frame of 151 amino acids with the translation start site ATG codon at base 67 and the stop codon TGA at base 520 (with reference to Figure 5.8). There are 66 bases of 5' untranslated region and 196 bases of 3' untranslated region. No poly A tail is present suggesting that the 3' region of the clone is incomplete. The open

Figure 5.5

Results obtained from a primary screen of the Asparagus cDNA library using initial PCR 450 bp product as probe. Plaques picked from this screen were used in a PCR reaction with commercially available primers to the vector to enable rapid identification of positives prior to plaque purification. Products of the PCR reaction were run on an agarose gel and then Southern blotted.

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a) Southern blot probed with the PCR 450 bp product. Lanes marked M contained 1 kb ladder as marker DNA. Lanes 1-10 refer to potential positives picked from the primary screen. Lanes 4,6,7 and 8 were seen to give positive signals. The filter was exposed to film for 3 hours.

b) Southern blot from above stripped and probed with the maize SOD2 gene. Only ianes 4,6 and 7 have given positive signals. The filter was exposed for 3 hours.




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Autoradiograph of a secondary library screen to obtain plaque pure samples. Numbers refer to the clone number assigned from the primary screen. The filters were probed with the 450 bp PCR product and left exposed to film for 16 hours.

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Restriction map of pSOD6 showing subcloning and sequencing strategy.

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Pst I/Bam HI/Xba I Eco RI/Pst I Direction of sequencing è Eco RI 330 bp fragment cloned into pSK-1 1 ----1 V Eco RI/Pst I with Eco RI Eco RI Hind III/Eco RI digested pSOD6 ----1 SOD coding region N-C 1 -390 bp fragment cloned into pSK-Eco RI Hind III/Eco RI Xho I/Hind III/Eco RV ŝ lambda zap - present in Bluescript pSK-Clone rescued from

Nucleotide sequence of *Asparagus officinalis* SOD, pSOD6, showing deduced amino acid sequence. Lines above the amino acid sequence show regions homologous to the primers used in PCR. The sequence shown is taken from the 720 bp *Eco* RI insert with reference to Figure 5.7 and therefore starts and finishes with an *Eco* RI site.

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G L H G F H V H A L G D T T N G C M S T GGGCTTCATGGTTTCCATGTTCATGCTCTTGGTGACACCACAAATGGGTGCATGTCCACT G P H F N P A G K E H G A P E D E N R H GGACCTCATTTCAATCCTGCTGGAAAGGAACATGGGGCACCTGAAGATGAGAATCGCCAT G D L G N V T A A E D G T A T F S I T А GCTGGTGACCTTGGAAATGTGACTGCTGCTGAGGATGGGACCGCTACTTTCAGTATTACT D S Q I P L T G P N S I I G R A V V V H GACAGTCAGATTCCACTCACTGGGCCGAATTCCATTATTGGAAGAGCTGTTGTTGTCCAT A D P D L G K G G H E L S K S T G N A GCTGACCCTGATGATCTTGGAAAGGGTGGACACGAGCTTAGCAAAAGCACTGGAAATGCT G G R V G C G I I G L Q A * GGCGGAAGAGTTGGCTGTGGGTATCATTGGACTTCÄGGCTTGAGCAATTCAAAACTTTGTT GTTGGCACTCGGAGTTGAATCGTGACGCGAGGATGATACTATTATGTATCACAAACACAT GCTTCTTTGATGTTTAGTGCTATGCGGAATAAGAAAACTTGTCGTGATATGCTGAATAAG

GAATTCGCGGCCGCTAGAAACCTCTCTCTCTCTCTCTCCCAAGGGGTCGCCTGAGATCACA

M V K A V A V V A G D V V K G H V F TAAAAAATGGTGAAGGCTGTTGCTGTAGTTGCTGGTGATGTCGTGAAGGGCCATGTTTTC

Р

L K

O E G D G P T T V T G S V S G

TTCAGCCAAGAGGGAGATGGCCCAACTACAGTAACCGGAAGTGTCTCTGGTCTGAAACCC

ECO RI

F

S

Eco RI

reading frame encodes a full length SOD sequence which has a predicted molecular mass of 15 kDa and an isoelectric point of 6.0, corresponding closely with DD1 which, on 2-D gel analysis of *in vitro* translated poly (A)⁺ RNA, had an approximate mass of 14 kDa and an isoelectric point of 5.6. Study of the pSOD6 translated amino acid sequence showed 100% homology with the N-terminal sequence of the DD1 protein and also 100% homology with the primers used in PCR (Figure 5.9).

An EMBL database search revealed that the open reading frame contained within pSOD6 had high homoiogy with many plant Cu/Zn SODs (Figure 5.10). At the protein level a higher homology with the cytosolic form (80-88%) was found than with the chloroplastic form (66%). Also, no evidence was found for a transit peptide that would direct import into the chloroplasts suggesting that pSOD6 encodes the gene for an asparagus cytosolic Cu/Zn-SOD.

5.5 Analysis of pSOD4

A second cione, besides pSOD6, identified as a putative positive for the gene for DD1 was pSOD4. Restriction mapping of pSOD4 indicated the presence of an internal *Eco* RI site. Since an internal *Eco* Ri site was also found in pSOD6 this suggested a relationship between the two clones with the difference being that pSOD6 was 720 bp in length while pSOD4 was approximately 740 bp. Approximately 250 bp of sequence data were obtained for the 5' and 150 bp for the 3' regions of pSOD4 so that a direct comparison with pSOD6 could be made. Sequence from the 5' region of pSOD4 had a 100% match with sequence from the 5' region of pSOD6 (Figure 5.11a), with base 8 of pSOD4 matching with base 102 onwards of pSOD6. The 5' untransiated region present in pSOD6 is absent from pSOD4 which aiso iacks the first 11 amino acids of the N-terminai sequence, but does contain a 100% match with the other 17 amino acids of the DD1 sequence (Figure 5.11b). The sequence of the 3' region obtained for pSOD4 was expected to contain approximately 30-50 base pairs of overiap with pSOD6 sequence. However, no match was found between the 150 bp of pSOD4 3' region of the exact length of the pSOD4 clone but could also indicate the presence of a second

Diagram showing how the sequence of pSOD6 compares with a) the DD1 N-terminal amino acid sequence, b) the 26 nt oligomer designed to the DD1 amino acid sequence, and c) the 28 nt oligomer designed to a highly conserved region present at the C-terminal end of plant cytosolic Cu/Zn SODs (White & Zilinskas, 1991).

: represents an exact nucleotide match

. shows a match with deoxyinosine

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a)

pSOD6	VKAVAVVAGDVVKGHVFFSQEGDGPTTV
DD1	VKAVAVVAGDVVKGHVFFSQEGDGPTTV

b)

pSOD6	5'-GTGAAGGCTGTTGCTGTAGTTGCTGG-3'

sense	5'-GTIAAGGCIGTIGCIGTIGTIGCIGG-3'
oligo	А

c)

pSOD6	3'-ACACCATAGTAACCTGAAGTCCGA-5'				
	:::::	:::::	: : : . : : .	::::	:
antisense	3'-ACACCAT	AGTAA	CCIGAIC	TCCC	ATGGC-5
oligo	G	А	А	т	
				Kpn	I site

Comparison of the deduced amino acid equence of the Asparagus SOD, pSOD6, with the amino acid sequences of cytosolic Cu/Zn SODs from maize SOD2 (Cannon *et. al.*, 1987), maize SOD4 (Cannon & Scandalios, 1989), pea (White & Zilinskas, 1991), cabbage (Steffens *et. al.*, 1986), tomato (Perl-Treves *et. al.*, 1988), and tobacco (Tsang *et. al.*, 1991). Conserved residues are enclosed in boxes. Residues involved in the formation of ligands to Cu are marked * while the aspartic acid residue that forms a ligand to Zn is marked ^.

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Comparisons of the sequence of pSOD4 with pSOD6 and with the DD1 N-terminal amino acid sequence.

a) Alignment of the nucleotide sequence of the 5' region of pSOD4 with homologous sequence from pSOD6. The full nucleotide sequence of pSOD6, to which the base numbers refer, can be found in Figure 5.8.

b) Alignment of the deduced amino acid sequence from the nucleotide sequence of pSOD4 shown in a) above with the N-terminal amino acid sequence from the DD1 protein.

c) Sequence from pSOD4 3' region that contained no homology to pSOD6. The figure is 5' to 3' with respect to Figure 5.15.

The line above a region of pSOD4 nucleotide sequence in a) corresponds to the region showing homology to the DD1 N-terminal amino acid sequence as seen in b).

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TATCGI	TCAACTGC	TTGTTAA	CTGATTTTTCAT	CTGCATGAGAT	CCTGGAGATA	CATTGA
	10	20	30	40	50	60
TGTCAA	CCTTGTAA	TGTGGAG	GGAAGCATCGCA	FTAGCTTAACT	CTCAAGCACA	AGCCAA
	70	80	90	100	110	120
			ECO RI			
TAACTGGCGGCCGCGAATTC		site				
	130	140				

C)

pSOD4	ISVKGHVFFSQEGDGPTTV
DD1	VKAVAVVAGDVVKGHVFFSOEGDGPTTV

b)

GGGCACCTGAAGATGAGAATCGCCATGCTGGTGCACCTTGGAAATGT ${\tt GGGCACCTGAAGATGAGAATCGCCATGCTGGTGCACCTTGGAAATGTGACTGCTGCTGAG}$

ACACCACAAATGGGTGCATGTCCACTGGACCTCATTTCAATCCTGCTGGAAAGGAACATG ACACCACAAATGGGTGCATGTCCACTGGACCTCATTTCAATCCTGCTGGAAAGGAACATG

 ${\tt CCGGAAGTGTCTCTGGTCTGAAACCCGGGCTTCATGGTTTCCATGTTCATGCTCTTGGTG$ ${\tt CCGGAAGTGTCTCTGGTCTGAAACCCCGGGCTTCATGGTTTCCATGTTCATGCTCTTGGTG}$

TGATATCCGTGAAGGGCCATGTTTTCTTCAGCCAAGAGGGAGATGGCCCAACTACAGTAA GTGATGTCGTGAAGGGCCATGTTTTCTTCAGCCAAGAGGGAGATGGCCCAACTACAGTAA

pSOD4 - top line, bases 1 to 226 pSOD6 - bottom line, bases 95 to 333

a)

SOD gene. Restriction maps of both clones showing the relationship between the two is presented in Figure 5.12. From the position of the internal *Eco* RI site and from the sequence data obtained it appears that pSOD4 lacks some of the 5' untranslated region present in pSOD6 while possessing a more extensive 3' untranslated region.

5.5.1 Genomic analysis of pSOD4 and pSOD6

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To determine whether pSOD4 and pSOD6 were in fact identical, a genomic Southern analysis was performed. If the clones were the same it was expected that they would hybridise to the same restriction enzyme fragments obtained from genomic DNA.

Asparagus genomic DNA was digested with *Hin* d iii, *Eco* Ri and *Bgl* II, run on duplicate agarose geis and prepared for Southern blotting. Sequencing had shown that the greatest divergence in the clones occurred in the 3' regions. Probes were therefore prepared using *Eco* RI digests and purifying the DNA fragments corresponding to the 3' regions, which were the 350 bp fragment from pSOD6 and the 420 bp fragment from pSOD4. The result is shown in Figure 5.13. Several bands have hybridised for each digest with both probes suggesting either a complex genomic organisation or the probes have hybridised to related SOD genes. However, what is apparent is that the pattern of hybridising bands differs for pSOD4 and pSOD6, most noticeably pSOD4 hybridises to bands at approximately 500 bp and 850 bp with the *Eco* Ri digest which cannot be seen with pSOD6, suggesting that they are different clones.

Since the clone pSOD6 contained a full open reading frame encoding SOD and showed 100% homology with the entire length of the DD1 N-terminai amino acid sequence, further studies concentrated on this clone.

5.6 In vitro transcription and translation studies of pSOD6

An in situ SOD activity assay showed the presence of six SODs in the asparagus ceil culture

Restriction maps of pSOD4 and pSOD6 showing the known relationship between the two clones.

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known region of homology from sequence data

Asparagus genomic Southern blots probed with pSOD6 and pSOD4 specific probes. A total of 10 μ g of genomic DNA were digested and loaded in each lane. DNA markers are shown in kb. Restriction enzymes used were *Hind* III (H3), *Eco* RI (RI) and *BgI* II. The hybridised filter was exposed to film for 136 hours.

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system. *In vitro* transcription and translation studies were used to identify which of the activity bands corresponded to that of pSOD6. The pSK⁻ vector enables the production of transcript from a piece of insert DNA using either the T3 or T7 promoters, depending on the orientation of the insert. The transcript can then be translated and the products run on a non-denaturing PAGE gel. A diagram showing the outline of the methodology used is shown in Figure 5.14.

The initial translation products from pSOD6 were divided into two with one aliquot run on a denaturing gel to ensure that a product of the expected size of 15 kDa was obtained (Figure 5.15a). The resulting signal, showing a product at 15 kDa, was faint as expected since [³⁵S]-methionine was used as a label and pSOD6 contains only two methionine codons, including the initial start methionine.

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The second aliquot of translation products was added to a sample of ciadode protein and run on a native gei. Cytosolic Cu/Zn SODs exist as a dimeric subunit structure with ligand formation to Cu and Zn ions required to give the correct conformation. The translation mix does not contain Cu or Zn ions so rather than altering reaction conditions, the cladode protein extract was added to the translation products in the expectation that Cu and Zn ions present in the extract would allow ligand formation and hence the production of the correct protein conformation in the labelied products. The native protein gel was run and stained for SOD activity (Figure 5.15b). The gei was then dried and exposed to film with the resulting autoradiograph shown in Figure 5.15c. By aligning the autoradiograph matched that of activity band 4. The clone pSOD6 was therefore correlated with that of SOD-4 (with reference to Figure 5.2a) and also provided further evidence that pSOD6 did indeed encode a gene for the enzyme superoxide dismutase.

5.7 Northern analysis of pSOD6 expression

Though the predicted isoelectric point and molecular mass of pSOD6 corresponded very closely with that of DD1, further confirmation of expression was required to determine

Diagram showing an outline of the *in vitro* transcription and translation technique used to ascertain which of the SOD activity bands corresponded with the Asparagus SOD gene, pSOD6.

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T7 promoter



Results of the *in vitro* transcription and translation experiment as outlined in Figure 5.14.

a) Translation products run on a 15% denaturing PAGE gel alongside marker proteins to determine the size of labelled products. The size of the marker proteins is shown.

b) Translation products, to which were added 20 μ g of ciadode protein, run on a 10% native PAGE gel alongside 20 μ g of cladode protein. The gel was stained for SOD activity prior to drying down. A picture of the dried gel is shown here.

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c) Gel from b) above dried down and exposed to film for three nights. The autoradiograph is shown aligned with the dried gel stained for SOD activity to enable direct comparison of activity bands with labelled bands.





- corresponding band on autoradiograph

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whether the asparagus SOD was expressed in the same way as the protein spots identified as DD1 on 2-D gel analysis. Using RNA isolated from cell cultures a northern gel blot was prepared and probed with pSOD6 insert. Expression was followed from ciadode up to day 7 after initial cell isolation (Figure 5.16a). Transcript was detectable from day 2 increasing slightly up to day 7 with no transcript present in cladode. This implies that pSOD6 is inducible in the cell culture system in a manner similar to that seen in the 2-D gel analysis of DD1 (see Figure 4.1). However, the SOD message appears to be upregulated by the cell culture system much later than the spot corresponding to DD1 for which upregulated message could be seen after 3 hours. The transcript level seen in the northern blot is also very much lower than that expected from the results of the 2-D gel analysis.

To determine whether pSOD6 could be induced as a consequence of wounding, a northern blot was prepared using RNA isolated from chopped up dark grown, etiolated seedlings. The result, Figure 5.16b, shows no apparent induction of the transcript in wounded seedlings. However, pSOD6 message does appear to be present to high levels in unwounded etiolated seedling and is also present in root tissue so any upregulation of message due to wounding may be "masked" by the high amounts of transcript constitutively present.

The presence of high quantities of transcript in dark grown seedlings and in root tissue suggests that pSOD6 transcript is associated with non-photosynthetic tissue and is perhaps induced in cell cultures as a consequence of incubating the cultures in the dark. Alternatively, induction may be due to a metabolic change resulting from a switch from a photoautotrophic to a heterotrophic metabolism. Roots and etiolated seedlings are heterotrophic and it is possible that the cultured cells switch to a heterotrophic mode due either to a lack of light, or by the presence of an external supply of sucrose in the cell culture medium. The effects of light and cell culture medium constituents on SOD activity were therefore examined.

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Northern blots showing expression of pSOD6 in ceil culture, wounded seedlings and roots.

a) Blot showing expression in cell culture. Clad refers to RNA extracted from cladodes while D1 to D7 refer to the number of days following the single cell isolation procedure. A total of 10 μ g of RNA were loaded per lane. The filter was probed with the pSOD6 720 bp *Eco* RI insert and the filter exposed to film for 3 weeks.

b) Blot showing expression in wounded dark-grown seedlings and in root. D0 to D5 refer to the number of days following the wounding procedure which consisted of chopping seedlings into 5 mm sections. The root RNA was obtained from a green plant. A total of 20 μ g of RNA were loaded per lane. The filter was probed with the pSOD6 720 bp *Eco* RI insert and the filter exposed to film for 2 weeks.

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Due to the low abundance of transcript in the northern blots, particularly in the blot shown in a), the autoradiographs were used as negatives to retain contrast and produce the reverse photographic images shown.







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5.8 Analysis of changes in SOD activity in response to light and cell culture medium constituents

The difficulty in designing an experiment to assess the effects of light and medium constituents was to try and separate these effects from any that may result from the initial cell culture preparation. The experiment was therefore performed on whole asparagus fronds (as described in 2.14.4). The cut ends of asparagus fronds were placed in solutions containing either sucrose, mannitol, sucrose and mannitol or in plain water. It was expected that the xylem tissues would draw up the solutions in the normal process of transpiration and supply the cells with the solutions in a manner reflecting the situation in cell cultures. Duplicates of each condition were placed either in the light or in the dark to see whether the effect of externally supplied sugars or the absence of light had the greatest effect on SOD activity.

After 4 days under these conditions, protein was isolated from the asparagus cladodes and and *in gel* SOD activity assay performed. The resulting gel is shown in Figure 5.17. In general, it can be seen that incubation in either the light or the dark has had very little effect. In fact, SOD activity for all the treatments appears to be greatest in the fronds placed in the light conditions, with the highest SOD activity present in fronds which were placed in the light with sucrose and mannitol. The activity profile of this sample was very similar to that of etiolated seedling tissue, suggestine that the supply of sucrose and mannitol have exerted a greater effect on SOD activity than that due to conditions of either light or dark.

5.9 Conclusions concerning the identity of DD1

The protein named DD1, initially found to be induced in the asparagus cell system, was discovered to be homologous to maize SOD2 on the basis of N-terminal amino acid sequence data. Study of SOD activity in asparagus cell cultures showed activity to change with increasing time in culture, with some SODs increasing and some decreasing in activity. After one week in culture the SOD activity profile observed is the same as that found in root protein and in dark grown etiolated seedlings. Possible wound-induced SODs were detected in

Results of an *in situ* gel SOD activity assay from an experiment to determine whether an increase in respiration is the cause of the induction of activity band SOD4 that was shown to correspond with the clone pSOD6. Asparagus seedlings were cut at the stem above the soil level and placed in the conditions described below for 4 days prior to protein islolation. A total of 20 μ g of protein were loaded per lane. In each case the sucrose concentration used was 1% (w/v) and mannitol 3% (w/v), corresponding to the concentrations found in asparagus cell cutture medium (table 2.2).

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lane 1 - water and light lane 2 - water and dark lane 3 - sucrose and light lane 4 - sucrose and dark lane 5 - mannitol and light lane 6 - mannitol and dark lane 7 - sucrose, mannitol and light lane 8 - sucrose, mannitol and dark

Lanes marked cl both contain 20 μg of cladode protein.



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wounded dark grown seedlings but these did not relate to any of the SOD activity bands seen in protein from the cell culture system.

The gene corresponding to the DD1 protein was cloned by using a combination of PCR and library screening. The clone analysed, pSOD6, showed a 100% match with the DD1 N-terminal amino acid sequence and was found to be homologous to many plant cytosolic Cu/Zn SODs. A second clone picked in the library screening process, pSOD4, was partially analysed. It showed many similarities to pSOD6, including homology with the DD1 N-terminal protein sequence, but was seen to be different when both clones were analysed on a genomic blot. The clone pSOD4 was also found to have a different 3' untranslated region when compared to the sequence of pSOD6. It is possible that the clones have the same coding region but differ in their untranslated regulatory regions and hence may exhibit differential transcriptional activation in response to varying oxidative stresses.

In vitro transcription and translation experiments indicated that pSOD6 corresponded with SOD-4 seen in *in gel* activity assays. This band increases in activity following single cell isolation and is found to be highly active in root, etiolated seedling tissue and in long term cell cultures. Northern experiments showed that pSOD6 transcript expression followed the same pattern as protein activity with transcript induced by the cell culture system but present in high amounts in a constitutive manner in roots and etiolated seedlings.

The SOD activity data and northern expression studies indicated that the SOD corresponding to the DD1 protein was highly associated with roots, dark grown seedling tissue, and was induced in the cell culture system in which cells are incubated in the dark. This data suggested that the SOD was either induced as a consequence of the dark, or was induced by some metabolic change resulting from a switch from a photoautotrophic to a heterotrophic metabolism. The effect of cell culture medium constituents on SOD activity indicated that it was possible that the external supply of sucrose in cell culture medium caused the induction of the asparagus SOD, with constitutive expression observed in etiolated seedlings and roots as a consequence of heterotrophic metabolism in these tissues.

Though the predicted isoelectric point and molecular mass of pSOD6 corresponded closely

with that of the protein spot identified as DD1 on 2-D gel analsis, expression studies of pSOD6 showed that while the transcript was induced in cell culture, the expression did not mirror that of DD1. The spot identified as DD1 was observed using 2-D gel analysis after only 3 hours whereas pSOD6 transcript is not detectable before day 2. This placed doubts as to whether the protein spot identified as DD1 on 2-D gels was the same as the protein spot which had been isolated to provide N-terminal amino acid sequence data.

The expression data, and also the results showing that pSOD6 was not wound-inducible, indicated that the promoter for pSOD6 would not be a worth while target for use in the genetic engineering of plants for resistance. However, continuing work on AoPR1, isolated as a consequence of the original DD1-34 clone (section 4.1) by Warner (1992), resulted in the isolation of a promoter which was found to be wound-inducible when transformed into tobacco plants. It was therefore decided to study the expression of the same promoter in potato, a potential target for genetic engineering.

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CHAPTER 6

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STUDY OF A WOUND-INDUCIBLE PROMOTER IN POTATO

6.1 Introduction and background to the AoPR1 promoter

Northern hybridisation analysis of the AoPR1 cDNA clone showed that the homologous transcript was induced both by the mechanical cell isolation process and by wounding seedling tissue, reaching a maximum steady state level 1 to 3 days following the initial stimulus in both systems (Warner *et. al.*, 1992). Utilising the technique of inverse PCR, a 1.1 kb region upstream of the AoPR1 coding region was cloned (Warner *et. al.*, 1993). This 1.1 kb fragment was shown to be a functional promoter by attaching it to the GUS reporter gene and transferring the whole construct into tobacco plants.

The GUS (β -glucuronidase) reporter gene originates from *E. coli* and is encoded by the *uid* A locus (*gus* A; Novel & Novel, 1973). The encoded protein is a hydrolase that catalyses the cleavage of a range of β -glucuronides, enabling its use in plant transformations in which background GUS activity levels are non-detectable (Jefferson *et. al.*, 1986; Jefferson *et. al.*, 1987). GUS enzyme activity can be easily localised using the histochemical stain X-GLUC which yields a blue dye following GUS-mediated hydrolysis, or quantitatively assayed using MUG which yields a fluorescent product, 4-MU, on hydrolysis which can be measured using a fluorimeter.

GUS activity in transgenic tobacco plants driven by the AoPR1 promoter was found to be induced by wounding with activity detectable within 3-10 hours and increasing up to 4-5 days (Warner, 1992), results similar to the northern expression data obtained. Expression was also found to be highly localised to the sites of damage. Developmental expression of GUS activity was observed in vascular tissue in regions where secondary thickening occurred, and in parts of the floral tissues including pollen (Warner, 1992). A deletion of approximately 200

base pairs from the 5' region of the promoter eliminated wound-inducibility of expression but retained some of the developmental expression. Salicylic acid was also found to induce GUS activity, acting in a synergistic manner when combined with the wounding stimulus.

The translated amino acid sequence of the AoPR1 cDNA shows homology to a group of cytosolic pathogenesis-related proteins (see section 1.6.3) which include a pea disease resistance protein, p149, a potato protein, pSTH2, a parsley PR1 protein, PcPR1-1, and a birch pollen allergen *Betv* 1 (Walter *et. al.*, 1990). Various members of the group are induced following pathogenic stress, wounding, and treatment with arachnidonic acid, an elicitor of the defence response.

Though the function of the AoPR1 protein is not presently known, the homologies with the cytosolic PR proteins strongly suggests an involvement in the defence response. The spatial and temporal induction pattern of the AoPR1 promoter GUS construct is also comparable with the induction of enzymes involved in the phenylpropanoid pathway (see 1.3), particularly of the PAL and CHS transcripts induced in wounded bean hypocotyls (Cramer *et. al.*, 1989; Ryder *et. al.*, 1987). Both enzymes are also active in the defence response.

Data from the wounding studies of the AoPR1 promoter in tobacco indicated that it may be suitable for genetic engineering purposes since it showed the required expression profile of rapid induction by day 1 following wounding, and then sustained activity. Further analysis of the promoter would be undertaken in potato, an economically important crop for which resistance was to be targeted against soft rot caused by *Erwinia* species. The presence of an AoPR1 homologue in potato, pSTH2, which is induced both by slicing potato tubers and by infection with *Phytophthora infestans* (Matton & Brisson, 1989), and the fact that the AoPR1 promoter was active in tobacco, indicated that the promoter would also be functional in potato, since both tobacco and potato are members of the Solanaceae family. Studies of the promoter in potato would concentrate on its wound-inducibility, the localisation of expression, and also its response to various pathogens.

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6.2 Transformation of potato plants

Two Agrobacterium strains containing AoPR1 promoter/GUS fusion constructs (Figure 6.1) were obtained from Simon Warner (Botany Department, Leicester University). The *Agrobacterium* strains were LBA4404 and pGV2260 which both contained the AoPR1/gus A fusion construct in the binary vector pBI 101 (Jefferson *et. al.*, 1987).

Potato tuber discs from the cultivar Desiree were transformed by the method of Sheerman and Bevan (1988). Some difficulty was encountered in removing the *Agrobacterium* strain pGV2260 which persisted in the initial antibiotic-containing plates. The difficulty was overcome by removing the least affected discs into $1 \times MS$ media containing cefotaxime at 750 µg/ml. Discs were left with occasional shaking for 30 minutes, dried on sterile filter paper and replaced on plates containing 400 µg/ml augmentin and 100 µg/ml kanamycin. No further growth of the *Agrobacterium* was observed. Transformants from each of the *Agrobacterium* strains were distinguished by using the prefixes LBA and pGV to denote their origin.

6.3 Confirming the presence of the *gus* A gene in putative transformed potato regenerants

6.3.1 PCR to amplify the gus A gene from genomic DNA

The presence of the reporter gene in kanamycin resistant plants was confirmed by using oiigonucleotide primers homologous to the *gus* A gene (designed by and obtained from Hayley McArdle, Leicester Biocentre) to amplify the gene by PCR from a preparation of genomic DNA. Samples of DNA were isolated from *in vitro* grown material from 7 potential transformants, the PCR reaction carried out and products run on an agarose gel (Figure 6.2a). The results of the PCR confirmed that pGV1, 2, 3 and 5, and LBA 4 contained the GUS gene while pGV4 and LBA5 did not and were therefore presumed to be escapes. The line pGV4 was used as a negative control in subsequent experiments.

Figure 6.1

Map showing the arrangement of the AoPR1 promoter/*gus* A construct. The construct is a translational fusion to the *gus* A reporter gene containing 30 amino acids from the AoPR1 cDNA (Warner, 1992). The vector used was pBI 101 (Jefferson *et. al.*, 1987), a binary vector containing a promoterless GUS gene which is used routinely in plant transformation to assess promoter activity.

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Potato lines obtained from transformation of the cultivar Desiree with the AoPR1 promoter/gus A construct.

a) Agarose gel showing the results obtained in a PCR reaction to determine the presence or absence of the GUS gene in genomic DNA isolated from putative transformants. A total of 250 ng of each primer homologous to the *gus* A gene (received from Hayley McArdle, Leicester Biocentre) were used in the PCR reaction which consisted of 30 cycles of 1 min denaturing at 95°C, 1 min annealing at 65°C, and 1 min extension at 72°C, in a total volume of 50 μ l. Template DNA used was 200 ng of genomic DNA and the positive control was 10 ng of pBI 101 DNA (Jefferson *et. al.*, 1987) which contains the full length *gus* A gene. PCR products were extracted and 5 μ l run on a 1.5% agarose gel. The expected fragment size was 438 bp.

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b) Table showing GUS activity results in the potato lines assessed in the PCR reaction described in a). The results are based on histochemical analysis and indicate only the presence or absence of a blue stain in the described conditions. Wounding was by stabbing using a yellow pipette tip.



b)

	root	stem	leaf	1 day wounded leaf	2 day wounded leaf	3 day wounded leaf
pGV1	-	+	-	+	+	+
pGV2	+	+	-	+	+	+
pGV3	-	-	-	+	+	a
pGV4	- 1	1	-		-	
pGV5	+	+	-	+	+	+
LBA4	+	+		+	+	+
LBA5	-		-	-		

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6.3.2 Histochemical localisation of GUS activity

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Results from the PCR were confirmed by histochemical assessment of GUS activity. Expression was studied in root, stem, and leaf to detect any constitutive activity, and also in leaf tissue that had been wounded for 1-3 days to assess wound-inducibility of GUS activity. Plant tissue was placed in buffer containing X-GLUC, left overnight, and then cleared in 70% ethanol. The results are shown in tabular form in Figure 6.2b.

From the results it can be seen that lines pGV4 and LBA5 showed no GUS activity, confirming the PCR results which showed that they did not contain the GUS gene. Lines previously identified as containing the GUS gene all show wound inducibility of expression although differences in activity were observed with some lines, notably pGV5, staining far more rapidly and intensely than other lines. All lines were induced by day 1 with induction sustained until day 3 except for pGV3 which showed no GUS activity in leaves which had been wounded for 3 days. No constitutive expression in leaves was noted though some did contain expression in the vascular tissue of young leaves. Expression in root and stem tissue varied between transformants, with pGV2, pGV5 and LBA4 showing constitutive expression in neither tissue. Differences in expression may reflect the different insertion sites of the promoter/*gus* A construct into the genomic DNA, with AoPR1 promoter activity modified by enhancer and silencer elements and by *cis* elements that determine tissue specificity.

6.4 Histochemical analysis of wound-induction in a representative line

The potato line pGV5 was chosen for histochemical analysis since it was found to give a strong result with an intense blue colour formed from the X-GLUC. This line also showed the most common pattern of expression with constitutive expression in both stem and root wound-inducible expression present day 1 to day 3 following wounding.

6.4.1 Expression in leaves

Leaves were detached from the plant, wounded using a yellow pipette tip and left on moist filter paper for 3 days before incubating in the presence of X-GLUC. Unwounded leaves were detached, placed on moist filter paper, and were stabbed immediately prior to incubation in the presence of X-GLUC. Constitutive GUS expression was observed in the vascular tissue of young leaves (Figure 6.3a) while wound-induced expression appears to be highly localised to the cells immediately surrounding the wound site (Figure 6.3b and c). The unwounded leaves showed no localised GUS expression around sites damaged immediately before placing in X-GLUC buffer.

6.4.2 Expression in tubers

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Potato tubers of different sizes, at varying stages of development, were cut into slices and immediately incubated in X-GLUC to observe constitutive expression. GUS activity in young tubers (Figure 6.3d i and ii) appears to be confined to bundles of vascular tissue, while in oider tubers, activity is more wide spread and is present in the vascular tissue and also in the cortex (Figure 6.3d iii and iv). Wound-inducibility of expression was assessed by slicing tubers into sections and leaving the sections on moist filer paper for 3 days prior to incubation in X-GLUC. Though staining in the vascular tissue remains strong, it can be seen that the tuber slices have stained a uniform blue (Figure 6.3d v, vi and vii) implying wound-inducibility in potato tubers.

6.5 Fluorimetric analysis of wound-induction in representative lines

The lines pGV1, pGV2 and pGV5 were chosen as representative transformants showing a range of ieveis of wound-inducibility of GUS activity. A potato line containing the CaMV 35S promoter attached to *gus* A (Jefferson *et. al.*, 1987; provided by Lee Rooke, Leicester Biocentre) was used as a non wound-inducible GUS expressing control in the leaf wounding

GUS histochemical results in leaves and tubers. All results shown are from the line pGV5.

a) Unwounded leaf showing constitutive GUS expression in the vascular tissue of a wounded leaf.

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b) Comparsion of an unwounded leaf (left hand side) with a leaf that was wounded for 3 days prior to staining for GUS activity (right hand side).

c) Picture showing greater detail of the localisation of GUS activity staining around wound sites.

d) GUS activity in tubers: i) and ii) GUS activity in young tuber slices; iii) and iv) expression in older tuber slices; and v), vi) and vii) expression in slices left for 3 days following slicing before staining for GUS activity.



experiments, while the untransformed line pGV4 was used as a negative control.

6.5.1 Wound-Inducible expression in leaves

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Leaves were removed from plants and wounded by stabbing with fine laboratory forceps to the same density on all samples, approximately 10 stabs per 1 cm². They were then placed on moist filter paper in sterile Petri dishes for up to 3 days before assaying for GUS activity using the substrate MUG. Unwounded controls were treated in the same manner but were not stabbed.

Results from the three lines containing the AoPR1 promoter/*gus* A fusion construct are shown in Figure 6.4. All three transformants show very little change in GUS activity day 1 after wounding, with activity in pGV1 and pGV2 increasing by 30-50 pmoles 4-MU/min/mg total protein while activity in pGV5 remains at background level. From day 1 to 3 a steady increase in GUS activity was observed in all three lines with levels rising to 615 pmoles 4-MU/min/mg total protein in pGV1, 859 in pGV2 and 1825 in pGV5. Activity became difficult to measure after 3 days, possibly due to lignification of the tissue leading to difficulty in extracting protein for assays. All three transformed lines therefore showed wound-inducible GUS activity, differing only in the quantitative measure of activity. The highest expressing line, pGV5, also showed the highest backgound activity levels at approximately 300 pmoles 4-MU/min/mg total protein. This background activity is probably due to constitutive expression present in the vascular tissues.

Figure 6.5 compares the responses to wounding of pGV5, pGV4 (the untransformed control) and the CaMV 35S/gus A construct. No detectable GUS activity was observed in the untransformed control. A slight increase in activity is detectable with the CaMV 35S GUS construct following wounding, with activity increasing from a background level of 430 pmoles 4-MU/min/mg total protein to 580 pmoles 4-MU/min/mg total protein 3 days following wounding, an increase of 35%. In the same time period, pGV5 increases in activity by 500%. The increase in GUS activity in the CaMV 35S construct following wounding may be due to increased penetration of substrate into the tissues and probably does not reflect any wound-

Bar charts showing the responses to wounding of the leaves of 3 independent transformants. Leaves were wounded by stabbing with fine laboratory forceps to the same density on all samples, approximately 10 stabs per 1 cm². GUS activity was measured using the fluorimetric substrate MUG. The average of 3 experiments was plotted with the standard error shown as bars.

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Line graph showing the response to wounding over 3 days of the transformed line pGV5, the control line CaMV GUS, and an untransformed control pGV4. GUS activity was measured using the fluorimetric substrate MUG. The average of 3 experiments was calculated and plotted with the standard error shown as bars.

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GUS activity in pmoles 4-MU

Wounding response of pGV5 compared with CaMV and an untransformed control, pGV4

inducibility of the 35S promoter.

6.5.2 Wound-inducible expression in tubers

Wound-induced expression in tubers was measured in two separate experiments. In the first experiment tubers of roughly equal size were removed from the transformed lines pGV1, pGV2 and pGV5, and from the control, pGV4. One tuber was left intact while the second tuber was wounded by stabbing through the skin with a narrow gauge needle. In the second experiment tubers were cut in half, with one half receiving wounds from needle stabs and the other half left alone. The second experiment was an attempt to eliminate individual differences in GUS expression that may exist between different tubers. In both cases the tubers were left on moist filter paper for 3 days.

The results from both experiments can be seen in Figure 6.6. Though background levels of GUS activity were relatively high in unwounded tubers compared to leaf tissue, varying from 980 pmoles 4-MU/min/mg total protein in pGV1 to 1700 in pGV2, a noticeable elevation of activity was seen in the wounded tuber equivalent to 2 to 4 times the background level. Very little difference in expression between the unwounded tuber half and wounded half were seen, particularly in pGV2 and pGV5 in which both halves showed high levels of GUS activity. This would appear to indicate that the act of cutting the tuber into halves was enough to effectively wound the entire tuber. This result indicated that the wounding stimulus travelled throughout the tuber. Experiments were therefore conducted to determine the extent the wound stimulus travelled from the original wound site, and whether the stimulus remained within one organ or could travel in a more systemic fasion.

6.6 Studies on the localisation of GUS expression

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The following experiments were designed to discover how far from the wound site elevated GUS expression could still be observed. One concern for the use of a wound-inducible promoter for the engineering of bacterial resistance in plants was that expression should be localised to the wound site, the potential site for infection from bacteria, to minimise unwanted

Bar chart showing the response to wounding of 3 independent transformed lines and an untransformed control, pGV4. Whole tubers of approximately equal sizes were wounded by stabbing with a needle and then left for 3 days before measuring GUS activity using the fluorimetric substrate MUG. The half unwounded and wounded tubers were halves of the same tuber and were used in an attempt to eradicate differences in GUS activity that may exist between different tubers.

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expression of the antibacterial substance. Histochemical data (Figure 6.3 b and c) indicated that *gus* A expression under the control of the AoPR1 promoter was highly localised to the wound site. Confirmation of this was to be obtained by measuring expression fluorimetrically. Analysis of systemic signalling would also be conducted by wounding a leaf and leaving the wounded leaf attached to the whole plant to see if *gus* A expression in this case remained localised to the wounded leaf and did not systemically affect other parts of the plant.

The following experiments were conducted using the line pGV5.

6.6.1 Analysis of localised signalling

Localisation of GUS expression within a leaf was measured by wounding a leaf at its tip, leaving the leaf for 3 days and then isolating protein for determining GUS activity from the wounded tip and from segments 5 mm apart away from the wound site.

The results are presented in Figure 6.7. GUS activity in the wounded tip was found to be high at approximately 14,000 pmoles 4-MU/min/mg total protein. immediately adjacent to the wound site, GUS activity fell markedly to only 2,680 pmoles 4-MU/min/mg total protein with activity decreasing still further as distance from the wound site increased. The large decrease in GUS activity away from the wound site indicates that expression is localised to the area of the wound site. The control unwounded leaf was found to have GUS activity twice that of normal background levels in the leaf tip but there was no gradient of activity down the ieaf which, apart from the tip, had normal background levels of approximately 300 pmoles 4-MU/min/mg total protein. The relatively high expression in the ieaf tip was presumed to be due to necrosis of the tip, since it had been observed in histochemicai analysis of leaf tissue that blue staining was visible around necrotic leaf tips.

6.6.2 Analysis of systemic signalling

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One leaf from the middle tier on a plant was wounded and left attached to the plant for 6 days.

Diagram showing the extent of the localisation of GUS activity following wounding. After wounding, a single detached leaf was left for 3 days before measuring GUS activity at increasing distances away from the wound site. The actual figures of GUS activity in a wounded leaf and in an unwounded control leaf are shown. Results are also presented in a graphical form. The line used was pGV5.

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 unwounded tissue

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 GUS activity in pmoles 4-MU/ min/mg of total protein

 At the end of this time leaves close to the wounded leaf and leaves connected only by the central stem were tested for elevated levels of GUS activity. The results are presented in diagrammatic form in Figure 6.8. No elevated levels of GUS activity were detected in any of the leaves that were not themselves wounded, indicating that GUS expression remained localised to the wounded leaf.

6.7 Response of the AoPR1 promoter to pathogen attack

The response of the AoPR1 promoter to pathogen infection was studied to see how it would differ, if at all, from the response to wounding which may, in turn, reveal insights into the elicitors involved in activating the promoter.

Histochemical staining was used to assess GUS activity rather than measuring activity with fluorimetric assays because initially it was not known where activity would be expressed. Though fluorimetric assays can give a quantitative measure they are unable to pinpoint precise locations of expression.

6.7.1 Infection with Potato Virus Y (PVY)

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PVY, a virus responsible for significant agricultural losses, is a member of the potyvirus group, having single stranded RNA enclosed in a flexible rod shape. Transmission is usually styletborne by aphids with cell to cell spread probably occurring by cytoplasmic streaming and systemic spread via the phloem. The virus is unable to enter a plant through an intact leaf surface with some kind of wounding which ruptures the cuticle necessary for infection to occur (Manners, 1982).

One leaf of 3 week old potato plants from the transformed lines pGV1, pGV2, pGV5, CaMV GUS, and the control pGV4, was inoculated with PVY by using sap taken from an infected plant and left attached to the plant. After approximately 10 days, symptoms consisting of small necrotic lesions were visible on the inoculated leaves. The virus spreads via the vascular

Diagram showing the results from an experiment performed to determine whether a wounding signal was transmitted systemically throughout the plant. A single leaf of the line pGV5 was wounded while attached to the plant and left attached. After 6 days, GUS activity in leaves from various positions on the plant was measured with the results as shown. Background activity levels in the line used were generally around 300 prooles 4-MU/min/mg total protein.

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GUS activity in pmoles 4-MU/ min/mg of total protein

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tissue wounded for 6 days tissue throughout the plant and after a further 3 to 4 days symptoms began to appear on the upper, uninoculated leaves consisting of areas of chlorosis which developed into necrotic regions along the vascular tissue.

Histochemical staining of inoculated leaves prior to the appearance of symptoms showed no detectable GUS activity. Once necrotic lesions had appeared (Figure 6.9a) GUS activity was clearly visible around the areas of dead tissue (Figure 6.9b). No staining of vascular tissue can be seen as the leaves were several weeks old and it had already been established that staining of vascular tissue was only visible in young leaves. Though the results are shown only from the one line, pGV5, the same localisation of expression could be seen in the other lines tested with differences apparent only in the intensity of staining. Some slight staining was noted in the CaMV GUS line but the stain was not directly localised around the areas of necrosis and may have been due to penetration of substrate into damaged tissue.

Initial secondary systemic symptoms caused by PVY consist of a chlorotic mottling visible on uninoculated leaves (Figure 6.9c), present particularly along regions of vascular tissue through which the virus spreads. GUS analysis of the chlorotic regions indicated the presence of some GUS activity (Figure 6.9d) which appeared to be centred along the vascular tissue.

6.7.2 Infection with Phytophthora infestans

Phytophthora infestans, a member of the Oomycetes, is the causal organism of potato blight. Infection naturally occurs by way of airborne spores which germinate in a droplet of water after a period of encystment. Entry into the plant is through the stoma with the fungus producing mycelium in the intercellular spaces of the host. Nourishment is through forked haustoria developed within the living cells. After a few days of vegetative growth reproductive structures, branched sporangiophores, are formed at which time the host tissue dies. *Phytophthora infestans* is therefore unusual in that initially it grows biotrophically but later switches to a necrotrophic mode of existence (Ingold, 1984).

GUS histochemical results showing the response of the AoPR1 promoter to infection by PVY.

a) A leaf from the untransformed line pGV4 showing typical lesions caused by infection with PVY.

b) Detail of an infected leaf from the line pGV5 stained to show the localisation of GUS activity.

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c) Leaves from pGV5 showing symptoms caused by secondary infection with $\ensuremath{\mathsf{PVY}}$.

d) Detail of GUS activity in a leaf of pGV5 showing secondary symptoms.



Young leaves were detached from plants and inoculated with varying dilutions of *Phytophthora infestans* zoomycetes. It was found that a 20 µl droplet containing 1000 zoomycetes was sufficient to initiate infection. Leaves were incubated under conditions of light and humidity to ensure maintenance of infection.

P. infestans lesions consist of a central region of necrotic tissue surrounded by a halo of living but infected tissue (Figure 6.10a). Since Desiree contains no major resistance genes, the lesion formed is not part of the hypersensitve response and was found to take approximately 5 days to appear. Histochemical analysis of a lesion (Figure 6.10b) showed that no GUS activity was detectable in the halo of living tissue surrounding the necrotic region. However, GUS activity did appear to be elevated in the tissue surrounding the halo.

6.7.3 Infection with Erwinia carotovora

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Erwinia carotovora is the major causal organism of bacterial soft rot in potato tubers, a serious storage problem. *Erwinia* is an opportunistic pathogen and infection is very much dependent on environmental conditions with some impairment of the host's resistance mechanisms required for successful infection. *Erwinia* is unable to penetrate the cuticle of plants directly with entry occurring naturally through wounds or lenticels present in tubers. The rot is caused by the secretion of plant cell wall degrading enzymes produced by the soft rot bacteria (Lyon, 1989).

Tubers, which had been harvested 5 months previously, were inoculated by vacuum filtration to ensure that bacteria entered via the lenticels. Infected tubers were then stored in both aerobic and anaerobic conditions, and at varying degrees of humidity achieved by placing tubers in dry containers, and in plastic bags which held varying quantities of water and which were left either loosely or tightly sealed. Once the soft rot had taken hold after approximately 3 to 5 days, which was determined by examining duplicate controls, tubers were sliced and stained for GUS activity. Tubers stored under dry, aerobic conditions did not develop any symptoms. Tubers which were stored under humid conditions, either aerobic or anaerobic, all developed areas of soft rot. Potato lines which were positive for GUS activity showed some

GUS histochemical results showing the response of the AoPR1 promoter to infection by *P*. *infestans* and *E. carotovora*.

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a) Leaf infected with P. infestans showing typical lesions.

b) Detail of a leaf from the line pGV5 infected with P. infestans and stained for GUS activity.

c) Slice of a tuber from the line pGV5 which had previously been harvested and stored for 5 months before staining for GUS activity.

d) Slice of a tuber from pGV5 which had previously been harvested and stored for 5 months before infection with *E. carotovora* and staining for GUS activity. Following infection, the tuber was kept in anaerobic, humid conditions.

Following slicing of the tubers shown in c) and d), slices were placed immediately in histochemical buffer containing X-GLUC to prevent expression due to wounding.



slight blue staining around the areas of tissue affected by soft rot (Figure 6.10 d) compared to the uninfected controls (Figure 6.10c).

6.8 Summary and conclusions

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Potato plants transformed with the AoPR1 promoter/gus A fusion construct showed woundinducibility of expression in leaves and tubers. Tubers showed constitutive expression in vascular tissue and in the cortex of older tubers while some lines also showed constitutive expression in roots, stems and the vascular tissue of young leaves.

Wound-induced GUS expression in leaves was found to be localised to a few cell layers bordering the wound site. Studies of the localisation of expression confirmed this observation with GUS activity confined to the actual leaves that were wounded with no systemic expression occurring. Wounding in leaves gave up to a 500% increase in GUS activity above background levels 3 days after the wounding stimulus was applied. In tubers, the increase in GUS activity above wounding was 100 to 300%. Background GUS activity levels in tubers was found to be much greater than background levels in leaves.

A small increase in wound-induced GUS expression was observed in the control plant tested containing the CaMV 35S/*gus* A construct. However, the increase was slight when compared to lines containing the AoPR1/*gus* A construct and was therefore thought to be due to greater penetration of substrate into the damaged tissue.

When challenged with various pathogens, the AoPR1 promoter did not respond to the presence of the pathogen itself but was activated once symptoms of disease, corresponding to cell and tissue damage, appeared. The stimulus for the AoPR1 promoter would therefore appear to correlate with damaged tissue, whether the damage is caused by mechanical wounding or by pathogen infection.

CHAPTER 7

DISCUSSION

7.1 Thesis aims

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An important aim in plant breeding is to improve disease resistance in plants. One way in which this may be achieved is through the use of genetic engineering. Increasing resistance to soft rot bacteria, which cause a substantial loss of many food crops, particularly attracts the use of genetic engineering since no natural plant resistance genes are available which may be bred into selected lines by conventional means. Many soft rot bacteria enter plant tissue through natural openings such as lenticels in potato tubers, but also through wound sites caused in the harvesting and handling of crops. One approach to engineering resistance in this instance is to induce the secretion of antibacterial substances at wound sites, in the expectation that this would prevent the multiplication and spread of any invading pathogenic bacteria.

The important components of a construct for engineering resistance using this approach include a wound-inducible promoter, a secretion signal sequence known to be active in plants, and a gene encoding an antibacterial protein. The aims of this individual thesis were therefore to study the secretion of an antibacterial substance, and to search for a suitable wound-inducible promoter.

A signal sequence known to be active in plants was already available in the form of the signal from wheat α -amylase. Study of the immune system of insects reveals that they have a group of proteins possessing high antibacterial activity which are induced and secreted into the haemolymph following bacterial infection. Homologous proteins are also found in mammalian systems and may be a universal system for defence against bacterial infections. These proteins have the advantage of requiring only one gene to ensure their production and the

porcine cecropin was therefore chosen as the antibacterial protein.

A synthetic cecropin gene was designed to the porcine cecropin protein sequence taking into account the codon usage of plants. Constructs were initially transferred into tobacco but though transcript could be detected on northern blots, no active protein was found. The constructs were therefore studied in yeast which would provide more rapid results than further analysis of the constructs in tobacco. Yeast was also chosen because it appeared to be a good system for the expression of heterologous proteins, and it had already been shown that the wheat α -amylase signal was fully functional in yeast cells.

Secretion of the cecropin gene would be under the control of a wound-inducible promoter. In order to protect wound sites, a promoter was required which was rapidly activated within a few hours following the wounding stimulus with activation sustained over a period of several days to ensure any pathogenic bacteria that may have entered the would site were not able to multiply and spread throughout the plant. Previous work on *Asparagus* cell cultures (Harikrishna *et. al.*, 1991; Warner *et. al.*, 1992) indicated that they may be a good model system for the isolation of wound-induced genes and hence wound-inducible promoters. Two-dimensional gel analysis of *in vitro* translated poly (A)⁺ RNA isolated following cell culture initiation, showed the presence of a group of polypeptides that were highly upregulated. Expression of one member of the group, DD1, was rapidly upregulated within 3 hours and expression maintained for several weeks following the initial wounding process. DD1 therefore appeared to be suitable for the isolation of a wound-inducible promoter and was pursued for further analysis. Protein was isolated and an N-terminal amino acid sequence obtained which, at that time, showed no homology to known proteins. One aim of the thesis was therefore to clone DD1 and to further assess its expression characteristics.

During the time of the project, a wound-inducible promoter isolated from the *Asparagus* system became available. The promoter was transferred into potato plants attached to a reporter gene so that its expression could be studied in response to pathogen attack as well as wounding. It would also be determined whether wound-induced expression led to systemic expression within the plant.

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This thesis is therefore concerned with the study of cecropin secretion in yeast, the isolation and expression analysis of the gene named DD1, and the analysis of a wound-inducible promoter in potato plants.

7.2 Secretion of cecropin in yeast

7.2.1 Summary and discussion of results

Yeast were transformed with a construct containing the synthetic cecropin gene attached to the wheat α-amylase signal sequence (pYWCE1, Figure 3.3a) under the control of a galactose inducible yeast promoter present in the expression vector pEMBLyex4. A second construct contained the same components with an additional VTE element (pYWCE2, Figure 3.3b) which had been shown to greatly enhance cecropin translation *in vitro* (Figure 3.1). The vector pEMBLEyex4 was also used to transform yeast to provide a non-cecropin producing control.

Initial tests concentrated on the detection of active, secreted cecropin by assaying media for antibacterial activity following promoter induction. Concentrated media were also tested but no antibacterial activity was found in either case. This was thought to be due to a dilution of any product by the media, despite the 20-fold media concentration achieved by the use of microconcentrators. A second test was therefore used in an attempt to detect antibacterial activity which involved spreading a bacterial lawn directly onto induced yeast colonies (Figures 3.4 & 3.5). This test was thought to be more sensitive due to the direct contact between the yeast and the bacteria with a halo of bacterial growth inhibition around secreting yeast colonies expected to be the result. However, it was discovered that acidification of the media by the yeast in itself inhibited bacterial growth, thus invalidating the test.

Northern blot analysis failed to detect the presence of a cecropin transcript (Figure 3.6b). This was initially a surprising result since when the same constructs had been transferred into tobacco, cecropin transcripts had been detected but no active product. The result could be due to the production of very low levels of heterologous mRNA in the yeast which could

either be caused by the instability of foreign mRNA or by inefficient transcription. Mellor *et*. *al.* (1985 & 1987) present evidence showing that low levels of heterologous mRNA are due to the lack of specific yeast sequences in the actual coding region of genes, leading to inefficient transcription. For example, it appears that the yeast phosphoglycerate kinase gene contains sequences within the coding region (Downstream Activator Sequence) to ensure efficient transcription (Mellor *et. al.*, 1987).

Low levels of heterologous mRNA could therefore lead to low levels of translation. The antibacterial plate well test used to detect cecropin required relatively high levels of expression (minimum of 3 μ g/ml, Figure 3.2a) to be detectable. It is therefore possible that small amounts of cecropin were being produced but at too low a level to be detected.

A major factor affecting both transcription and translation, especially of foreign genes, in yeast is codon usage. Evidence exists showing that highly expressed genes in several species show a strong codon bias (Ernst, 1988) and though heterologous proteins have been expressed in yeast without altering codon usage, this may be one of several factors that affect efficiency of expression. For example, a wheat α -gliadin gene has been expressed in yeast (Neill *et. al.*, 1987) but low protein yields were obtained, presumed to be due to the codon usage pattern which was distinctly different from that of yeast.

Examination of the wheat signal sequence and the synthetic cecropin gene sequence reveals the presence of several codons which are rarely used by *S. cerevisiae*. For example, the yeast avoids the use of codons containing 100% GC content (Bennetzen & Hall, 1982) but the codon GCC occurs 6 times out of a total of 57 codons in the wheat signal/cecropin gene construct. Though yeast contain tRNAs for all possible codons, the abundance of particular iso-accepting tRNAs corresponds with the codon bias found (Ikemura, 1982). Codon bias is especially apparent with highly expressed genes so foreign genes showing a low codon bias will normally be expressed at very low levels. A change in codon bias greatly affects gene expression as shown by Hoekema *et. al.* (1987) who found that when they changed 39% of the major codons in the highly-expressed *PGK1* gene to minor ones, the yield of PGK protein decreased 10 times. The decrease was due to some decrease in mRNA level and also a decrease in translation. One can envisage that a lack of certain rare iso-

accepting tRNAs could lead to ribosomal pausing with concomitant degradation of mRNA. Conversely, Kotula and Curtis (1991) optimised the codons in an immunoglobulin kappa chain gene from mouse for expression in yeast and found a 50-fold increase in protein yield. In this instance, mRNA levels remained the same but the rate of translation increased 5-fold.

It would therefore appear that a codon bias optimised for expression in plants, such as that in the synthetic cecropin gene, was not ideal for expression in yeast. It is still possible that very small amounts of cecropin were synthesised but at levels too low to detect by the methods used.

Northern blot analysis of RNA isolated from pEMBLyex4 and pYWCE1 following promoter induction showed that the *ura* plasmid marker rapidly decreased up to 2 hours following induction in pYWCE1 (Figure 3.6c). This decrease was shown to be due to a drop in plasmid copy number since total cell number remained the same over the time course (Figure 3.7). The decrease in plasmid copy number could be due to attempts by the yeast to minimise the use of rare codons. If promoter induction is leading to synthesis of cecropin there will be a marked drop in the amount of rare iso-accepting tRNAs available in the cell. This situation may prove to be toxic if some rare charged tRNAs are required for the synthesis of key metabolic enzymes. A rapid loss of excess plasmid, retaining only enough copies to enable growth on uracil deficient media, may therefore be occurring to minimise possible toxic effects resulting from a rapid depletion of rare tRNAs.

Addition of the VTE element to the cecropin construct, to produce the plasmid pYWCE2, appeared to have a deleterious effect on yeast. No high copy number transformants containing this plasmid could be selected, neither could RNA in sufficient quantities for a northern blot be obtained due to the low density to which the cells grew in selective media. The plasmid was also found to be highly unstable when grown in either selective or non-selective media (Figure 3.8). These phenomena indicate that cecropin was possibly being produced but was also toxic to the cells. Previously it was concluded that yeast containing pYWCE1 were possibly producing cecropin but in too low an amount to be detected. If translation were to be greatly enhanced by the VTE element there are two consequences: the first is that translation enhancement leads to a complete depletion of the pool of available

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rare iso-accepting tRNAs, ceasing production of key metabolic enzymes that may contain rare codons; the second is that before the pool of rare tRNAs is completely depleted, enough cecropin is synthesised to exert toxic effects on the yeast cells. A deleterious effect was seen on yeast containing pYWCE2 whether the promoter was induced or not. However, some promoter activity under non-inducing conditions could be due to incomplete repression of the galactose inducible promoter, or to a deficiency of glucose in the medium leading to derepression of the promoter. Any slight promoter activity could then be greatly enhanced by the VTE element.

Product toxicity appears to be a general characteristic of proteins that insert into cell membranes. Some examples include the expression of polyoma virus middle T antigen in which the toxicity of the protein was thought to be due to non-specific insertion and disruption of the intracellular membranes of the host yeast. During growth, the yeast either lost the plasmid or truncated proteins were produced that were non-toxic (Belsham et. al., 1986). One example shows several of the same characteristics as yeast containing pYWCE2 and that is the expression of human immune interferon-y. In this case cells failed to grow to high density in culture and the vector plasmid was highly unstable in both selective and nonselective conditions (Fieschko et. al., 1987). These examples would appear to indicate that yeast containing pYWCE2 are synthesising a toxic product which is affecting the host. Cecropins exert their effect on cell membranes. They are highly soluble in water where they exist as random coil structures which is the form adopted in insect haemolymph. When brought into contact with bacterial membranes, cecropins fold into an amphipathic helical structure (Steiner, 1982) which, as aggregates, have the potential to form large pores in bacterial membranes (Christensen et. al., 1988). A positive surface charge or incorporation of cholesterol into membranes decreases sensitivity to cecropins up to 60-fold (Christensen et. al., 1988) hence the reason why eukaryotic cells are far more insensitive to cecropins than bacterial membranes. Intracellular membranes have a far lower cholesterol content and could therefore be highly sensitive to the presence of cecropin. It can be envisaged that yeast containing pYWCE2 produce some cecropin which, instead of being secreted, causes pore formation in intracellular membranes and thereby proves to be toxic.

Cecropins are naturally secreted into insect haemolymph. A major difference between the

cecropin clones isolated from insects and the synthetic gene used in the yeast constructs is the possession of a pro sequence as well as a signal sequence (van Hofsten *et. al.*, 1985; Lidholm *et. al.*, 1987). The importance of the pro sequence has been established in several cases including the expression of thaumatin in yeast in which the expression of the preprothaumatin gene was far higher than the expression of genes encoding partly mature forms (Overbeeke *et. al.*, 1989).

The importance of pro sequences has also been shown in the expression and secretion of defensin. The defensins are similar to cecropins in being small, basic proteins that are induced in insects in response to antibacterial infections. High levels of expression in yeast were achieved by making fusion constructs with the yeast pheromone mating factor α (MF α 1) secretion signal and pro sequence attached to a synthetic defensin gene designed to incorporate the yeast preferred codon bias (Reichhart *et. al.*, 1992).

Successful expression and secretion of up to 600 μ g/ml of cecropin A has also been achieved by using a baculovirus vector with *H. cecropia* pupae as host (Hellers *et. al.*, 1991). In this instance, the cecropin A cDNA containing the entire preprocecropin sequence was used. Hellers *et. al.* (1991) initially used the clone for cecropin B which lacked a pro sequence but though they were able to detect mRNA they found no protein product, implying that the pro sequence is essential for product synthesis and secretion. It is possible that the pro sequence is required to prevent formation into active amphipathic helical structures while in close contact with intracellular membranes until secretion has occurred.

The general conclusion is therefore that cecropin was probably being produced by the yeast but low yields, due to product toxicity and presence of rare codons in the cecropin gene, prevented detection. Lack of a pro sequence possibly led to poor or no secretion and a lethal effect on the host yeast cells.

7.2.2 Future work regarding secretion of cecropin

One of the conclusions of the yeast work was that cecropin may have been produced but

could not be detected. The assays themselves could be improved by using buffered plates in the overlay test to eliminate inhibition of bacterial growth caused by acidification of the medium by the yeast. Addition of small amounts of lysozyme to the plates, in amounts too small to exert an antibacterial effect alone, could also increase the sensitivity of the test (Professor Peter Ham, Keele University, personal communication).

if lack of detection of cecropin were due to low yield, there are many ways in which yield could be improved. These include changing the codon bias of the synthetic gene to reflect that found in highly expressed genes, for example, high yields of defensin were possible by using a yeast codon bias (Reichhart *et. al.*, 1992). The secretion signal used can be changed to improve secretion efficiency. For example, use of the signal sequence from yeast α -factor mating type has been found to result in the efficient secretion of heterologous proteins (Brake *et. al.*, 1984; Bitter *et. al.*, 1984). A change of host from *S. cerevisiae* to the yeast *Pichia pastorls* which has been found to give high levels of expression of heterologous proteins (Cregg *et. al.*, 1987; Tschopp *et. al.*, 1987; Digan *et. al.*, 1989) may also improve yield.

However, the aim of the yeast work was not to ultimately achieve high expression levels of cecropin in yeast, but to try and determine if the constructs needed altering in order to achieve detectable expression levels in plants. When the synthetic cecropin gene and wheat α -amylase secretion signal were transformed into tobacco plants under the control of the CaMV 35S promoter, cecropin transcripts were detected on northern blots but no protein product could be isolated or detected. The major difference between the tobacco and yeast work was that in yeast the synthesis of cecropin was under the control of an inducible promoter. Since the yeast work indicated that the synthesis of cecropin within ceils may be toxic due to potential effects on internal cell membranes, the synthesis of cecropin under the control of the selection of transformants that were synthesising cecropin successfully. Only transformants containing a defective translation of cecropin may have been selected. The yeast work indicated that cecropin was possibly being synthesised and comparisons with other successfully expressed and secreted proteins suggested that a major problem with the constructs, which may have led to cell toxicity, was the lack of a pro sequence. Successful expression and secretion of

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cecropin from plant cells may therefore be achieved by the addition of a pro sequence to the cecropin gene, and also by placing the synthesis of cecropin under an inducible promoter so that even if the production of cecropin within cells proves to be toxic, this will not prevent the selection of transformants that are capable of synthesising cecropin.

7.3 The cloning of DD1/asparagus SOD

7.3.1 Summary and discussion of results

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Previous analysis of poly (A)+ RNA isolated from the asparagus cell culture system at varying days following the initial cell isolation procedure had identified an induced message which appeared to have the expression characteristics required of a promoter to be used in the genetic engineering of plants for bacterial resistance (see section 4.1). This induced message was named DD1 and was seen to be rapidly activated by the wounding process with expression sustained for several days (Figure 4.1). Protein isolated from 2-D gels enabled the synthesis of an antibody to the DD1 protein and also the production of an N-terminal microsequence of 28 amino acids. The microsequence initially showed no homology to known proteins. Screening of an asparagus cDNA expression library using the DD1 antibody failed to identify a positive clone, most likely due to the low titre of the antibody. Using oligonucleotides designed to the N-terminal amino acid microsequence as probes also failed to identify clones containing homology to the N-termInal amino acid sequence. However, sequence analysis of one of the three clones identified by the 26 nt oligomer, pDD1-5, was found to have high homology to at least two glycine-rich proteins (Figure 4.10) which are involved in cell wall strengthening and are induced in response to wounding (recently reviewed by Showalter, 1993).

Later database searches also revealed that pDD1-5 had high homologies to several keratin genes. Of the total of 292 bp of nucleotide sequence obtained for pDD1-5, 63.4% identity over 142 bp at the nucleotide level was shown to the *Xenopus laevis* mRNA for a 51 kD cytokeratin type I (Hoffmann *et. al.*, 1988), and 61.7% identity in a 167 bp overlap to the

mouse epidermal 67 kD type II keratin mRNA (Steinert *et. al.*, 1985). The homologies are confined to the N-terminal regions of the keratin genes which are highly glycine-rich and are thought to be responsible for the formation of an insoluble structure following an interaction with epidermal matrix proteins (Steinert *et. al.*, 1984). The relationship between the structure and function of plant GRPs and animal cytokeratins has previously been noted by Rohde *et. al.* (1990) who found homology between a *Hordeum vulgare* GRP and a mouse cytokeratin.

Though the clone pDD1-5 was not shown to have homology to the DD1 N-terminal amino acid sequence, the discovery that it had homology to a class of wound-inducible genes, the GRPs, does Illustrate that the asparagus model cell culture system may enrich for wound induced genes when, out of 3 clones effectively isolated at random, one of them showed homology to known wound-inducible genes.

Continuing database searches with the DD1 N-terminal amino acid sequence eventually revealed a homology of 82% with the maize SOD2 gene (Cannon *et. al.*, 1987; Figure 5.1). This positively identified DD1 as a cytosolic Cu/Zn SOD which enabled a targeted PCR approach to be used to isolate the corresponding clone. A clone, pSOD6, was obtained which showed 100% homology with the N-terminal amino acid sequence (Figure 5.9a).

The discovery that the DD1 protein from which the N-terminal amino acid sequence data was obtained was in fact a cytosolic Cu/Zn SOD is not completely surprising. SODs are induced in response to an increase in the amount of free oxygen radicals present (Tsang *et. al.*, 1991; reviewed by Bowler *et. al.*, 1992) which increase in response to a variety of stresses. Initiation of cell cultures leads to a variety of stresses, some of which lead to an alteration in the pattern of SOD activity in cell cultures (Bauw *et. al.*, 1987; Figure 5.2). Harikrishna *et. al.* (1991) noted that initiation of asparagus cell cultures led to a vast increase in oxygen uptake 2-5 days following the cell isolation process, indicating that an increase in respiration had occurred. One source of oxygen radicals in ceils is the electron transport chain operating in the mitochondria. It is expected that an increase in respiration would lead to an increase in SOD activity. An increase in mitochondrial SOD activity would be expected and this is the case found in tobacco cell suspensions (Bauw *et. al.*, 1987), in which it was concluded that the

increase in respiration was caused by the presence of sucrose in the culture medium (Bowler *et. al.*, 1989). A general change in the oxygen radical balance in cells may also cause an increase in the activity of cytosolic SODs, such as the induction of the cytosolic Cu/Zn SOD seen in the initiation of asparagus cell cultures.

Expression studies of pSOD6, encoding the gene for the asparagus cytosolic SOD, showed an increase in message in cell cultures 2 days after the initial isolation procedure (Figure 5.16a). This time period of induction correlated to some extent with that seen for the induction of DD1 in 2-D gel analysis of in vitro translated poly (A)+ RNA (Figure 4.1). However, the transcript level seen with pSOD6 was very much lower than that expected for DD1 which was calculated to constitute up to 1% of the total message population (Fioroni, 1989). The low transcript level of pSOD6 was also seen during library screening in which it was found to be present at a frequency of only 1 in 10,000 in an asparagus cDNA library which had been constructed using message obtained from days 1-3 cell culture RNA. The expression analysis of pSOD6 and comparison with the 2-D gel analysis therefore indicated that it was unlikely pSOD6 was a member of the DD1 group of proteins identified in the first few days following cell culture initiation. Analysis of the pSOD6 amino acid sequence translated from the nucleotide sequence (Figure 5.8) reveals that it contains only 2 methionine residues including the initial methionine. Since [35S]-met was used to label the in vitro translated products for the 2-D gel analysis, it seems unlikely that the labelled products for pSOD6 would be visible amongst other highly labelled proteins. This observation is correlated by the in vitro transcription and translation analysis of pSOD6 alone. Using only pure pSOD6 message but again with [35S]-met as a label, a product was obtained but it was very faint (Figure 5.15).

The observation that pSOD6 was highly unlikely to be one of the DD1 group of proteins seen on the 2-D gel analysis places some doubt as to the origin of the DD1 antibody (Fioroni, 1989). The antibody was prepared by isolating a protein sample corresponding to DD1 coordinates on a 2-D PAGE gel using protein prepared from 6 week old cell cultures. The antibody was polyclonal in nature and was found to bind to all 5 members of the DD1 related group present on day 2 following cell isolation.

Close examination of the work performed by Fioroni (1989) revealed that the protein sample from which the N-terminal amino acid sequence data was obtained, which showed the identity to SOD, originated from protein isolated from 2 month old cell cultures. The protein samples used to prepare the antibody and the N-terminal sequence data therefore originated from different sources. It is highly possible that the antibody was prepared to a protein totally separate from the protein from which the N-terminal sequence was obtained but which had a similar molecular weight and isoelectric point to the DD1 protein. The identification of DD1 on 2-D gels relied on pinpointing the appropriate molecular weight and isoelectric point. Any variation in the way the gels were run would have resulted in great difficulty in identifying particular spots, especially when the abundance of spots in and around the DD1 group at 14-16 kD and isoelectric point 5.0-6.0 is considered (Figure 1.2). Confirmation that the DD1 group of polypeptides are not related to SOD could be obtained by seeing if a SOD antibody binds to the DD1 group on 2-D gel analysis.

The DD1 group of *in vitro* translated proteins seen on day 2 using 2-D gel analysis may correspond to a different group of proteins. Fioroni (1989) suggested that they may belong to a group of PR-related proteins which show similar molecular weights and expression characteristics. It may eventually be found that the PR-like gene, AoPR1, will correspond to one of the spots assigned to the DD1 group.

Further analysis of pSOD6 expression concentrated on determining whether the induction of message seen following cell culture initiation was due to wounding or was induced as a consequence of the cell culture system. Successful wound induction in clones isolated following upregulation by the cell culture system has been shown by observing upregulation in wounded darkgrown seedlings (Warner, 1992). This approach was therefore used to see if the pSOD6 clone was upregulated by wounding. However, northern analysis of wounded and unwounded dark grown seedling tissue showed that pSOD6 transcript was already present in relatively high levels in dark grown etiolated seedling tissue (Figure 5.16b). No increase in message was seen following wounding but it is possible that any response to wounding was masked by the high levels of transcript present.

High levels of pSOD6 transcript were also seen in the root tissue of green plants. In gel SOD

activity assays gave very similar profiles for protein isolated from cell culture material, from roots, and from dark grown etiolated seedlings (Figure 5.2). This result, combined with the results from the northern expression analysis of pSOD6 appeared to suggest that some factor other than wounding was responsible for the upregulation of pSOD6 seen in cell culture material.

The one factor that these tissues have in common is growth in the dark. The isolation of single cells in the cell culture system involves grinding green photosynthetically active cladode tissue and then placing isolated single cells into cell culture medium and incubating in the dark. After a few days incubation the cells lose their photosynthetic capacity and revert to a heterotrophic mode of existence (Harikrishna *et. al.*, 1992). Roots and dark grown seedlings do not photosynthesise and are heterotrophic. The potential cause of induction of pSOD6 is therefore the change from leading a phototrophic existence in the light to a heterotrophic mode in the dark. The association of cytosolic Cu/Zn SODs with tissues that are not normally involved in photosynthesis has been noted with the tomato Cu/Zn SOD (White *et. al.*, 1990).

Dark induced genes have been isolated and include a senescence-associated gene isolated from radish cotyledons (Azumi & Watanabe, 1991), and phytochrome regulated genes (Lissemore & Quail, 1988; Kay *et. al.*, 1989). However, examination of the function of SOD and the stresses involved in its upregulation suggest that it is not the light or dark in itself that is responsible for induction but the consequences of the light or dark, particularly an increase in superoxide radicals, that leads to an increase in SOD transcript and activity.

Superoxide radicals are constantly produced within the cell. in the chloroplasts this most commonly occurs when an electron from an excited chlorophyll molecule or ferredoxin is transferred to molecular oxygen (Asada & Takahashi, 1987), while in the mitochondria oxygen radicals are formed by the electron transfer chain (Loschen *et. al.*, 1974). In the cytosol, enzymic reactions are responsible for superoxide radical production with enzymes such as xanthine oxidase, aldehyde oxidase, and other flavin dehydrogenases capable of generating superoxide as a catalytic by-product (Fridovich, 1986). These superoxide radicals are

removed by a combination of non-enzymic and enzymic mechanisms, the most effective of which are the enzymes SOD and catalase which, in combination, convert the superoxide radical to water and molecular oxygen (recently reviewed by Scandallos, 1993). Any perturbation in the normal cellular processes will disturb the balance between the rate at which superoxide radicals are formed and removed. An increase in the amount of superoxide radicals will lead to an increase in the mechanisms involved in their removal in order to prevent irreversible cellular damage. Conditions that lead to an increase in photosynthesis generally increase the production of superoxide radicals in the chloroplasts (Rabinowitch & Sklan, 1980; Wise & Naylor, 1987) with a corresponding increase in SOD activity in the chloroplasts, while increases in respiration lead to an increase in oxygen radicals in the mitochondria (Bowler *et. al.*, 1989) with concomitant increase in mitochondrial SOD activity.

Cytosolic SODs appear in all cases to increase in response to stress, even if the stress appears to be mainly confined to either the chloroplasts or mitochondria. For example, cytosolic SODs have been found to increase in response to paraquat (Matters & Scandalios, 1986), wounding and drought stress (Perl-Treves & Galun, 1991), and to heat shock (Tsang *et. al.*, 1991). This may indicate that the increase in cytosolic SOD is a generalised response to increases in oxygen radicals. In fact, interchangeability between Cu/Zn SODs and MnSODs has been observed in yeast. Greco *et. al.* (1990) found that when *S. cerevisiae* is grown under conditions of limited copper, the amount of Cu/Zn SOD mRNA, activity and protein decreased while a corresponding increase in MnSOD activity and mRNA was observed.

in the case of the asparagus cytosolic Cu/Zn SOD, pSOD6, an increase in transcript is observed when cells are placed in culture medium, which is reflected by an increase in the activity band SOD-4 which was found to correspond to pSOD6 (section 5.6). By day 8 the SOD activity profile of protein from cultured cells is almost the same as that from dark grown etiolated seedlings and root (Figure 5.2). When whole asparagus fronds are supplied with sucrose, the SOD activity profile is also representative of that in roots and etiolated seedlings (Figure 5.17). Both dark grown etiolated seedlings and roots depend on sugar breakdown to provide energy while cultured cells are supplied with sugar in the culture medium. The correlation of the presence of the asparagus SOD transcript with cultured cells, dark grown

etiolated seedling and root, all tissues which are undergoing increased respiration when compared with photosynthetically active green tissue, leads to the conclusion that it may be increased respiration that causes an induction of the asparagus SOD. Wounding would be expected to increase the activity of this SOD since wounding leads to an increase in respiration but it is possible that this increase was masked in northern hybridisation analysis of wounded seedling tissue by the presence of high levels of endogenous asparagus SOD transcript.

Library screening that resulted in the cloning of pSOD6 also identified a second clone, pSOD4, that showed close similarities to pSOD6. The clones were seen to be identical at the N-terminal end of the coding region but differed in the 3' untranslated regions (Figures 5.11 & 5.12). Other cases in which two SOD clones with almost identical sequences were isolated also exist. Isin *et. al.* (1990) isolated two clones for pea chloroplastic Cu/Zn SOD which were found to differ in their 3' untranslated regions. The difference was due to a 37 bp insert found to be characteristic of transposable element insertion sites. Cannon and Scandalios (1989) isolated two genes for maize cytosolic Cu/Zn SOD, *Sod4* and *Sod4A*, for which the proteins were biochemically indistinguishable. The genes were found to have only two amino acid differences in their coding regions but differed to a greater extent in both their 5' and 3' untranslated regions. A similar case has recently been reported concerning the isolation of two cDNA clones for cytosolic Cu/Zn SOD in rice (Sakamoto *et. al.*, 1992) but in this instance greater amino acid differences were seen in the coding regions than that reported by Cannon and Scandalios for the maize clones.

Cannon and Scandalios (1989) suggest that the possession of two genes with the same coding region could confer an evolutionary advantage. If the two genes were arranged in tandem on the chromosome they could provide intramolecular recombination sites which would allow the removal of mutations through recombination events. A genomic Southern blot using an oligonucleotide designed to the N-terminal region of pSOD6 indicated that two related genes may be arranged in tandem in asparagus (section 4.3.2, figure 4.5). Confirmation of this requires further analysis, for example, by isolating overlapping genomic clones and showing that the two genes were indeed arranged in tandem.

7.3.2 Future work regarding SOD activity and expression in asparagus cell cultures

Though *in vitro* translation studies showed pSOD6 to correspond with the activity of SOD-4, this result still requires further confirmation, which could be achieved by isolation of the protein corresponding to SOD-4 and seeing if protein sequence obtained corresponds with the translated DNA sequence of pSOD6.

The isolation of two clones, pSOD6 and pSOD4, which showed homology at the 5' ends indicates that the northern blots showing expression of pSOD6 may show the combined expression profiles of both clones. Further sequencing of pSOD4 is required to determine to what extent the two clones are homologous and if hybridisation conditions can be found that are capable of differentiating between the two transcripts. In the case of maize, where two very closely related cytosolic SODs were isolated, transcripts could be differentiated by using probes originating from the 3' regions of the cDNA, where the clones were found to differ the most (Cannon & Scandalios, 1989). Since pSOD4 and pSOD6 differ in their 3' regions it may be possible to use the same approach to determine expression specific to the clones.

Though pSOD6 was shown not to be wound-induced, there is a possibility that woundinduced SODs are present as shown by the presence of induced SOD activity bands in chopped up seedlings (Figure 5.2). These could be cloned by screening the library with pSOD6 as a probe under conditions allowing hybridisation to ciones that are similar in sequence, an approach used by Cannon and Scandallos (1989) to clone cytosolic Cu/Zn SOD4 with maize cytosolic Cu/Zn SOD2 as a probe. Study of expression profiles would enable wound-induced SODs to be identified and their potential for use in the genetic engineering of resistance analysed.

The aim of cloning the gene corresponding to the spot identified as DD1 on 2-D gel analysis was eventually to enable the cloning of a promoter which would be rapidly activated by wounding with activity sustained for several days for the purposes of engineering bacterial resistance in plants. The clone identified as having 100% homology with DD1 protein microsequence was shown to be a cytosolic Cu/Zn SOD which was probably induced by

respiration and not directly by wounding. The promoter for this gene would therefore be unsuitable for use in genetic engineering since it would respond to a general stimulus rather than to the more specific wounding stimulus. However, though the promoter may only be useful for studies of the regulation of SOD, the SOD gene itself may prove to be a worthwhile target for engineering purposes.

Pathogen attack causes an increase in superoxide radical formation with a corresponding increase in SOD activity. For example, *Pseudomonas syringae* infection of tobacco induces MinSOD (Bowler *et. al.*, 1989). Some pathogens also exert their toxic effects through an oxygen radical mechanism, for example, many fungi of the genus *Cercospora* produce the toxin cercosporin. Alone, the toxin does not damage the plant but in the presence of light cercosporin reacts directly with molecular oxygen to produce either singlet oxygen and /or superoxide radicals leading to toxic effects on the plant cells. Williamson and Scandalios (1992) showed that in maize though SOD transcript levels accumulated dramatically in response to the toxin, total SOD activity and individual SOD isozyme levels remained constant, suggesting that protein turnover may be involved in the response of SOD to oxygen radicals.

The activity of SOD in pathogenicity varies according to the compatibility or incompatibility of the interaction. The involvement of superoxide radicals in the hypersensitive response in the incompatible interaction has been discussed in section 1.9.1. Basically, evidence is accumulating showing that superoxide and SOD are involved In establishing either a compatible or incompatible interaction between plant and pathogens. It appears that superoxide radicals are required for the hypersensitive response to occur in an incompatible interaction. If SOD activity is rapidly induced following pathogen infection, superoxide radicals are removed, there is no hypersensitive response and a compatible interaction is established (Zacheo & Bleve-Zacheo, 1988; Hwang *et. al.*, 1991). In contrast, the induction of SOD activity is delayed or induction occurs to a far lower extent in an incompatible interaction, allowing the establishment of a hypersensitive response.

it may therefore be possible to engineer resistance to pathogens in plants through manipulating SOD activity in such a way that it follows the pattern of an incompatible response. This would require that SOD activity is not induced following pathogen attack to allow the formation of a hypersensitive response. This could be achieved by using antisense to SOD genes attached to a promoter that is rapidly activated following pathogen infection. This would decrease SOD activity levels, allowing superoxide radicals to establish a resistance response. An alternative approach would be to engineer the increased production of oxygen radicals at infection sites to stimulate the establishment of a hypersensitive response, perhaps by engineering enzymes that form superoxide radicals as a by-product, such as xanthine oxidase or aldehyde oxidase (Fridovich, 1986).

Though the promoter for the asparagus gene, pSOD6, is unlikely in itself to be useful in engineering resistance, the study of the function of SOD and particularly its involvement in pathogenicity, has revealed possible alternative approaches to the engineering of resistance in plants to pathogens.

7.4 Expression analysis of a wound-inducible promoter in potato

7.4.1 Summary and discussion of results

The isolation of the AoPR1 promoter (Warner *et. al.*, 1993) provided a promoter which showed the required expression characteristics of rapid induction with sustained activity for use in the engineering of plants for bacterial resistance. Activity of this promoter was therefore analysed further using the GUS reporter gene in potato, a target for engineering resistance to bacterial soft rot.

All transformed potato plants obtained showed wound-inducible GUS expression (Figure 6.2b), varying mainly in quantitative expression (Figure 6.4). Some variation was observed in expression seen in root and stem tissue with some lines showing constitutive expression in both tissues and other lines showing either no constitutive expression or expression in only one of these tissues. Differences in expression of the reporter gene under the control of the same promoter are presumed to be due to the actual site of insertion of the AoPR1

promoter/gus A construct into the potato genome. However, the results did indicate that all transgenic potato lines obtained containing the AoPR1 promoter/gus A construct showed wound-inducibility of expression. This indicates that the wound-inducible element of the promoter is strong and is affected only quantitatively by its insertional position. Wound-inducible expression was found to be highly localised to a few cell layers surrounding the wound site. This same pattern of expression was observed following infection by various pathogens in which expression of the gus A reporter gene was highly elevated around sites of dead and damaged tissue caused by the infection (Figures 6.9 b & 6.10 b, d).

Many genes have been isolated which are upregulated both by wounding and pathogen attack and many of these are involved in the general plant defence response. For example, chalcone isomerase, an enzyme that catalyses a step common to the synthesis of flavonoid pigments and isoflavonoid phytoalexins, is upregulated in bean following infection with *Colletotrichum lindemuthianum* and also by mechanical wounding (Mehdy & Lamb, 1987). Chitinase in bean is also upregulated by the same two stimuli (Hedrick *et. al.*, 1988). Other enzymes upregulated both by wounding and infection include phenylalanine ammonia lyase (Cramer *et. al.*, 1989; Lois *et. al.*, 1989), 4-coumarate CoA ligase (Schmelzer *et. al.*, 1989), and a tomato anionic peroxidase gene (Mohan *et. al.*, 1993), an enzyme thought to cause polymerisation of phenolic residues into cell wall polymers.

The activation of some genes by both wounding and pathogen attack is not surprising. Many pathogens gain entry into plants through wound sites so it is to the advantage of the plant to have a defence system activated by wounding. Some of the genes that are activated also serve a common purpose, for example, the upregulation of genes involved in strengthening the cell wall such as the extensins (Rumeau *et. al.*, 1990) serves to protect a wound site from desiccation and also acts to prevent the entry and spread of pathogens.

Some genes differ in their response to wounding and pathogen invasion. For example, mRNA for a proline-rich protein from bean was found to decrease in response to treatment of cell cultures with fungal elicitor, but increased following wounding (Sheng *et. al.*, 1991). In some cases a differential response to wounding and pathogen infection has been shown to be due to the possession of a multigene family in which individual members of the family

respond to either wounding or pathogen challenge. This has been shown to be the case with 3-hydroxy-3-methylglutaryl coenzyme A reductase (Yang *et. al.*, 1991), and with the β -glucanase genes in which wounding strongly induces the basic glucanase genes but has little effect on the acidic isoforms (van de Rhee *et. al.*, 1993).

Differences in the response of some genes to pathogen infection are due to the compatibility or incompatibility of the reaction which is dependent on the possession of resistance genes in the plant and corresponding virulence genes in the pathogen (see section 1.9.1). For example, an incompatible interaction in French bean showed rapid, localised accumulation of PAL and CHS transcripts, while in the compatible interaction, induction of these transcripts was delayed and not localised (Bell *et. al.*, 1986). The induction of some genes, however, shows no difference between a compatible and incompatible interaction, for example the β -glucanases and chitinases in potato (Schröder *et. al.*, 1992). Transcript accumulation for many genes involved in the defence response showing incompatible dependent and independent responses were studied by Jakobek and Lindgren (1993) who eventually concluded that there are two mechanisms involved in the induction of defence transcripts, a general mechanism which responds equally to both wounding and pathogen stimuli, and a more specific mechanism associated with the incompatibility of a reaction with the induction of a hypersensitive response.

A phenomenon commonly associated with the hypersensitive response is systemically acquired resistance (SAR) in which defence related genes are activated at distances away from the initial site of infection (see section1.8.3). SAR therefore requires the transmission of a signal from the affected site to other, non-affected, parts of the plant. Pathways involving salicylic acid (eg. Malamy *et. al.*, 1990; Kauss *et. al.*, 1992), jasmonic acid (eg. Farmer & Ryan, 1992; Grimes *et. al.*, 1992), and ethylene (eg. Ecker & Davis, 1987; Weiss & Bevan, 1991) are thought to be involved (reviewed by Maiamy & Klessig, 1992; Enyedi *et. al.*, 1992). However, no evidence was found showing that wounding or pathogen attack activated the AoPR1 promoter in a systemic manner (Figure 6.8). Salicylic acid was found to activate the promoter only when wounding was applied at the same time (Simon Warner, personal communication). SAR is most commonly associated with the hypersensitive reaction in plants which is the result of an incompatible interaction between plant and pathogen. Since the potato cultivar

used, Desiree, has no major resistance genes, the effect of an incompatible interaction, which may lead to SAR, could not be studied. However, results obtained from infection of the transgenic potato plants showed that the AoPR1 promoter did not respond to the presence of the pathogen itself, but reporter gene expression only became detectable once visible cell damage had occurred (Figure 6.9d). This indicates that it may be cell damage caused either by mechanical wounding or through the growth and replication of a pathogen that activates the AoPR1 promoter.

Possible elicitors leading to activation of the AoPR1 promoter may therefore include cell wall fragments derived from either plant or pathogens (reviewed by Ryan & Farmer, 1991; Farmer *et. al.*, 1991; see section 1.8.2). During the course of infection by, for example, *E. carotovora*, hydrolytic enzymes are released by the pathogen which degrade the plant cell wall. These include polygalacturonase, pectinesterase, pectin lyase and cellulases (reviewed by Lyon, 1989) which release specific plant cell wall fragments. Pectin has been shown to regulate plant defence responses. For example, the addition of pectic fragments to a suspension of castor bean cells resulted in lignin synthesis detectable within 3 hours (Bruce & West, 1989). The induction of plant β -glucanase and chitinase enzymes by pathogen infection (for example, Schröder *et. al.*, 1992) results in the release of elicitor active fragments from pathogen cell walls. These include the β -glucans and it has been shown that a β -1,3-glucanase induced in soybean seedlings as a result of pathogenic infection releases phytoalexin elicitor active fragments from the walls of the pathogen (Ham *et. al.*, 1991). β -Glucan elicitor binding sites in the membrane fractions of soybean roots have also been identified (Schmidt & Ebel, 1987).

β-Glucanases, induced as a response to both wounding and pathogen attack, are localised both to the cell wall and to the vacuole (van den Bulcke *et. al.*, 1989; Mauch & Staehelln, 1989). It is therefore possible that the cell wall localised β-glucanases are involved in the activation of further β-glucanases and other defence genes through the production of βglucan elicitors, as suggested by Simmons *et. al.* (1992). The vacuole localised enzymes, which include chitinase (Boller & Vögeli, 1984) as well as β-glucanase, may form a final line of defence against pathogens once the cell lyses (Mauch & Staehelin, 1989). The vacuole contains several hydrolytic enzymes (Boller & Kende, 1979) and it is possible that rupture of

the vacuolar membrane by wounding or pathogen attack releases enzymes that in turn release elicitor active fragments from plant and pathogen cell walls. The effect of cell wall derived elicitors on AoPR1 promoter driven GUS activity has not been analysed so further conclusions conerning the activation of this promoter cannot be made at this stage.

The available information concerning the AoPR1 promoter at present indicates that it is activated as a result of a general mechanism in response to cell damage caused either mechanically or by pathogen invasion. AoPR1 promoter/gus A constructs need to be transferred into potato lines containing major resistance genes in order to assess the response of the promoter to an incompatible interaction before further conclusions can be made concerning the activation of this promoter. Preliminary data from the analysis of transgenic tobacco containing the AoPR1/gus A construct conducted by Dr. Luis Mur at Leicester University does, however, indicate that the incompatibility or compatibility of a pathogen interaction makes no difference to the activity of the AoPR1 promoter. Activation of the promoter, leading to reporter gene expression, can only be observed once cell damage has occurred.

7.4.2 Future work involving the use of the AoPR1 promoter in engineering disease resistance

Following wounding in tobacco, the induction of the *gus* A reporter gene under the control of the AoPR1 promoter was detectable within 3 hours with activity increasing up to 4-5 days (Warner *et. al.*, 1992). Expression was also highly localised to the actual wound site with no systemic induction observed following the wound stimulus. This pattern of expression was therefore that required of a promoter to be used for the engineering of bacterial resistance in plants by the wound-inducible secretion of an antibacterial substance.

However, the AoPR1 promoter was also found to be expressed in the vascular tissue at sites of secondary thickening and in parts of the floral tissues, including the pollen, when expression of a reporter gene was studied in tobacco (Warner, 1992). Constitutive expression in the vascular tissues of young potato leaves was also noted with high

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expression in tubers. A promoter is required that responds only to wounding since the expression of a foreign protein in various parts of the plant is obviously undesirable, particularly expression in pollen which may lead to concerns about the escape of toxic proteins into the food chain.

Many promoters direct expression which is not confined to one particular stimulus. For example, the PR proteins in tobacco not only accumulate in response to pathogen infection but are also present during flower development (Lotan *et. al.*, 1989). If the same gene product serves many purposes, it is advantageous to the plant to direct the expression of that product using a promoter which responds to different situations. This means that it is highly unlikely that a promoter will be found that responds purely to wounding.

Deletion analysis of the potato PI II gene promoter is beginning to identify regions of the promoter that confer wound-inducibility (Keil et. al., 1990), and a sequence has been identified that binds nuclear protein extract from wounded tomato leaves (Palm et. al., 1990). A deletion of approximately 200 base pairs from the 5' region of the AoPR1 promoter was found to eliminate wound-inducibility of expression but retained some of the developmental expression (Warner, 1992). This data illustrates that promoters are constructed of regions which respond specifically to different stimuli. It may therefore be possible to design a promoter which retains the required characteristics but eliminates unwanted expression. A wound-inducible element could then be attached to enhancers to provide entirely specific promoters with predictable activity. The problem of insertional position affecting expression could be eliminated by the use of locus control regions (LCRs) or scaffold attachment regions (SARs). These are sequences of DNA which have been found to confer positionindependent expression of the chicken β -globin gene in transgenic mice (Reitman et. al., 1990), and of the GUS reporter gene in tobacco (Breyne et. al., 1992) when attached to the 5' end of promoters. Designing promoters using this building block approach will only become possible once more analysis of promoters, including the AoPR1 promoter, takes place. Analysis will include making deletions of regions of the promoter from both 5' and 3' ends and also from central regions, and also mutations of regions identified as conferring specific properties. This analysis will probably require several years of work but crude chlmaeric promoters may be constructed at present using regions already identified as conferring

wound-inducibility and attaching them to minimal promoters such as the -90 bp region of the CaMV 35S promoter (Fang *et. al.*, 1989).

Desirable elements of the AoPR1 promoter which should be identified and retained are regions conferring wound-inducibility of expression and localisation of expression so that wide spread induction of a toxic protein is not a problem. The induction of the AoPR1 promoter by pathogens also appears not to depend on the pathogen but the damage that it inflicts on a cell. A promoter that is not affected by the compatibility or incompatibility of an interaction is required since its activity would be more predictable. Further work analysing the activity of this promoter in relation to incompatible Interactions is needed to confirm that it is not affected by the presence of the pathogen itself.

Work analysing the effects of various elicitors on AoPR1 activity should also be conducted. The induction of the AoPR1 promoter by an elicitor would enable a crop of harvested potatoes to be sprayed prior to storage to ensure the immediate production of an antibacterial protein. The soft-rot bacteria are soil dwelling and are therefore given most opportunity to infect potato crops at the time of harvesting which not only may wound the crop but also harvests the bacteria in conjunction with the potato crop. If the entire crop were induced to synthesise the immediate production of an antibacterial protein this may prevent infection and so eliminate any future problems with soft rot that are normally encountered during storage. Spraying with an elicitor will also provide a second strategy to combat soft rot should the wound-inducible element fail to confer protection.

7.5 Final conclusions

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A major aim of plant breeders is to improve disease resistance in plants. Traditional plant breeding methods require the identification of a resistant line which is then crossed with a desired line to obtain the required line with improved resistance. However, for some bacterial infections of plants, such as soft rot caused by *Erwinia* species, no resistant lines are available and alternative methods have to be employed to increase resistance in plants. It is in such a

situation that the use of genetic engineering provides a complementary tool to the plant breeder. One approach to engineering resistance in potato to soft rot is to induce the secretion of an antibacterial substance at wound sites which provide entry points for the bacteria. Elements of this approach were examined in this thesis, including the secretion of the antibacterial substance, cecropin, and the analysis of a suitable wound-inducible promoter. Future continuation of this work will require that constructs are made with the wound-inducible AoPR1 promoter attached to a full cecropin sequence (containing pre and pro sequences) with the wheat α -amylase secretion signal. Once transgenic potato plants are obtained, they can then be assessed for any increased resistance to *E. carotovora* infection.

Recent papers indicate that this approach to engineering resistance will probably be successful. For example, a major component of this approach is the use of a wound-inducible promoter to target cecropin to the sites where it is most likely to encounter the soft rot bacteria. A similar targeted approach was used by Fehér *et. al.* (1992) who produced Potato Virus X (PVX) resistant potato plants by expressing PVX coat protein under the control of the ethylene-inducible carrot extensin gene promoter. Virus infection leads to an increase in endogenous ethylene production which then activates the promoter, ensuring that coat protein is produced when required.

Another property of the proposed route for increasing resistance was the use of a cecropin gene which had been modified for maximum expression in plants by altering codon-usage. This approach has also been adopted by two groups who have both modified the *cryIIIA* δ -endotoxin gene from *B. thuringiensis* to reflect plant codon usage (Adang *et. al.*, 1993; Perlak *et. al.*, 1993). Both groups have shown that transgenic potato plants were obtained with increased resistance to the Colorado potato beetle under laboratory (Adang *et. al.*, 1993) and field conditions (Perlak *et. al.*, 1993).

An important aspect of the proposed approach to engineering resistance was the secretion of the antibacterial protein. Düring *et. al.* (1993) have recently shown that potato plants containing T4 lysozyme attached to the barley α -amylase secretion signal under the control of the CaMV 35S promoter possess increased resistance to soft rot caused by *E. carotovora*. This example illustrates very strongly that it is possible to engineer increased resistance to

soft rot bacteria. The approach outlined in this thesis, however, involved the use of a woundinducible promoter in the expectation that this would restrict the production and secretion of antimicrobial substances to sites where they will be most effective, rather than having a more constitutive production of the substance throughout the plant.

The most striking aspect of the approaches people have used to engineer resistance in plants is that they all use some component of the plant's natural defence system. For example, the defence genes are inducible either by wounding or pathogen attack; some defence gene products, such as the chitinases and β -glucanases, are secreted into the intercellular fluid; and antimicrobial substances such as the phytoalexins are produced. It is therefore by studying the plant's own highly successful defence system that further approaches will be revealed that enable the genetic engineering of increased resistance to be achieved.

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