Isolation Of Antibody Fragments Recognising Phytopathogen Secreted Enzymes And The Expression Of scFvs In Transgenic Tobacco.

Thesis Submitted For The Degree Of
Doctor Of Philosophy
At The University Of Leicester

By

Neil Gavin Taylor BSc (Hons) (Leicester)
Department Of Botany
University Of Leicester

November 1997.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Acknowledgements.

I would like to thank my supervisor Prof John Draper for his advice and help in this project. I am particularly grateful to Dr Simon Firek for his extensive help and advice throughout the years (and of course his constant good humour!). I would like to thank Dr Meran Owen for his advice on growth of hybridoma lines, and to Dr Garry Whitelam for useful discussions. I am also indebted to all those who have supplied materials, including Prof George Salmond for the pelC clone, Louis Shain for the gift of *C. parasitica* PG, and members of Dr Greg Winters labs for their helpful discussions. I would also like to thank Dr Jon Ride for supplying *B.cinerea* PG, and also for interesting discussions. Thanks also go to all the people that have I worked in a lab with, particularly Rob Darby, Mark Skipsey, Dave Martin and Luis Mur.

Particular thanks go to my family, for constant support both morally and financially. I would also like to thank all my friends from outside the department for keeping me sane.

Abstract

Isolation Of Antibody Fragments Recognising Phytopathogen Secreted Enzymes And The Expression Of scFvs In Transgenic Tobacco.

Neil Gavin Taylor

The expression of antibody fragments has been suggested as a possible mechanism for the introduction of disease resistance into transgenic plants. Important pathogenicity factors of certain phytopathogens are secreted cell wall degrading enzymes. Pectate lyases and polygalacturonase are important cell wall degrading enzymes secreted by *Erwinia carotovora* and *Botrytis cinerea* respectively. The blocking of activities of these enzymes could offer an opportunity for reducing the ingress of these pathogens. Pectate lyase C from *E. carotovora* was purified to near homogeneity by cation exchange chromatography following overexpression in *E.coli*. Hybridoma lines recognising this enzyme were obtained and an scFv (single chain Variable fragment) isolated from one of these lines. This scFv did not recognise pectate lyase. The purified enzyme was used to inoculate mice to construct libraries of scFvs derived from spleen mRNA. These libraries did not yield any scFvs recognising pectate lyase. A synthetic human scFv library was then screened with pectate lyase and *Botrytis cinerea* polygalacturonase (PG), and scFvs recognising PG isolated. These scFvs were tested against a range of antigens to determine specificity. One scFv, C1, was found to bind PG but did not bind to control antigens bovine serum antigen, hen egg lysozyme or pectate lyase. Neither did this scFv bind to PG isolated from another fungal pathogen, *Cryphonectria parasistica*. scFv C1 was found to bind to a carbohydrate epitope on the *B. cinerea* polygalacturonase .
For the strategy of expressing scFvs to introduce disease resistance to be successful, scFvs must accumulate extracellularly in tissue of transgenic plants, where they would come into contact with phytopathogen secreted enzymes. Two unrelated scFvs were introduced into tobacco and targeted to the cytosol and the apoplast. Despite the presence of transcript, no scFv protein was detected by an antibody recognising a peptide TAG made as a translational fusion with the scFvs.

Table Of Contents.

Figures.

Tables

Chapter 1: Introduction.

1.1 Plant Disease.

In nature, the occurrence of plant disease does not normally represent a noticeable problem. When plants are grown as crops however, huge losses are incurred due to the symptoms of many different diseases. It is the sheer intensive nature of agriculture that gives rise to many of these problems. In a natural environment spread of disease may be prevented by the presence of mixed vegetation, but in a crop field the close proximity of the same variety of plant allows the rapid development and spread of disease. It is hard to quantify the financial losses caused by plant disease, but the extent of research into the breeding and development of plants showing resistance to important diseases gives an indication of the magnitude of the problem.

1.1.1 Fungal Plant Pathogens.

Possibly the most important group of plant pathogens are represented by the fungal plant pathogens. Although most of the estimated 100 000 species of fungi are saprophytic in their nutrition and feed solely on dead material, approximately 8000 can cause disease on plants (Schafer 1994). There are several possible steps that may be crucial in a fungus being pathogenic including initial attachment of the fungus to the plant surface, germination and the formation of infection structures, penetration of the host plant and colonisation of the host. The level of knowledge of these stages in the pathogenesis of fungi is rather rudimentary. This is largely because until recently, efficient methods of gene cloning have not been widely available for fungi, resulting in only a basic understanding of the molecular biology of these species. There is also a great diversity in the fungi that are plant pathogens. This is in contrast to the situation in bacterial plant pathogens, where relatively few closely related species are the causal agents of most bacterial plant diseases. Add to this the fact that some economically important fungal plant pathogens such as rusts and mildews are obligate pathogens (and are hence difficult to cultivate in the laboratory), and it is easy to understand why the level of knowledge is so basic.

An important example of a fungal plant pathogen is *Botrytis cinerea*. This species not only attacks the flowers, fruits and vegetables of a wide range of

species in temperate regions as a necrotroph, but also commonly establishes latent infections which often leads to post-harvest grey mould (McNicol *et al.*,1985). The host range of this pathogen is very broad, and extends from soft fruits such as strawberry and raspberry, through pomaceous species such as pear, to crops such as cucumber. *Botrytis cinerea* is considered to be the most important soft fruit pathogen. The mode of infection of this fungus is similar to many bacterial pathogens in that it relies on the secretion of several enzymes which macerate plant cell walls. The most important of these is thought to be polygalacturonase, which is the first enzyme secreted by many fungal pathogens when grown on isolated cell walls (Johnston and Williamson 1992). It has also been seen that endo-polygalacturonases are constitutively expressed in ungerminated and germinating conidia of *Botrytis cinerea*. Several groups have isolated the different PG isoforms from a number of different isolates, and it appears that the number of isoforms vary widely, depending on the isolate used and the age of the culture from which the enzymes were purified. This variability in the type and number of isoforms reflects the wide variation in the phenotype and aggressiveness of this species, which in turn reflects its very broad host range (Johnston and Williamson 1992).

1.1.2 Viral Plant Pathogens.

Viruses are simple pathogens usually consisting simply of one or more nucleic acid molecules encased within a protein or lipoprotein case. Viruses are only able to replicate within a suitable host, where their replication is dependent on the protein-synthesising machinery of that host. The nucleic acid making up the viral genome can be either single or double stranded, and DNA or RNA. Due to the simplicity and small size of these genomes however (approximately 10^4-10^6 bp), they have been well studied. The known functions of plant viral gene products can be collected into several different groups. The structural proteins are merely the coat proteins of the simple viruses, and the core proteins of those viruses possessing a more complex structure. Enzymes produced by viruses include proteases in those viruses requiring processing of polyproteins created by the translation of the whole, or a segment, of the genome. There are also enzymes produced that act as a nucleic acid polymerase in some manner. These polymerases can, for example, copy a whole RNA genome in which case it is known as a replicase, or copy full length genomic RNA into genomic DNA - a reverse

transcriptase. In several groups of viruses, a viral encoded protein has been shown to be essential for movement within the host plant, in either a cell to cell or systemic manner.

As stated previously, the infecting virus relies on functions provided by the host in order to multiply and spread. Viruses use amino acids and nucleotides synthesised by the host in order to build viral proteins and nucleic acids, and energy from the host is required for the polymerisation involved in this synthesis, and this is usually provided in the form of nucleotide triphosphates. In order for synthesis of viral proteins, the ribosomes, tRNAs and other enzymes and factors involved in protein synthesis must be supplied by the host. Although most viruses supply a polymerase activity appropriate to their nucleic acid synthesis, some still rely on host proteins to become involved in genome replication. This reliance on the host reflects the simplicity of viruses as compared to fungi or bacteria. Despite this simplicity, they cause massive economic losses of crop plants. The symptoms shown by susceptible hosts to viral infection are varied, and the major symptom caused by specific viruses are usually reflected in the name of the virus, for example tobacco mosaic virus, tobacco ringspot virus and barley stripe mosaic virus. The sequestration of host raw materials into the production of viruses is an obvious way in which viral infection could lead to disease, and is indeed probably a factor when the plant is under some nutritional stress. It is however unlikely that this is a major factor in disease induction unless there is a pre-existing nutritional stress. One important and common symptom of viral infection is the stunting of growth. Viral infection may cause stunting by affecting the synthesis, translocation or effectiveness of plant hormones. Indeed in most situations, virus infection decreases the concentration of auxin and giberellin and increases that of abscisic acid. Plants may also become stunted if there is a reduction in the availability of fixed carbon. This may occur by direct effects on the photosynthetic apparatus, effects on stomatal opening or by reduction of the translocation of fixed carbon in the phloem. Mosaic diseases caused by viral infection are common, and the mosaic arises from dark green islands of tissue being present. These islands appear to be normal, but are resistant to super infection, and at present no convincing evidence has been shown to support the various theories that have been put forward for this. (For a comprehensive treatise of plant virology see Matthews 1991).

1.1.3 Bacterial Plant Pathogens.

The study of bacterial plant pathogens has been greatly aided by the fact that the majority of plant pathogenic bacteria are gram negative bacteria belonging to the Enterobacter family. This renders them very similar to the most studied bacterium, *Escherichia coli.* Thus the molecular techniques that have been developed over the many years that *E. coli* has been studied can largely be applied, either directly or with minor alterations, to the study of these phytopathogens. Thus the understanding of the pathogenesis of these bacteria is relatively advanced. Although the bacteria in general are quite similar and closely related, their mode of action in producing an infection and subsequent disease of a host plant can vary greatly, and a brief overview of the range of bacterial pathogens will be given here. As the virulence factors produced by these plant pathogens cannot always be closely correlated to the type of disease caused, these will be discussed separately.

1.1.3.1 Types Of Bacterial Diseases.

There are several different types of diseases caused by bacterial pathogens, leading to a wide range of symptoms and degree of severity. Necrotic diseases, such as those caused by *Pseudomonas syringae* pv *phaseolicola* on bean causing halo blight, result in localised cell death sometimes followed by the death of whole stems or blossoms (Sigee 1993). Vascular wilts are caused by invasion and colonisation of the vascular tissue. The symptoms of these diseases, such as wilting and dwarfing may only become apparent in dry weather where water supply is limiting. An example of this disease type is caused by infection of a wide range of species by *Pseudomonas solanacearum* (Sigee 1993). A related type of disease is yellows disease, caused by infection of the phloem, resulting in limited flow of photosynthetic products. Tumour diseases are caused by oncogenic bacteria such as *Agrobacterium tumefaciens,* which causes crown gall tumours on a wide range of dicotyledenous plants (Hooykaas and Beijersbergen 1994).

Soft rot diseases are caused by bacteria which are, generally, due to their mode of action, not particularly specific in their host range. The symptoms of rotting are caused by the release of cell wall degrading enzymes by the bacteria, thus releasing the contents of the cells (Perombelon 1987). Soft rot diseases are important in that they are not just found in growing tissues, but they are also important storage diseases. Storage conditions of, for example, potatoes result in the resistance of the plant tissue being reduced (largely due

to a lack of phytoalexin accumulation), but the aggressiveness of the pathogen is not affected (Perombelon 1987). Examples of soft rotting bacteria are *Erwinia chrysanthemi* and *Erwinia carotovora* subsp *carotovora*, both of which cause soft rotting of potato tubers along with blackleg, the symptoms of which include systemic vascular necrosis and wilting (Barras *et al.*,1994). These bacteria will be discussed in more detail later.

1.1.3.2 Pathogenicity And Virulence Factors.

The expression of pathogenicity and virulence factors by the bacterium is a major aspect of disease induction. Pathogenicity can be described as the fundamental ability of a pathogen to cause disease while virulence is the degree to which that disease affects the health of the plant. One of the most important pathogenicity factors is the ability of the bacteria to disrupt the host plasma membrane and cause the release of water, cations and nutrients into the extracellular medium, creating an improved environment for bacterial growth. Although this ability is possessed by most bacterial phytopathogens which also appears to be involved in eliciting a hypersensitive response in an incompatible reaction, it does not appear to be a feature of those bacterial plant pathogens which cause tumour diseases (Hooykaas and Beijersbergen 1994). The virulence of a plant pathogen relates to those factors determining the speed of growth and spread of the bacteria, and the extent of destruction of host tissue. Although there are clearly many different factors which define the virulence of a pathogen, the major virulence factors and their roles in plant disease have been studied and will be discussed here.

1.1.3.2.1 Toxins.

The release of toxins by plant pathogens is a wide ranging phenomenon. Toxins are non-enzymatic metabolic products released by the bacteria that cause damage to the host plant. These toxins can have wide ranging physical and biochemical effects such as chlorosis, water soaking, necrosis, wilting and growth abnormalities. These actions are not essential for growth and spread of the bacteria *in planta*, but may create an improved environment for the bacteria by, for example, causing remote cell damage, thus releasing water and nutrients at some distance from the bacterium. Examples of toxins are phaseolotoxin, produced by *P. syringae* pv *phaseolicola*, which affects the ornithine cycle by inhibition of the enzyme ornithine carbomyl transferase (Hartman *et al.*,1986), and syringomycin, produced by *P. syringae* pv *syringae*,

which affects plasmalemma function in infected plant tissues by the stimulation of a plasmalemma proton pump ATPase, thus altering the pH gradient across the membrane (Xu and Gross 1988).

1.1.3.2.2 Extracellular Enzymes.

The release of extracellular degradative enzymes is a feature of many plant pathogenic bacteria, particularly those involved in soft rot diseases. Pectolytic enzymes are particularly important in the development of diseases caused by these bacteria, as the pectic substances in the middle lamella of the plant cell wall are exposed to any bacteria which may be in the extracellular space. Examples of bacteria producing these enzymes are *Erwinia carotovora* subsp *carotovora*, *Erwinia chrysanthemi* and *Pseudomonas solanacearum*. There are several pectolytic enzymes produced by these pathogens, including polygalacturonases, pectate lyases, pectin lyases and pectin methylesterases. The multiplicity of many of these enzymes may reflect differences in the substrates available in different hosts and / or cooperative action for maximal maceration of the host tissue (Barras *et al.*,1994). These pectolytic enzymes will be discussed in greater detail later. Other extracellular enzymes are also produced by plant pathogenic bacteria, but their function and importance have not been dissected in such fine detail. For example the role of proteases secreted by *Xanthomonas campestris* pv *campestris* in pathogenesis is not clear, but protease lacking mutants exhibit delayed symptom development (Tang *et al.*,1987). Likewise cellulases produced by *Pseudomonas solanacearum* are important but not essential in disease development (Roberts *et al.*,1988).

1.1.3.2.3 Extracellular Polysaccharide.

The role of extracellular polysaccharide (EPS) in the development of symptoms in plant disease may be many fold. Bacteria producing EPS include *Erwinia amylovora*, *E. stewartii* and *Pseudomonas solanacearum*. Initially in infection the EPS may prevent recognition of the pathogen by the plant, as it may mask receptor sites. An additional possibility is that the initiation of a hypersensitive response may be prevented by this lack of recognition and thus an incompatible reaction be prevented. EPS may also play a role in the initiation and maintenance of water soaking, and the restriction of water movement by occlusion of xylem vessels. A change in the carbohydrate utilisation in the plant may also occur if a large quantity of EPS is produced,

by use of the plant carbohydrate and its sequestration into a plant insoluble form (Denny 1995).

1.1.4 The Plant Pathogen Interface.

The determination about whether a particular pathogen will cause disease symptoms on a given plant depends on the interaction between pathogen encoded factors with plant encoded factors. The occurrence of an incompatible reaction (ie resistance to that pathogen) is accompanied by a multi component defence response including a hypersensitive reaction, chemical 'weapons' such as phytoalexins and hydrolytic enzymes being produced, and structural barriers such as lignin and hydroxyproline-rich cell wall proteins being deposited (Dixon *et al.*,1994). This incompatible reaction is initiated in response to recognition of pathogen avirulence determinants, also known as elicitors. This recognition may be induced specifically, such as in an interaction between the pathogen's avirulent genotype and the host's resistant genotype, or non-specifically by biotic or abiotic elicitors.
Elicitors may be released from the pathogen prior to or during ingress into the host, for example pectic oligomers released by enzymatic digestion of pectic components of the plant cell wall, or they may be integral components of the pathogen surface that require some host activity before they may be released, such as B-glucans and chitin. Elicitors may also be synthesised and released in response to signals from the host (Hahn 1996). In the case of pectic oligomers being produced by the action of pathogen encoded enzymatic activity, the nature of the oligomer surrounding the bacteria may influence the final outcome of the interaction. If oligomers with a relatively high degree of polymerisation (DP) (10-15 residues) are present, the defence systems of the plant will be activated and an incompatible reaction will occur. If the oligomers are of a low DP however, this causes the production in the pathogen of inducers of pectinases, and hence a compatible, or susceptible reaction will proceed (Barras *et al.*,1994). Therefore in this case, the activity of the pectic enzymes produced by the pathogen within the plant environment will apparently determine whether disease will occur, and this will be discussed further later.

1.1.4.1 Non-Host Resistance.

The outcome of an interaction between a single pathogen and host or non-host plant tissue relies on the products of two classes of pathogen genes. The

disease specific (dsp) genes are concerned only with the development of disease in a host plant. The hypersensitive reaction and pathogenicity (hrp) genes are required however for both the development of disease within a host plant but also with the initiation of a hypersensitive reaction within a non-host plant. This can be seen in hrp- mutants where partial or complete loss of disease is seen in host plants, but these mutants also exhibit a failure to induce a hypersensitive reaction in non-host tissue (Roine *et al.*,1997). It has recently been reported that *avr* genes encode proteins which are involved in the production of a bacterial pilus, and that this may act either as a channel for protein transfer or as a cell to cell contact between pathogen and plant (Roine *et al.*,1997).

1.1.4.2 Race - Cultivar Interactions.

The situation where a specific cultivar of a species is susceptible to a specific race of pathogen but not other races of that same pathogen means that a more complex interaction is seen. This depends on the specific interaction between a pathogen avirulence gene and a plant resistance gene, and has been termed a gene-for-gene interaction. The outcome of an interaction between dominant avirulence and resistance genes is incompatible. If either the resistance or avirulence genes are recessive however, a compatible reaction will take place, leading to development of disease. Due to the necessity of the presence of both genes in a dominant state, it has been suggested that there is a direct interaction between their gene products. This gene-for-gene interaction was first described for the interaction between the fungus *Melampsora lini* and rust disease of flax (Flor 1955), but has now been seen in many other fungal race cultivar interactions, and in bacterial pathogens, such as the interaction of *Pseudomonas syringae* pv *glycinea* and different soybean cultivars (Staskawicz *et al.*,1984). The molecular basis of the interaction between R-gene products and their corresponding avr gene products has been the subject of intense study since the recent cloning of R-genes from plants which demonstrate resistance to bacterial, fungal or viral pathogens (Mindrinos *et al.*,1994, Hammondkosack *et al.*,1996). The transfer of these genes between plant species may increase the opportunities for plant disease resistance, as will be discussed later.

1.1.5 Erwinias as Plant Pathogens.

The genus *Erwinia* has long been seen as somewhat of a depository for plant pathogenic or plant associated bacteria that fit into no other genus. Because of this, the genus contains bacteria of a wide range, both in the habitat they occupy and the symptoms they cause. The range of species show many different characteristics. *Erwinia amylovora*, the causal agent of fire blight in rosaceous species, produces copious amounts of extracellular polysaccharide, as does *E. stewartii* which causes plant wilting (Denny 1995). *E. herbicola* produces many substances including antibiotics, bacteriocins, pigments and ascorbic acid (Sigee 1993). *E. carotovora* and *E. chrysanthemi* are the main causes of soft rotting of potatoes (Barras *et al.*,1994). As can be seen, the variation is quite extensive, but possibly the most interesting and important of the species are the soft rotting *Erwinias*.

1.1.5.1 The Soft Rotting Erwinias.

E. carotovora subsp *carotovora* (Ecc) is the major causal agent of bacterial soft rots of vegetables and fruits. It exhibits a broad host range, and is widespread in the environment (Perombelon 1987). Like the other members of the soft rotting Erwinia group, Ecc produces a wide range of extracellular degradative enzymes. These include pectate lyase (pel), endo-polygalacturonase (pectate hydrolase - peh), cellulase (cel), pectin methylesterase (pem), pectin lyase (pnl) and protease (Koutojansky 1987). Biochemical and genetic analyses have been carried out on these extracellular enzymes to try to determine their role in pathogenicity. Possibly the most interesting enzymes within this range are the pectate lyases and the endo-polygalacturonase. These enzymes attack the α-1,4-glycosidic linkages in pectate, polygalacturonase by hydrolysis to leave saturated products, and the pectate lyases by B-elimination to give unsaturated products. It is interesting that there is so much apparent enzymatic redundancy in these species, but this may reflect the need to act in different hosts which may provide different conditions, or the need for cooperative action in order to achieve maximum maceration of the plant tissue.

1.1.5.2 Pectate Lyases.

The pel genes of Ecc have been at the centre of research into the pathogenicity of this bacteria for many years. The different strains of Ecc produce a number

of different isoforms of pectate lyase, and these have been shown to be important in the pathogenesis of these organisms (Collmer and Keen 1986; Barras *et al.*,1994). Mutants that are unable to secrete pectate lyases have been shown to be avirulent. Indeed, single pectate lyase isoforms from the closely related bacteria *E. chrysanthemi* when expressed in *E. coli* have been shown to convert *E. coli* to a macerating activity on potato tubers (Tsror *et al.*,1991). Ecc strain SCRI193 (Salmond *et al.*,1986) produces four major pectate lyase isoenzymes (Plastow *et al.*,1986). Two of these forms represent extracellular enzymes, pelC and pelD, and the other two forms, pelA and pelB are periplasmic. These differences in cellular location are reflected in that these sets of genes have been placed into different gene families (Hinton *et al.*,1989).

1.1.5.3 Secretion Of Extracellular Enzymes.

The secretion of extracellular enzymes is obviously an important factor in determining pathogenicity. Four types of secretory pathways exist in plant pathogens, which will be outlined in turn.

1.1.5.3.1 Type I secretion pathway.

A characteristic of proteins secreted by the type I pathway, such as protease secreted by *Erwinia chrysanthemi* is the lack of an N-terminal signal sequence. There is evidence to suggest the presence of a C-terminal sequence that defines secretion, but this signal has not been well defined in sequence terms, and is not cleaved off upon secretion. Proteins secreted by this pathway do not exist as free periplasmic intermediates, which suggests a one step secretion pathway, possibly by the use of a channel or a gated pore (Salmond 1994).

1.1.5.3.2 Type II secretion pathway.

This 'general secretory pathway' (GSP) is a two-step secretion system which secretes a wide range of proteins, including the pectinases produced by a wide range of pathogens. The first step in this process is the export across the inner membrane. For this to occur, an N-terminal signal peptide is required which is cleaved off by a signal peptidase on its way through the inner membrane. This first step involves gene products present in a wide range of gram negative bacteria, including *E.coli*. The export of the protein into the periplasm results in a free periplasmic intermediate, and these proteins can undergo limited or complete folding. Indeed, the formation of disulphide

bonds in the periplasm may be an important factor in the further secretion of the protein. The second step in secretion of proteins by this pathway is transport across the outer membrane. This step is mediated by the out system of proteins, of which there are 13 in *Erwinia carotovora* encoded by a cluster of *out* genes. These proteins, are, somewhat surprisingly, mainly found spanning the inner membrane. *Erwinia out* mutants exhibit reduced virulence, as the exoenzymes accumulate in the periplasm. It is interesting to note that in these mutants, proteases are still secreted by the type I pathway, confirming that it is a distinct pathway. There is no apparent signal for type II secretion across the outer membrane, but it could be the case that a patch secretion motif (ie one generated by partial folding of the proteins) acts to direct secretion. Considering the similarity between the *out* genes in different species, such as *Erwinia carotovora* and *Erwinia chrysanthemi,* it is surprising that secretion of one species enzymes from the others system cannot occur. This further suggests the presence of some sort of secretion signal being present in the protein itself (Salmond 1994).

1.1.5.3.3 Type III and IV secretion pathways.

These secretion pathways are more specialised than the general secretory pathway, type III resulting in the sec independent secretion of harpins, molecules which elicit the hypersensitive response and also determine pathogenicity. For example in *Erwinia amylovora,* the causal agent of fireblight, *hrpN-* mutants result in an inability to cause a hypersensitive response in tobacco along with a lack of pathogenicity in pear (Salmond 1994). The type IV pathway is responsible for the secretion and targeting of T-DNA from *Agrobacterium tumefaciens* (Salmond 1994).

1.1.6 Regulation Of Expression Of Pectate Lyase.

1.1.6.1 Cell Density Dependent Expression.

It has been noted in all the soft rotting *Erwinia* species that in culture, the production of the extracellular enzymes only occurs once the bacteria have reached the stationary phase of growth. This is now known to be due to the effect of a freely diffusible autoinducer. These autoinducers were first described in the marine bacterium *Vibrio fischeri.* Upon reaching a certain cell density, these bacteria fluoresce. It was subsequently found that it was not the cell density that was important but the concentration of a homoserine

lactone derivative, whose production was directed by the expression of the *lux*I gene, which itself is inducible by the homoserine lactone derivative, leading to the conclusion that the diffusible signal molecule was an autoinducer. These autoinducers have now been found in many species, controlling conjugation in *Agrobacterium*, extracellular enzyme production in *Pseudomonas* and nitrogen fixation in *Rhizobium* (for a review of quorum sensing by bacteria see Fuqua *et al.*,1996). The autoinducer produced by *Erwinia carotovora* induces the production of all the extracellular enzymes and also the antibiotic carbapanem. This autoinducer (termed OHHL) induces expression of the *lux*I homologue *exp*I. *ExpI* mutants are avirulent and unable to colonise plant tissue, but these activities can be restored by the addition of exogenous OHHL, and this supply needs to be continuous in order to achieve successful proliferation. The current hypothesis concerning the importance of this autoinducer is that it only allows expression of the extracellular enzymes when there is a large enough population of the bacteria in order to mount a successful challenge to the plant defences. As many autoinducers are derived from a similar structure, it has been suggested that the coordinated production of the antibiotic carbapanem may serve to eliminate other autoinducer producers or users in order to prevent confusion as to the population present (Barras *et al.*,1994).

1.1.6.2 Response To Plant Components.

It has also been noted in the laboratory growth of *Erwinia carotovora* that there is little exoenzyme production in a simple growth medium, but if plant extracts are added to this medium then the expression of those enzymes increases. This plant inducibility is controlled by the *aep*A and *aep*B genes (activate extracellular protein production). It is possible that *aep*B controls the synthesis of aepA, and they both act in a cascade like network. This level of regulation of the expression of the extracellular enzymes also serves to prevent the unnecessary production of these enzymes when the bacteria are not *in planta* (Barras *et al.*,1994).

1.1.6.3 Response To Pectin Degradation.

In *Erwinia chrysanthemi*, there has also been found a co-regulation of the expression of all genes involved in pectin catabolism, involving both the depolymerisation by the extracellular enzymes and the subsequent catabolism of the monomeric subunits. The kdgR transcriptional repressor has been

found to control expression of all these genes along with the *out* genes, which allow secretion of the extracellular enzymes. There therefore appears to be a close link between the extracellular and intracellular steps of pectin catabolism (Barras *et al.*,1994). At present no similar system has been proved to exist in *Erwinia carotovora*, but there are kdgR recognition motifs found upstream of the *pel, peh* and *aep* genes, leading to the suggestion that a similar system is present (Barras *et al.*,1994).

1.1.6.4 Hierarchical Control Of Expression.

The array of regulatory systems found in the *Erwinia* species may seem to be somewhat redundant, but control by a number of systems allows the pathogen to sort, classify and finally respond appropriately to a broad range of stimuli. This is well illustrated by the two pathways shown to be present in Ecc. The autoinducer system provides an 'indigenous' metabolic control system, whereas the aep system responds to the presence of plant components. There are interactions between these systems, shown by the fact that exp- mutants (deficient in autoinducer) remain exoenzyme deficient even in the presence of plant stimuli (Pirhonen *et al.*,1993). Thus there appears to be a hierarchical control of production of these enzymes, allowing production only in the most appropriate conditions.

1.1.7 Signal Exchange Involving Pectic Oligomers.

As described earlier, the degree of polymerisation (DP) of pectic oligomers produced may determine the outcome of a plant-pathogen interaction. As most of the pectic oligomers produced are the result of the action of pathogen produced pectinases, the efficiency of these enzymes would appear to determine the outcome. An example of this is the action of *Erwinia chrysanthemi* pelC in tobacco. *In vitro* this enzyme produces low DP oligomers, but activates plant defence responses in tobacco. However, the activity of pelC *in planta* is approximately 100 fold-lower than it is in vitro, due to the apoplastic pH of 6. Thus mostly high DP oligomers will be produced and a defence response induced. In a tobacco cell suspension however, there is a very rapid proton influx upon treatment with high DP oligomers, leading to an alkalinisation of the extracellular medium. In this higher pH, it is possible that the pelC activity will be higher and hence produce produce low DP oligomers and then mount a successful infection (Barras *et al.*,1994). So hypothetically, the pH of the medium could determine

whether infection occurs or not.
A change of extracellular pH has not been reported upon infection with *Erwinia carotovora*, but there are results suggesting that a similar system may be present. Upon treatment of tobacco with whole culture or separate purified enzymes, plant defence genes are activated. This is not the case when tobacco is injected with live cells (Barras *et al.*,1994). Perhaps in the first case oligomers of high DP are present, and in the second case the cumulative effect of the different pel isoenzymes has outcompeted and suppressed the plant defence inducing response.
As has been described, the interaction between plant and pathogen, involving components and signalling mechanisms from both sides, may have a significant effect on the outcome of any infection.
The recent advances in technology which have allowed the introduction of foreign genes into plants has increased the number of strategies available to increase the resistance of plants to certain pathogens.

1.2 Plant Genetic Engineering.

For several centuries, the improvement of plant crops by conventional breeding techniques has been widespread. This technique however is slow, as the crossed progeny must be back-crossed many times in order to achieve the original phenotype with just the desired change, and it only allows the crossing of related species. Over the past ten years however, the rapid advances seen in molecular biology have resulted in the development of reliable methods for the introduction of foreign genes into plants. As this method can introduce a desired trait in one step, and as it allows a gene from any source (bacteria, plant or animal) to be introduced, the advantages are many fold. Plants have an enormous regenerative capacity, and many plant cells can be grown in culture from which whole plants may be regenerated. Most plant transformation techniques also result in the stable integration of the gene into the plant genome, thus genetic recombination by crossing of transgenic plants can introduce new genes into plants, and plants homozygous for a particular gene can be generated by self fertilisation. The resulting seed can then be stored almost indefinitely under ambient conditions. Finally, the potential yield of a recombinant protein from a transgenic plant is massive, as production can be scaled up to agricultural proportions, thus reducing costs (Ma 1995).

Most transgenic plants are generated for specific research purposes, but broader objectives have also been followed for the improvement of crops or the production of foreign proteins.

1.2.1 Pathogen Resistance.

Much work has been carried out on the production of transgenic plants showing enhanced resistance to a range of pathogens. Due to the levels of understanding of the different types of pathogens as described earlier, much of the work has been focused on viral resistance. These techniques have generally involved the introduction of viral sequences into the plant genome (for a comprehensive review of this technology see Beachy 1997).

1.2.1.1 Coat Protein-Mediated Resistance.

Expression in plants of viral coat proteins has been shown for a number of combinations of plant and virus to confer resistance to viral infection, as seen by delay or absence of systemic disease symptoms. This was first demonstrated in transgenic tobacco plants expressing the coat protein (CP) from tobacco mosaic virus (Abel *et al.*,1986). This resistance only extends to viruses closely related to that from which the coat protein was taken. In TMV resistance, the degree of resistance is correlated to the level of CP expression. Two models for this resistance have been proposed. The re-encapsidation theory involves the plant produced CP binding to viral RNA as soon as viral CP is removed. Viral disassembly will be absent or delayed if levels of CP in the cell are above a threshold level. This theory is consistent with the observation that the degree of resistance is related to the expression level of CP. It also explains the specificity, in that if the CP cannot bind to the RNA of the infecting virus, no resistance is possible. The prevention theory involves the binding of plant encoded CP to putative cellular receptors for viral entry or disassembly. This binding then prevents the binding of viral CP and thus the entry to the cell or formation of disassembly complexes. This explains how in some cases relatively low levels of CP can provide viral protection in transgenic plants, and specificity depending on the specificity of the viral receptor. The expression of CP in plants has resulted in the description of several examples of resistance to viral infection (Fitchen and Beachy 1993).

1.2.1.2 Replicase-Mediated Resistance.

Transgenic tobacco expressing a TMV replication related protein were found to exhibit high levels of resistance to TMV and closely related strains. This resistance has also been seen in plants transformed with related sequences from a number of different viruses. These transgenes have encoded defective or functional components of viral replicase complexes. Results have suggested that the number of infected cells is the same in transgenic or wild type plant tissue, but that viral replication is reduced markedly in the transgenic plants. Although replication is not completely halted, it is impeded severely enough to prevent systemic infection of the plant (Fitchen and Beachy 1993).

1.2.1.3 RNA-Mediated Resistance.

Most of the examples of RNA-mediated resistance were discovered in control plant lines used in studies of CP mediated resistance. In experiments with a few viruses, both anti sense and translationally impaired sense RNAs conferred protection. Although a mechanism has not been confirmed for any of these examples, possible explanations include binding to the genome to inhibit replication or translation and competition for viral or host factors essential for replication.

1.2.1.4 Satellite RNA Resistance.

Virus satellites and satellite RNAs depend on helper virus for replication and are not related by sequence similarity to the virus. In experiments with the satellite RNA from tobacco ringspot virus (TRV), the accumulation of satellite increased dramatically following TRV infection, presumably because of replication of the transcriptionally derived RNAs. The transgenic plants showed a marked delay in symptoms, and virus accumulation was reduced (Gerlach *et al.*,1987).

1.2.1.5 Fungal Resistance.

Work towards providing resistance against fungal pathogens by the use of genetic engineering is at a much less advanced stage than that giving viral resistance. Approaches have been made by over-expressing endogenous genes, or by the expression of foreign genes in transgenic plants. Some success has been seen with both approaches.

Transgenic potatoes over-expressing the potato osmotin-like protein have demonstrated increased tolerance to the fungus *Phytopthera infestans*, the

causal agent of late-blight (Zhu *et al.*,1996). Increased tolerance to the oomycete fungi *Peronospora tabacina* and *Phytopthera parasitica* var *nicotianae* which cause downy mildew diseases has been demonstrated in transgenic tobacco plants over expressing the pathogenesis-related protein PR1a (Alexander *et al.*,1993).

The expression of a bean chitinase gene in tobacco has shown to increase the tolerance of the transgenic plants to growth in soil containing the fungus *Rhizoctonia solani,* but these plants show no enhanced resistance to fungi such as *Pythium aphanidermatum,* which lack chitin in their cell walls (Broglie *et al.*,1991). The combinatorial expression of two genes to increase disease resistance has met with some success. For example, transgenic tomato plants carrying both the tobacco chitinase and glucanase genes show greatly increased resistance to Fusarium wilt disease than plants expressing one or other of the genes alone (Jongedijk *et al.,* 1995). This approach has also led to some success in higher resistance being shown by the expression of fungal cell wall hydrolases alongside the barley ribosome inactivating protein (Jach *et al.*,1995). It is likely that this combinatorial approach will lead to a more durable resistance against a constantly evolving pathogen population. The expression of foreign phytoalexins in transgenic plants has also led to resistance being demonstrated. Transgenic tobacco carrying the grapevine stilbene synthase gene show pathogen induced accumulation of resveratrol and increased resistance to *Botrytis cinerea* (Hain *et al.*,1993).

1.2.1.6 Bacterial Resistance.

Relatively little success has been reported in the construction of transgenic plants resistant to infection by bacterial pathogens. The expression of bacteriophage T4 lysozyme in transgenic tobacco with the barley α amylase signal peptide to direct secretion to the apoplast resulted in increased resistance to *Erwinia carotovora* ssp *atroseptica* (During *et al.*,1993). The generation of active oxygen species by the transfer of the *Aspergillus niger* glucose oxidase gene into transgenic potato has given strong resistance to *Erwinia carotovora* as well as *Phytopthera infestans* and *Verticillium dahliae* (Wu *et al.*,1995). The transfer of cloned resistance genes between species is also an approach that has led to some success. For example transfer of the tomato *Pto* gene to tobacco leads to hypersensitive response based resistance to *Pseudomonas syringae* pv *tabaci* carrying the *avrPto* gene (Rommens *et al.*,1995). Resistance genes that recognise all or multiple races of a pathogen may be of even greater use. For example the

Bs2 gene from pepper recognises a common virulence determinant of *Xanthamonas campestris* pv *vesicatoria* (Kearney and Staskawicz 1990). Similarly, the *Xa21* gene from rice gives resistance to all known races of *Xanthomonas oryzae* pv *oryzae*, and transgenic rice carrying this gene are resistant to 29 different isolates of this pathogen (Wang *et al.*,1996). One limitation to the transfer of resistance genes from one species to a distantly related species is the possibility that the signal transduction pathways neccessary for induction of resistance may be R-gene specific and therefore these pathways may be absent in all but the most closely related species. This approach however is likely to increase in importance and effectiveness as more resistance genes are cloned and this will allow the 'pyramiding' of resistance genes to give resistance to a range of different pathogens.

1.2.1.7 Expression Of Antibodies In Transgenic Plants.

The discovery that the expression of antibodies in plants was possible (Hiatt *et al.*,1989) led to suggestions that they may be deployed in a capacity to give resistance of plants to certain pathogens. This has given rise to a large interest in the expression of antibodies in plants. Before this can be discussed, a basic description of antibody structure and function is necessary.

1.3 Monoclonal Antibodies.

Since 1975, when the first discovery was made that a mammalian cell line could produce a single species of antibody (Kohler and Milstein 1975), the use of monoclonal antibodies in all biological sciences has been widespread. The discovery that this technique - hybridoma technology, was general meant that monoclonal antibodies have since been made which bind to all manner of biological molecules, including proteins, carbohydrates, nucleic acids and hapten antigens. Even antibodies with catalytic properties have been isolated (Roitt 1994). This broad range of potential antigens has led to the widespread use of monoclonal antibodies in research and even in human health care (Waldmann 1991). The fact that a monoclonal antibody is a single species of antibody has been vital in this development, as it also means that they only bind to a single epitope on the antigen, and this has allowed the differentiation of even the most similar of molecules.

Hybridoma technology was initially extended by the use of somatic cell genetics,

which allowed antibody mutants to be selected (Rudikoff *et al.*,1982) and the production of antibodies of dual specificity (Milstein and Cuello 1983). The fact remains however that the production of monoclonal antibodies is a time-consuming and labour-intensive activity, and this encouraged the development of different techniques for the production of antibodies of single specificity. Great advances have been made since the advent of gene technology, but to understand this the structure of an immunoglobulin molecule must be discussed.

1.3.1 Immunoglobulin Structure.

The most common of the five types of antibody, and hence the form of antibody used most in antibody manipulation is the IgG molecule, and the structure of this will be discussed. An IgG molecule is a Y-shaped molecule in which the stem of the Y is responsible for triggering effector functions in the host and the arms responsible for recognition of the antigen (Fig 1.1). The antibody molecule consists of two copies of two types of polypeptide chains. The light chain consists of a variable (VL) and constant (CL) domain. The heavy chain contains one variable (VH) and three constant domains (CH1, CH2, CH3). In each case the variable (amino terminal) domain is the one which contributes the antigen binding activity. All six domain types are similar in sequence and structure, containing 100-120 residues folded into two antiparallel sheets. The fact that these domains are so well defined and are so distinct is of great use in the manipulation of antibodies, as different domains can be used separately or swapped between antibodies. The effector domains can largely be ignored for the purposes of this discussion, as the most exciting advances have been made in the engineering of the variable regions which determine antigen recognition and binding.

The two variable regions for each arm of the molecule (a heavy variable domain (VH) and a light variable domain (VL)) each consist of a largely conserved polypeptide chain with three regions of hypervariability. The variable loops in VL are called L1, L2 and L3, and those in VH are H1, H2 and H3. These complementarity determining regions (CDRs) largely define the antigen binding capability by combining with the CDRs on the partner chain to form an antigen binding 'pocket'. Because of the antigen binding ability being defined by this one domain, most of the emphasis of antibody engineering has been focused onto this region (For a review of immunoglobulin structure see Roitt 1994).

1.3.2 Immunoglobulin Derivatives.

A range of antibody fragments have been produced from the whole IgG molecule (Fig 1.2). Antigen binding (Fab) fragments contain the amino terminal two domains of the heavy chain with the entire light chain, and can be produced by either proteolytic digestion of whole IgG molecules or by the use of genetic engineering (Roitt 1994). These fragments can be reduced further by the removal of the constant domains to leave only the variable domains in the form of a variable fragment (Fv) (Winter and Milstein 1991). These two chains can be linked with a synthetic polypeptide linker chain to produce a single chain variable fragment (scFv) (Winter and Milstein 1991). These molecules have been the focus of a large research effort in recent years, which will be reviewed later. Single domain antibodies (dAb) consisting of either a lone heavy or light chain can still recognise antigens (Ward *et al.*,1989), as can the even smaller minimum recognition units (mru) (Taub *et al.*,1989) which are simply a single CDR. In order to make use of gene cloning techniques, the organisation of the immunoglobulin gene must be understood.

1.3.3 Immunoglobulin Gene Structure.

The modular assembly of antibodies is also seen in the organisation of V genes, as the individual domains are encoded by separate exons. During lymphocyte differentiation, DNA rearrangements allow the formation of an entire V fragment and splicing produces an entire antibody mRNA (Fig 1.3) (Roitt 1994). This DNA rearrangement leads to a large proportion of the diversity found between antibodies.

1.3.4 Diversity Of Antibodies.

One of the remarkable aspects of the immune system is the diversity of antibodies that may be produced, even though in the germ line there are relatively few variable gene segments. For example, in human heavy chains, the first two CDRs are drawn from a pool of approximately 50 VH gene segments (Tomlinson *et al.*,1992) which then combine with D segments and JH segments (Ravetch *et al.*,1981) to create the third hypervariable loop. This loop is by far the most variable of the three CDRs in both sequence and length (2-26 residues) (Winter *et al.*,1994). This variability is due to imprecise joining of the segments allowing different reading frames to be used, also nucleotides can be inserted or deleted at the junctions and the D segments can recombine as D-D fusions (Sanz 1991). In human light chains, a similar situation exists where the

first two CDRs are drawn from approximately 30Vκ and 30 Vλ segments (Williams and Winter 1993), and the third loop being created by the joining with Jκ and Jλ segments. This third loop is not as diverse in structure as those of the heavy chain, being 7-11 residues in λ chains (Martin 1996) and normally 6 residues in κ chains (Martin 1996). A similar situation to this exists in mice, from which most hybridoma lines are derived. It has been proposed after the study of crystal structures and nucleotide sequences, that there are a small number of 'canonical structures' for each of the five loops excluding H3. Most examples of these loops have been shown to belong to one of these groups. For example in mice as many as 95% of the L2 loops are believed to conform to a single canonical structure, while 70% of L1 structures are accounted for by four canonical structures. It must be noted however that these 'conserved' loop structures concern only the conformation, and once decorated with the many side chains that exist, can still be highly variable. No canonical structures have been defined for H3 chains, due to the huge divergence of size and sequence (Chothia *et al.*,1992; Chothia and Lesk 1987). Thus most of the sequence (and hence structural) diversity of antibodies is encoded by the heavy chains.

1.3.5 Development Of An Immune Response.

In an animal, immunisation with an antigen leads to the production of high affinity antibodies. This happens in two distinct steps. Initially, a fast step leads to the production of antibodies with a relatively low affinity (10^5-10^7 M^{-1}) (Roitt 1994). These antibodies are derived from the repertoire that already exists, as described above. Those cells that recognise the antigen proliferate and undergo the second stage of antibody production, that of affinity maturation (Roitt 1994). The main tool of this process is the hypermutation of the genes from these cells followed by selection of the cells which produce antibodies of higher affinity. This is a Darwinian process that involves point mutations followed by selection driven by the requirement of antigen for cell survival. Thus, those cells producing antibodies of increased affinity survive and undergo further rounds of selection. This mutation leads to a huge potential repertoire from which new antibodies may be selected. As immunisation proceeds, additional high affinity antibodies are produced with V-D-J combinations that are rarely found in the primary repertoire (repertoire shift) (Berek and Milstein 1987). These antibodies produced by a combination of affinity maturation and repertoire shift can have

extremely high affinity (>$10^{10}M^{-1}$) (Roitt 1994).

1.4 Expression Of Antibodies In Plants.

Recently, considerable research efforts have been exerted towards the aim of producing antibodies in transgenic plants. The first description of a transgenic plant expressing an antibody was made in 1989 (Hiatt *et al.*,1989). This work involved the production of tobacco lines expressing solely the heavy chain or the light chain (each with their respective murine signal sequences) followed by the cross pollination of these lines to give progeny expressing both chains. The progeny indeed contained assembled antibody, which was shown to have the same affinity and specificity as the original mouse antibody. Levels of intact antibody were seen to be higher than the levels of individual chains seen in the parental lines, and even the crossing of parental lines expressing greatly differing amounts of heavy and light chain resulted in progeny showing the same level of each chain. This suggests that the coexpression of both chains increases the yield of each chain. It is likely that this is due to instability of lone chains. The yield of intact antibody was found to be up to 1% of total soluble protein. It was also found that the immunoglobulin signal sequences, targeting secretion to the apoplast, were necessary to see any accumulation of antibody, and constructs lacking these sequences demonstrated detectable transcript levels but very little detectable antibody. This suggested that cotranslational insertion into the lumen of the endoplasmic reticulum was necessary for chain assembly and stability. The absence of antibodies in the cytoplasm may be due to the reducing environment, which is unfavourable for disulphide bond formation, and due to the lack of chaperonins. It is also interesting to note that further work in the replacement of murine signal sequences with signal sequences derived from barley α amylase resulted in mis-targeting to the chloroplast of some immunoglobulin (During *et al.*,1990). These reports however demonstrated the ability of plants to produce functional antibodies, and initiated much work in this area.

1.4.1 Multimeric Immunoglobulin Expression In Plants.

Other studies have shown the feasibility of producing even more complex multimeric immunoglobulins in plants. Transgenic plants have been produced which express a secretory IgA. This antibody consists of four

different chains. Initially, IgA light and heavy chains were expressed separately and then crossed to give a plant expressing an IgA. This was then crossed to a plant expressing a J chain, which facilitates the formation of a dimeric IgA, and this plant was further crossed to a plant expressing a secretory component. The result is a plant expressing four different chains which assemble in the correct manner to produce a secretory IgA (Ma 1995), which is the form of antibody most common in the mouth due to its increased stability. The antibody produced can be used in the prevention of colonisation of the bacterium *Strep. mutans* which is the cause of dental caries (described later). Due to the previously described complexity of assembly of whole antibodies, several groups have been working towards the production of smaller antibody fragments in transgenic plants.

1.4.2 Expression Of Single Antibody Domains In Plants.

The first report of the successful expression of an antibody fragment in transgenic plants was made in 1991 with the production of an isolated VH domain in plants up to a level of 1% total soluble protein (Benvenuto *et al.*,1991). Although this construct contained a signal peptide to direct secretion, very little VH was found in the apoplast, and several possible explanations have been made for this (Benvenuto *et al.*,1991). More interest has however focussed upon the production of scFvs and Fab fragments in plants.

1.4.3 Expression Of Fab Fragments.

The first description of Fab fragments in plants came in 1993 with the expression of the complete and Fab fragment of an antibody which recognises human creatine kinase (Deneve *et al.*,1993). The accumulation of Fab up to 0.8% total soluble protein when secreted by the aid of the *Arabidopsis thaliana* 2S2 storage protein signal peptide was seen, but this was not higher than the accumulation of intact antibody, as had been expected due to the difference in complexity. This was possibly due to a higher turnover of the Fab. This high level of expression was only found in primary callus tissue, whereas the expression levels in leaf tissue was around 0.05% total soluble protein in each case (Deneve *et al.*,1993). Further work with this antibody and Fab fragment has shown accumulation in Arabidopsis to be in the extracellular space (De Wilde *et al.*,1996).

1.4.4 Expression Of scFvs.

In 1992 the first report was made of the intracellular expression of an scFv in transgenic tobacco (Owen *et al.*,1992). An scFv was cloned from a hybridoma line expressing antibodies against phytochrome and expressed at levels up to 0.1% total soluble protein. As described later, this scFv was active and showed an effect in the plants. This was the first report of deliberate expression of an antibody fragment inside a plant cell showing that an active antibody could be produced, as the previous reports used secretion signals to direct secretion. It is perhaps surprising that active scFv can accumulate in the cytosol, as an scFv has 2 intramolecular disulphide bonds, which have been assumed to be necessary for folding and functionality (Glockshuber *et al.*,1992). Disulphide bonds would not be expected to be formed in the cytosol, due to its reducing nature. However, other reports of the intracellular expression of an scFv have since been made (Tavladoraki *et al.*,1993).

1.4.4.1 Effect Of Sub cellular Location.

The anti-phytochrome scFv was then investigated to determine its accumulation and activity upon secretion. An N-terminal signal sequence, derived from the tobacco pathogenicity-related protein PR1a was fused to the scFv, and its cellular location in transgenic tobacco determined (Firek *et al.*,1993). It was found that the scFv did accumulate in the apoplast, at a much higher level than previously seen with the intracellular expression. This difference in expression levels was seen even though the transcript for the secreted scFv was present at a much lower level. Although differences in translatability could have caused this difference in scFv accumulation, it was considered by the authors that the difference was more likely attributed to the lower protein stability in the cytoplasm, possibly due to its interaction with its labile antigen (phytochrome) (Firek *et al.*,1993). Other reports have been made of differences in expression levels of antibodies in transgenic plants, and these will be reviewed in Chapter 6.

1.4.5 Applications Of Plant Produced Antibodies.

1.4.5.1 Mass Production Of Antibodies.

The prospect of harvesting huge quantities of monoclonal antibodies or their derivatives holds great potential for the economic production of almost limitless amounts. An estimate was made in 1990 that the expression of an

antibody in soybean at a level of 1% total protein would result in costs of approximately US $100 per kilogram (Hiatt 1990). This is a greatly reduced cost compared to traditional hybridoma techniques. However, the high purity of antibodies required for, for example, therapeutic purposes, would raise these costs dramatically. Developments are being made however in the area of molecular carriers such as oleosins to enhance the ease of purification of expressed proteins (Vanrooijen and Molony 1995). It has recently been reported that an scFv accumulated to relatively high levels in seeds upon expression by a seed specific promoter (and secretion to the apoplast), and that storage of seeds for one year under ambient conditions has no effect on scFv level or activity (Fiedler and Conrad 1995). It is still envisaged that the large scale production of antibodies would make their use in other areas than therapeutics, for example in industrial processes, where high purity is not required and previous high cost has precluded their use.

1.4.5.2 Passive Immunisation.

One area that currently requires the large quantities of antibodies that plants can potentially produce is that of the *in vivo* application of therapeutic antibodies. An example of this is a monoclonal antibody which prevents the colonisation of the bacterium *Strep. mutans*, the cause of dental caries in humans. Application of this antibody to adult teeth has shown to confer resistance to this pathogen. The production of this antibody has been confirmed in plants (Ma 1995) and these plant antibodies have been delivered in food and have prevented colonisation and disease (Ma 1995). An added advantage for the use of plant produced antibodies is the ease of storage of seeds, which can then be grown when necessary to produce the antibody. An attractive proposition, with the continuing increase in the number of crops that can be transformed, is the use of plant antibodies in developing countries where the existing farming infrastructure could be used for antibody vaccine production rather than the building of new pharmaceutical factories.

1.4.5.3 Production Of Modified Antibodies.

For many therapeutic antibodies, much emphasis has been placed on the production of small antibody fragments. As discussed, these have been shown to be functional when produced in plants. In other cases however, the constant region is necessary in order to maintain bivalent binding (Ma *et al.*,

1990) or because the mode of action requires complement binding or other functions determined by the constant regions. It has also been suggested that alterations in the constant region can be made, for example the addition of an extra constant domain in order to enhance Fc mediated functions (Ma 1995).

1.4.5.4 Improvement Of Plant And Crop Performance.

The ability to express antibodies in plants has been seen as a novel method by which to improve crops. The fact that antibodies can also be targeted to different cell compartments, for example intracellular, apoplastic, chloroplastic or plasma membrane bound, means that many different processes could be targeted.

One area in which there has been considerable activity is the attempt to produce antibodies in plants which confer some sort of disease resistance. Although this resistance will be due purely to the inactivation or sequestration of molecules, rather than the immune response as in animals, sòme success has been achieved. One example of this is the production of an scFv in plants which recognises a coat protein epitope of the artichoke mottled crinkle virus (AMCV). Transgenic *Nicotiana benthamiana* plants expressing this scFv showed increased resistance to AMCV, as seen by a delay in disease symptoms and a reduction in the amount of virus coat protein accumulation (Tavladoraki *et al.*,1993). The mechanism of this action is not known, but it is possible that the scFv binds to an essential Ca2+ binding site on the coat protein and so prevents uncoating of the virus or recoating of progeny virus (Tavladoraki *et al.*,1993). Another example of viral resistance by the expression of antibodies is shown by the expression of a full size antibody that recognises a tobacco mosaic virus (TMV) epitope present only on the surface of intact tobacco mosaic virions. In *in vitro* studies, preabsorption of this antibody with virus results in strongly reduced infectivity. The two chains were expressed from the same T-DNA in a tandem arrangement, including their native murine leader peptides to direct secretion to the apoplast (Voss *et al.*,1995). These plants exhibited a reduction in viral infectivity of 48%, and the level of infectivity decrease was seen to be related to the amount of antibody produced in other transgenic lines (Voss *et al.*,1995). All lines showed equimolar amounts of heavy and light chain, even though in some lines the amount of light chain transcript was up to three times that of the heavy chain transcript. It was suggested from this that, as in animal B-cells, the amount of functional intact immunoglobulin in plants is

determined by the expression of heavy chain (Voss *et al.*,1995).
Further developments in the field of virus resistance encoded by antibodies is likely to be the expression of antibodies in plants that bind non-structural components of viruses, such as replicases and proteins involved in virus transmission. This area has already been shown to be viable in animal cells expressing antibody fragments directed against the HIV reverse transcriptase, resulting in resistance of those cells to HIV infection (Maciejewski *et al.*,1995).
Attempts have also been made to introduce resistance to root knot nematodes by the expression of antibodies in plants. In this work, the entire heavy and light chains were cloned from a hybridoma line producing antibodies recognising the stylet secretions of the root knot nematode *Meloidogyne incognita*. These secretions include chemical signals which alter the plant cell cycle and also cell differentiation. These chains were expressed separately in Xanthi tobacco with native leader sequences, and expressing lines crossed to give plants producing full size antibody (Baum *et al.*,1996).
Although the hybridoma line used secreted IgM antibodies, production of the two chains without a joining component resulted in their association in an IgG form. Expression of this antibody in leaf tissue resulted in no difference in root knot nematode parasitism. A number of explanations for this have been given, including the suggestion that the stylet secretions may be injected directly into the cytosol, so avoiding contact with the antibodies which were secreted to the apoplast (Baum *et al.*,1996).
It is possible that plant expressed antibodies may prove to give a broader range of resistance to pathogens than some other methods. Due to the multiplicity of many enzymatic pathogenicity factors (for example *Erwinia carotovora* pectate lyases as described earlier), an antibody expressed in plants that inactivates one of these forms of the enzyme by binding to the active site may also inactivate other forms of the enzyme due to the similarity that must occur at active sites. This resistance may also spread to related species which attack plants in the same way. Similarly, the effect of antibodies directed against the active site of a host protein, such as polygalacturonase involved in fruit ripening, may affect more than one form of the enzyme. Previous work using antisense genes (Gray *et al.*,1992) has resulted in the reduction in activity of one form of polygalacturonase, but due to sequence differences, this antisense gene has not been active against other foms. The conservation at the peptide level may however be sufficient for a plant antibody to inactivate more than one form of the enzyme.

1.4.5.5 Immunomodulation.

Intracellular expression of antibodies has been shown to modulate the metabolism of the expressing cell, for example the decrease in alcohol dehydrogenase activity in yeast expressing an anti ADH antibody (Carlson 1988). It may similarly be possible to alter plant metabolism by the expression of catalytic antibodies, or enzyme or hormone inactivating antibodies. The first example of this was the expression of an scFv that recognises the hormone phytochrome (Owen *et al.*,1992). This scFv was expressed in transgenic tobacco and was shown to be functional by passing extracts over a phytochrome sepharose column. Seeds derived from a high expressing plant were shown to display aberrant light mediated germination behaviour, suggesting that the action of phytochrome was being modulated (Owen *et al.*,1992). Another example of modulation of hormone action has been demonstrated (Artsaenko *et al.*,1995). Here an scFv was expressed in plants which binds the plant hormone abscisic acid (ABA). This scFv was retained in the lumen of the endoplasmic reticulum (by the addition of a C-terminal KDEL retention signal along with an N-terminal signal peptide). The result of expression of this scFv was a wilty phenotype, similar to the phenotype of ABA deficient mutants (Artsaenko *et al.*,1995). The mechanism for this is not known, but it is possible that binding and sequestration of ABA in the endoplasmic reticulum reduces the amount of free ABA elsewhere in the cell. Interestingly however, overall ABA levels were shown to be higher in transgenic than in wild type plants (Artsaenko *et al.*,1995).

1.5 Scope Of This Thesis.

As described earlier in this introduction, there are very few examples of plants engineered to be resistant to bacterial pathogens. Recent advances in technology involving the cloning and expression of antibody genes allows a different approach to be taken. It is possible that the expression in transgenic plants of an antibody or antibody fragment which causes interference with a pathogenicity factor may affect the pathogenicity of that organism and increase the chances of the plant mounting a succesful resistance to that pathogen. At the time that this work was initiated (late 1991), there were no published examples of the expression of scFvs in transgenic plants. There were also very few examples of the cloning of scFvs from either hybridoma lines or from phage display libraries. The work described in this thesis

involves attempts to isolate (from either conventional hybridoma lines or phage display libraries) and express scFvs against bacterial and fungal enzymatic pathogenicity factors which act to degrade plant cell walls. If these scFvs reduce the activity of these enzymes then their expression in plants may lead to some resistance to these pathogens. The success of such a strategy also relies on the ability to secrete these scFvs into the apoplast, as their expression within a cell is unlikely to have any effect on these extracellular enzymes. Thus there is also work described in this thesis involving the determination of whether secretion of scFvs is possible, and whether they accumulate in the apoplast to concentrations likely to affect pathogen ingress. All this work aims towards the development of a novel disease resistance strategy in plants by the expression of totally foreign proteins.

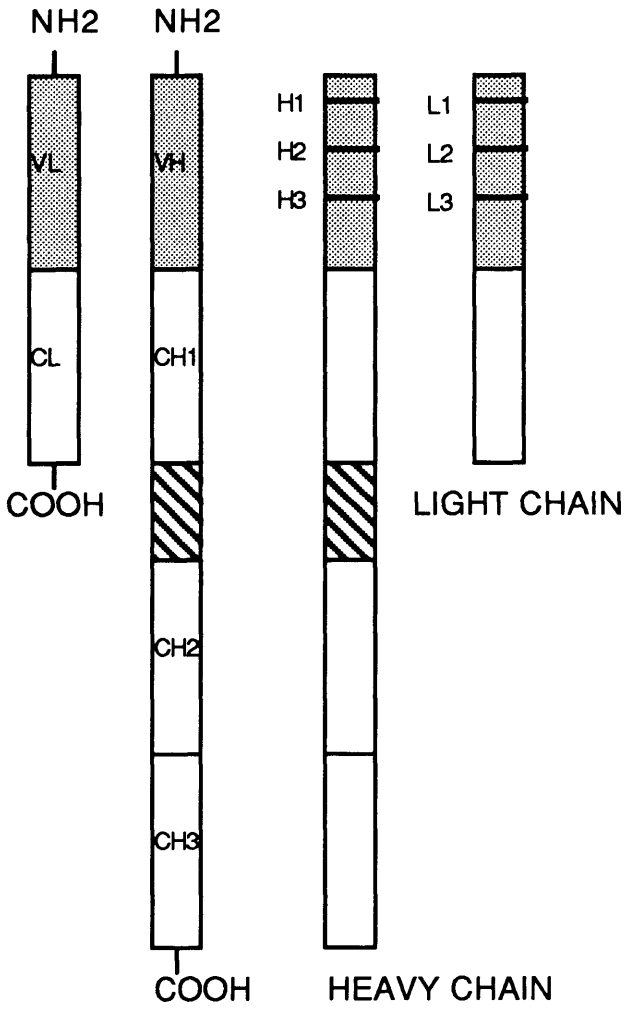


Fig 1.1. Schematic Diagram Of An IgG Molecule.

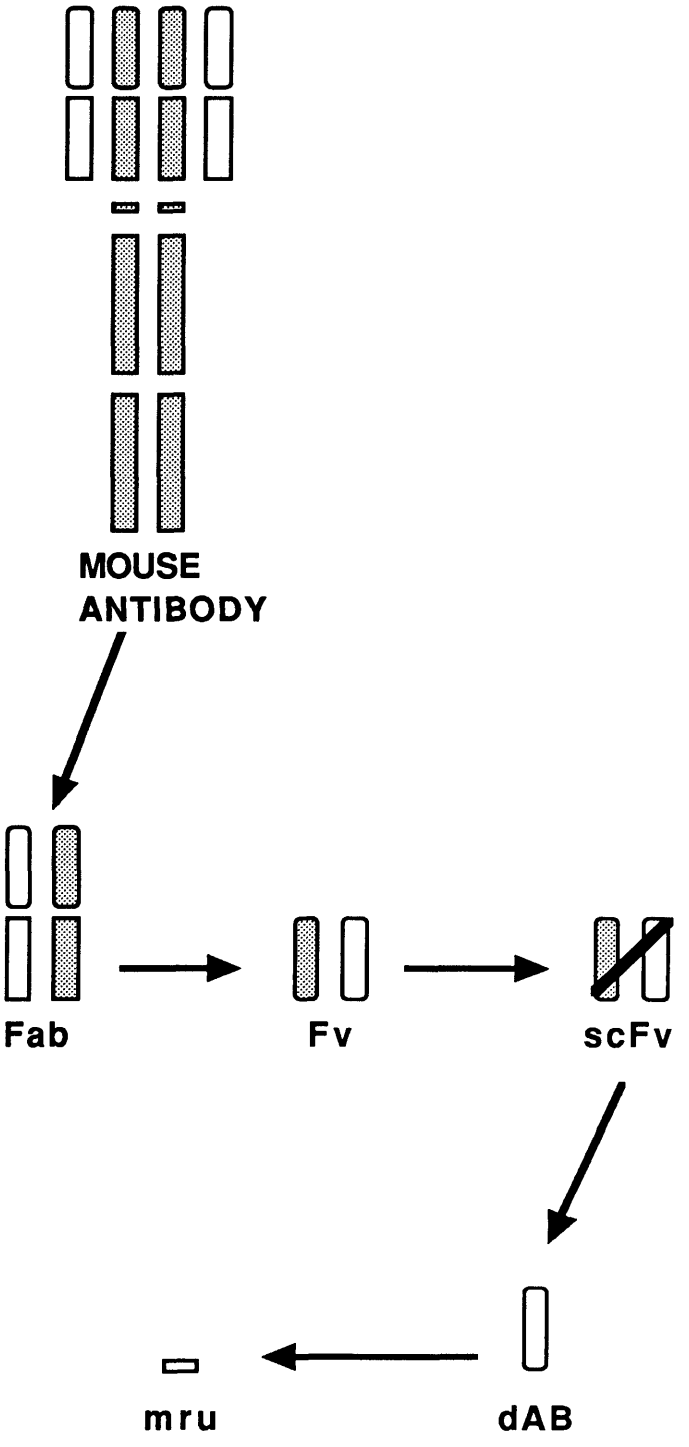


Fig 1.2. Derivatives Of An IgG Molecule.
Fab - antigen binding fragment. Fv - variable fragment. scFv - single chain variable fragment. dAB - single domain antibody. mru - minimal recognition unit.

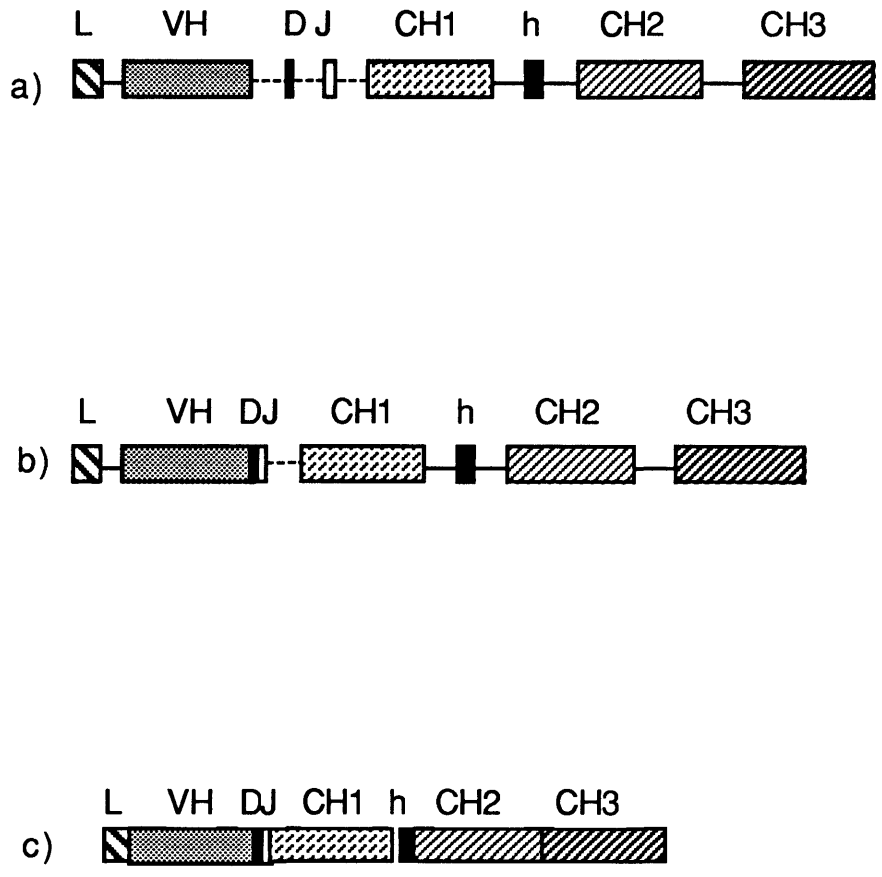


Fig 1.3. Immunoglobulin Gene Structure.
a) - unrearranged VH gene.
b) - rearranged VH gene.
c) - immunoglobulin mRNA.
L - leader sequence. VH - heavy variable region.
D - D segment.
J - joining segment. h - hinge.
CH - heavy constant regions.

Chapter 2 : Materials And Methods.

2.1 Sources Of Molecular Biological Reagents, Enzymes And Plant Tissue Culture Chemicals.

Molecular biology grade chemicals and reagents were purchased from BDH Ltd or from Sigma Chemical Company Ltd. Enzymes were from BRL, Boehringer Mannheim, Stratagene or Pharmacia. Tissue culture salts and hormones were purchased from Flow Laboratories or Sigma. Agar and agar-based media were from Difco Laboratories.

2.2 Bacterial Culture And Storage.

2.2.1 Strains And Genotypes.

Escherichia coli:

XL1-Blue : *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [*F' proAB, lacIqZΔM15, Tn10* (tetr)]. (Bullock *et al* 1987).

TG1 : K12, *Δ(lac-pro), supE, thi, hsdD5* [*F' traD36, proA+B+, lacIq, lacZΔM15*]. (Ward *et al* 1989).

HB2151 : K12, *ara, Δ(lac-pro), thi* [*F' proA+B+, lacIq, lacZΔM15*]. (Carter *et al* 1985).

K38 : *thr, leu, lacY, thi, supE, hsdR, tonA, trxA.* (Tabor and Richardson 1985).

Agrobacterium tumefaciens :

LBA4404, rifr binary construct host strain. (Hoekema *et al* 1983).

2.2.2 Growth Of Bacterial Cultures.

Liquid cultures were inoculated from well isolated bacterial colonies into liquid media containing the appropriate antibiotics using a sterile pipette tip. From glycerol stocks, a flame sterilised loop was dipped into the frozen stock and streaked onto an agar plate containing the appropriate antibiotics. Cultures were grown overnight, at either 30°C or 37°C for *E. coli* and 28°C for *A. tumefaciens*, liquid cultures being grown in a shaking incubator. Media containing agar was allowed to cool to 50°C before the addition of antibiotics and pouring into petri dishes.

2.2.3 Storage Of Bacterial Cultures.

Agar plates were stored for a maximum of 4 weeks at 4°C. Glycerol stocks were made in cryotubes from overnight cultures by mixing with an equal volume of

60% glycerol in liquid media. This stock was then flash frozen in liquid nitrogen and stored at -80°C.

2.2.4 Antibiotics.

The following antibiotics were commonly used for the selection of plasmids, episomes or bacterial strains carrying resistance genes, at the following final concentrations.

Antibiotic.	*E.coli.*	*A.tumefaciens.*
Ampicillin	100µg/ml	-
Kanamycin	25-100µg/ml	50µg/ml
Tetracycline	12.5µg/ml	-
Rifampicin	-	50µg/ml
Augmentin	-	400µg/ml

2.3 Cloning And Manipulation Of Plasmids In E.coli.

2.3.1 Large Scale Prepararion Of Plasmid DNA.

100ml of an overnight culture was centrifuged at 10000rpm in a GSA rotor (Sorvall) in a Sorvall RC5B centrifuge for 15 minutes at 4°C. The pelleted cells were resuspended in 3ml of solution I (50mM glucose, 10mM EDTA, 25mM Tris.HCl pH 8.0) and cooled on ice. After the addition of 6ml of solution 2 (200mM NaOH, 1%SDS) the solution was mixed well by rolling the tube. After a 5 minute incubation on ice, 3ml of ice-cold 3M Potassium Acetate pH 4.8 was added, mixed as above and incubated on ice for 15 minutes. After centrifugation at 10000rpm at 4C for 15 minutes in an SS34 rotor (Sorvall), the supernatant was filtered into a fresh tube through 2 layers of muslin. The nucleic acids were then precipitated by the addition of 10ml propan-2-ol and spinning at 10000rpm for 10 minutes at room temperature in an SS34 rotor. The pellet was then resuspended in 300µl of dH_2O and transfered to an eppendorf tube. 300µl of ice-cold 5M LiCl was added to precipitate RNA and the mixture centrifuged at 13000 rpm in a minifuge (Eppendorff). The supernatant was then transferred to a fresh tube and an equal volume of propan-2-ol added. After spinning as above, the pellet was resuspended in 500µl dH_2O and 20µl of 10mg/ml RNase added. After 30 minutes at room temperature, the solution was extracted with phenol,phenol/chloroform

and finally with chloroform. 200µl of 5M Ammonium Acetate and 1ml of ethanol was added, and incubated at room temperature for 10 minutes. After centrifugation at 13000 rpm for 5 minutes, the pellet was washed with 70% ethanol, vacuum dried and resuspended in 200µl of dH_2O.

2.3.2 Small Scale Preparation Of Plasmid DNA.

1.5ml of an overnight culture was centrifuged for 3 minutes in a minifuge at 13000rpm. The bacterial pellet was then resuspended in 100µl of solution I and to this 200µl of solution 2 was added and the tube inverted several times until the solution cleared. Following the addition of 150µl of 3M Potassium Acetate pH4.8 and inversion of the tube, 10µl of 10 mg/ml RNase was added followed by 30 minutes incubation at room temperature. After phenol/chloroform extraction the plasmid DNA was precipitated from the aqueous phase by the addition of 2.5 volumes of ice-cold ethanol. After centrifugation, the pellet was washed with 70% ethanol before being vacuum dried and resuspended in 50µl of dH_2O.

2.3.3 Further Purification Of DNA.

In order to achieve pure enough DNA for automated sequencing, DNA was subjected to a further incubation with 10µl per µg DNA of 10mg/ml RNase at room temperature for 60 minutes followed by addition of an equal volume of 1.6MNaCl 13%w/v PEG8000. After centrifugation at 13000rpm for 5 minutes, the pellet was resuspended in 100µl of dH_2O and subjected to phenol, phenol/chloroform and chloroform extractions. To the aqueous phase was added 1/10th volume 3M sodium acetate pH4.5 and 2 volumes of ethanol. After 30 minutes at -20°C, the sample was spun at 13000rpm for 5 minutes, and the pellet washed with 70% ethanol before vacuum drying and resuspension in dH_2O.

2.3.4 Restriction Digests.

These were performed according to the manufacturers instructions using the buffers supplied with the enzymes.

2.3.5 Agarose Gel Electrophoresis.

Agarose gels were prepared by dissolving agarose in 1X TAE buffer (40mM Tris-acetate, 1mM EDTA pH8.0) by boiling and addition of ethidium bromide to a final

concentration of 5µg/ml. The agarose concentrations used varied from 0.6% to 2%, (2% for fragments <0.5kb, 1.2% for DNA <5kb, 0.6% for DNA >5kb). 1/10th volume of DNA loading buffer (25% w/v Ficoll 400, 0.25% w/v bromophenol blue) was then added to the DNA samples before pipetting into the wells. DNA markers (1kb ladder, Gibco) were co-electrophoresed with all samples. (Band sizes in bp 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298, 220, 201, 154, 134, 75). The gels were run submerged in TAE buffer containing 1µg/ml ethidium bromide at 50-150 volts. DNA was then visualised and photographed under UV light.

2.3.6 Reverse Transcription.

25µl first strand reactions were set up containing 10 pmoles oligonucleotide primer, 140mM KCl, 50mM Tris-HCl pH8.1, 8mM $MgCl_2$, 0.5mM each dNTP, 10mM dithiothreitol, 10 units RNAsin and 10 units AMV reverse transcriptase. RNA template was added after heating it to 65°C for 3 minutes and quenching on ice, and the reaction incubated at 42°C for 60 minutes. The reaction was then heated to 99°C for 2 minutes, quenched on ice and stored at -20°C until required.

2.3.7 PCR Amplification.

100µl PCR reactions were prepared with 20pmol of each oligonucleotide primer, an appropriate quantity of template DNA, 50mM KCl, 10mM Tris-HCl pH8.3, 2.5mM $MgCl_2$, 0.01%w/v gelatine, 200mM dNTPs and 5units of *Taq* DNA polymerase and overlaid with mineral oil. They were then subjected to amplification using the appropriate conditions in a Perkin Elmer Cetus DNA Thermal Cycler.

2.3.8 Isolation Of DNA From Agarose Gels.

2.3.8.1 Electroelution.

The desired DNA fragment was excised from an agarose gel and placed in the chamber of an electroelution tank which already contained 1X TAE to the level of the elution reservoir. The collection chamber of the tank was then filled with high salt buffer (3M sodium acetate pH7.9, 0.01% w/v bromophenol blue) to retain the DNA when 100V was passed through the apparatus. After 60 minutes, the high salt buffer containing the eluted DNA was removed from the collection chamber and the DNA precipitated by addition of 2 volumes of ice-cold ethanol. After 30 minutes at -20°C, the DNA was pelleted by centrifugation and washed

with 70% ethanol before vacuum drying. The pellet was then resuspended in the appropriate volume of dH_2O for further use.

2.3.8.2 Gel Slot.

After separating the desired DNA fragment on an agarose gel, a narrow slot was cut the full width of the DNA fragment immediately in front of the desired fragment. The gel was then returned to the tank from which some buffer had been removed so as to reach the top of the gel but not to submerse it. The slot was then filled with 20% w/v PEG8000, 2MNaCl solution and the gel run for an appropriate time in order for the band to run into the slot but not beyond. The PEG/NaCl was then removed from the slot and viewed on a transilluminator to visualise the presence of the DNA. The DNA was then precipitated using ethanol and washed with 70% ethanol to remove excess salt. After vacuum drying and resuspension in dH_2O, the DNA was ready for further use.

2.3.9 Ligation Of DNA Fragments.

Purified DNA was mixed in a 3:1 molar ratio of insert to vector before the addition of 5X ligase buffer (250mM Tris.HCl pH7.5, 35mM $MgCl_2$, 5mM DTT) and 1 unit of T4 DNA ligase in an appropriate volume. Ligations were left at 15C overnight before transformation into bacteria.

2.3.10 Transformation Of *E.coli*.

2.3.10.1 Preparation Of competent Cells.

An overnight culture was diluted 1/100 in fresh medium and this culture was grown for 2-2.5 hours until the OD600=0.5-0.7. The cells were then pelleted by spinning at 4000x*g* for 20 minutes in a pre-chilled centrifuge, and the cells resuspended in 1/10th volume ice-cold sterile 50mM $CaCl_2$. After 60 minutes on ice, the cells were recentrifuged and resuspended in 1/50th original volume sterile 50mM $CaCl_2$, 20% v/v glycerol. After aliquoting into 100µl aliquots, the cells were flash frozen and stored at -80°C.

2.3.10.2 Standard Transformation Procedure.

An aliquot of cells was thawed on ice before the addition of DNA and mixing. After 30 minutes on ice, the cells were held at 42°C for 2 minutes before the addition of 1ml of liquid media. After 10 minutes on ice, the cells were incubated at 37°C for 60 minutes before plating out.

2.3.10.3 Preparation Of Electrocompetent Cells.

Cells were grown as for normal competent cells, stored on ice for 30 minutes and spun at 4000x*g* for 20 minutes in a prechilled centrifuge. The cells were then carefully resuspended in 1/10th volume of sterile dH_2O. After recentrifugation, this step was repeated. The cells were then resuspended in 1/100th original volume of 20% glycerol before aliquoting into 40µl portions, flash freezing and storing at -80°C.

2.3.10.4 Electroporation.

A vial of electrocompetent cells were thawed on ice, and DNA added. (A maximum of 1µl of ligation mix can be added due to the presence of salt, but where required the ligation mix was ethanol precipitated before washing in 70% ethanol and resuspending in dH_2O). The cells and DNA were then transferred to a pre-chilled 0.2cm cuvette which was banged on the desk to ensure the contents were at the bottom before placing into the electroporation chamber of a BioRad Gene Pulser which was set to 25µF, 200ohms and 2.4kV. Immediately after the pulse, 1ml of liquid media was added to the cuvette, the cells removed to an eppendorf tube and incubated at 37°C for 60 minutes before plating out.

2.3.11 Identification Of Colonies Carrying Recombinant Plasmids.

Certain vectors used in this thesis allow the use of a colour assay to identify clones carrying inserts, due to the multiple cloning site being within the lacZα gene. The product of this gene, the α-subunit of B-galactosidase complements a host mutation and allows the catabolism of the substrate X-GAL (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside) upon induction by IPTG (isopropyl-B-D thiogalactopyranoside) to form a blue product. Interuption of this gene with a DNA insert destroys this activity, so recombinant colonies appear white, not blue, when grown on a plate containing X-GAL and IPTG. 20µl of 40mg/ml IPTG in water and 20µl of 40mg/ml X-GAL in dimethylformamide were spread on plates and allowed to dry before spreading the cells to allow this colour selection.

2.3.11.1 PCR Amplification Direct From Colonies.

In cases where colour selection was not possible, or when a number of different inserts could be present, single colonies were picked with a sterile tip, streaked

onto a fresh plate and then dipped into a 20µl PCR reaction using either primers which anneal within the vector, or those specific for the DNA insert of interest. After amplification, the products were analysed on an agarose gel to confirm the presence of an insert the correct size.

2.4 Cloning And Manipulation Of Plasmids In *Agrobacterium tumefaciens.*

2.4.1 Transformation Of Agrobacterium With Plasmid DNA.

Transformation of *Agrobacterium* was achieved by following the electroporation method for *E.coli*, with the following ammendments. As *Agrobacterium* grows more slowly than *E.coli*, the inoculum for the preparation of cells was 1/100th of a 2 day old culture, and was left to grow overnight to achieve the correct cell density. The Gene Pulser was set to 25µF, 2.5kV and 600ohms, to give an optimum time constant of 13 msec. The cells were left to recover for 2 hours at 28°C before plating.

2.5 DNA Sequencing.

DNA sequencing was carried using Taq DyeDeoxy Terminator Cycle Sequencing performed on an Applied Biosystems 373A DNA sequencing system using ABI dye-labelled dideoxy nucleotides as terminators and AmpliTaq DNA polymerase. 1µg of template DNA (prepared by alkaline lysis and PEG purification) was added to 9.5µl of reaction pre-mix and 3.2pmol of primer. After making the volume up to 20µl with dH_2O and overlaying the reaction with a drop of mineral oil, the reaction was subjected to 25 cycles of 96C for 30 seconds, 50C for 15 seconds and 60C for 4 minutes in a Perkin Elmer Cetus 480 thermal cycler. To remove unincorporated terminators, the mixture was made up to 100µl and extracted first with chloroform, and subsequently twice with phenol/H_2O/chloroform (86:18:14). The aqueous phase was taken and the extension products precipitated using 15µl of 3M sodium acetate pH 4.5 and 300µl ethanol. After 30 minutes at -20C, the DNA was pelleted by centrifugationin a minifuge for 5 minutes at 13000rpm and washed with 70% ethanol before being vacuum dried. The dried pellet was then passed on to the automated DNA sequencing service operated at Leicester University where samples were run and data returned for computer analysis using the Wisconsin Genetics Computer Group (GCG) programmes (Deveraux *et al* 1984).

2.6 Overexpression And Purification Of Pectate Lyase.

2.6.1 Induction Of Expression.

Clones carrying the heat inducible T7 RNA polymerase were grown at 30C to prevent unwanted expression from this promoter. After growth of cultures to an OD600 of approx. 0.8, induction by shifting to 37°C for 30 minutes was carried out. After a further 2 hours at 30°C, proteins were extracted and used for analysis.

2.6.2 Preparation Of Cellular Fractions.

2.6.2.1 Total Cell Fraction.

The total cell fraction was prepared by resuspending pelleted cells in 1X denaturing SDS-PAGE buffer (see 2.11.2.1) and boiling for 5 minutes.

2.6.2.2 Soluble And Insoluble Fractions.

Extracts were prepared by resuspending pelleted cells in 1/10th volume dH_2O and sonicating on ice for 3 periods of 30 seconds. After centrifugation, the pellet constituted the insoluble fraction and the supernatant represented the soluble fraction.

2.6.2.3 Periplasmic Fraction.

After centrifugation, the pelleted cells were resuspended in 250µl per 100ml culture of 5g/l NaCl. After the addition of 4 volumes of chloroform and vortexing, the sample was incubated at room temperature for 15 minutes. 10 volumes of 10mM Tris.HCl pH8.0 was then added, the sample mixed and allowed to settle. The aqueous phase was then removed to a fresh tube and constituted the periplasmic fraction.

2.6.3 Cation Exchange Chromatography For Pectate Lyase Purification.

A 1cm by 7cm column of degased Biogel-CM (Biorad) equilibrated with 0.1mM $CaCl_2$, 4mM Tris.HCl pH8.0 was used to separate proteins on the basis of their isoelectric point. After application of the periplasmic fraction in equilibration buffer, the column was washed with three column volumes of this buffer. The pectate lyase was then bulk eluted with 500mM NaCl, 5mM Tris.HCl pH 8.0, and 1.5ml fractions collected.

2.6.4 Pectate Lyase Assays.

2.6.4.1 Agar Plate Assay.

Duplicate colonies were stabbed out on agar plates containing 0.4% PGA (polygalacturonic acid) and 0.5mM $CaCl_2$ and grown overnight. After flooding one of the plates with enough 10% copper acetate to cover the colonies, the plate was incubated at room temperature for 60 minutes. Excess copper acetate was then drained off and pectate lyase expressing colonies detected by the presence of a clear halo on a blue background.

2.6.4.2 Slide Assay.

Microscope slides were poured with a thin layer of molten substrate agar (0.5% agarose, 0.1% PGA, 50mM Tris.HCl pH 8.5, 1.5mM $CaCl_2$) and allowed to solidify. Small drops of pectate lyase containing samples were then applied to the gel and allowed to incubate at room temperature for 60 minutes. After flooding with 0.05% ruthenium red for 30 minutes, the stain was rinsed off with dH_2O and pectate lyase activity shown by the presence of a clear area on a red background.

2.6.4.3 Spectroscopic Assay.

100µl of an enzyme dilution was added to 900µl of assay mixture (0.11%w/v polygalacturonic acid, 110mM Tris-HCl pH 8.5) and the absorbance increase at 230nm measured on a spectrophotometer (Perkin-Elmer Lambda 5UV/VIS).

2.6.5 Polygalacturonase Assay.

Polygalacturonase activity was determined by the increase in reducing groups. 500µl of a diluted enzyme solution was added to 500µl of 0.5%w/v polygalacturonic acid in 0.2M sodium acetate buffer pH5.5 and incubated for 60 minutes at 30°C. Samples were then made up to 1.5ml with distilled water and mixed with 1.5ml DNSA reagent (1%w/v dinitrosalicylic acid, 0.2%v/v phenol and 0.05%w/v sodium sulphite in 1% w/v NaOH) and boiled for 15 minutes before cooling. After the addition of 500µl 40%w/v sodium potassium tartrate.$4H_2O$ solution, the absorbance was measured at 576nm on a spectrophotometer (Perkin-Elmer Lambda 5UV/VIS).

2.6.6 Treatment With Periodate.

ELISA plates coated with the appropriate antigens were washed with PBS (10mM phospate buffer, 2.7mM KCl, 137mM NaCl) and filled with 20mM $NaIO_4$ in 50mM sodium acetate buffer in the dark for 60 minutes and the reaction stopped by copious washing with PBS. Blocking and probing was then carried out as described.

2.7 Culture Of Hybridomas.

2.7.1 Growth Of Hybridoma Cell Lines.

Hybridoma cell lines were recovered from the freezing medium by gentle centrifugation and gentle resuspension in culture medium (45%v/v RPMI 1640 containing 25mM hepes and L-glutamine, 45%v/v 1X Dulbeccos modified eagles medium and 10%v/v fetal calf serum). This cell suspension was then diluted into 10ml of the same medium and grown at 37°C with 5% carbon dioxide until the cells were confluent. At this stage, an aliquot was taken and diluted ten-fold in fresh medium.

2.7.2 RNA Extraction From Hybridoma Cells.

To pelleted cells, 10 volumes of lysis buffer (4M guanidine monothiocyanate, 100mM sodium acetate pH5.2, 5mM dithiothreitol and 0.5%w/v SDS) were added and the sample vortexed vigorously. The sample was then extracted with an equal volume of phenol/chloroform and total nucleic acids precipitated from the aqueous phase by the addition of 1/10th volume 3M sodium acetate pH 5.2 and 2 volumes of ethanol (stored at -20°C). After 60 minutes at -20°C, the nucleic acids were pelleted by centrifugation at 13000x*g* for 30 minutes at 4°C. The pellet was resuspended in DEPC treated water and after the addition of 7 volumes of 4M LiCl, stored overnight at 4°C After centrifuging for 60 minutes at 7500x*g* at 4°C, the pellet was resuspended in 3M LiCl and the tubes respun for 30 minutes. The pellet was then resuspended (in 0.2%w/v SDS, 50mM EDTA pH8.0) and RNA precipitated by the addition of 1/10th volume 3M sodium acetate pH5.2 and 2 volumes of -20°C ethanol. After storing overnight at -20°C, the RNA was pelleted by centrifuging at 7500x*g* for 30 minutes at 4°C and resuspended in dH_2O.

2.8 Use Of Experimental Mice.

2.8.1 Inoculation.

Female BALB/c mice weighing 10g each were inoculated intravenously with

50μg of filter sterilised antigen in PBS on days 0, 4, 7 and 11. After a further 3 days a test bleed was taken for analysis. The mice were then left for a further 14 days before administration of a final 'boost' and 3 days after this injection the mice were sacrificed and the blood and spleen extracted. All this work was carried out by the staff of the BioMedical Services at Leicester University.

2.8.2 Serum Extraction From Experimental Mice.

Serum was extracted from fresh blood by allowing it to clot for 2 hours at 37°C before being left at 4°C overnight. The blood was then centrifuged at 13000rpm in a minifuge (Eppendorff) for 2 minutes and the supernatant serum fraction taken.

2.8.3 Preparation Of Spleen Cell Suspension.

Spleen were cut into 4 pieces and placed into a sterile tea strainer resting in 10ml cold Dulbeccos modified eagle medium containing 5%v/v fetal calf serum (DMEM/FCS) and mashed gently with the plunger of a 50 ml syringe. The resulting cell suspension was transfered to a centrifuge tube and left to stand for 3 minutes. The supernatant was then transfered to a clean tube and spun for 10 minutes at 0°C at 400x*g* with no brake. The pellet of cells was then resuspended in 5ml DMEM/FCS and layered carefully onto 5ml Ficoll 1.077 in a 15 ml centrifuge tube. After spinning at 400x*g* for 25 minutes at room temperature with no brake, the lymphocytes at the interphase which showed themselves as a white band were harvested and centrifuged at 400x*g* for 10 minutes at room temperature. The cells were then washed twice in PBS/TNC/BSA (80%v/v PBS, 10%v/v (3.8%w/v tri-sodium citrate) and 10%v/v (30%w/v bovine serum albumin)) and once in PBS before they were used in an RNA extraction as described in section 2.7.2.

2.9 Tobacco Transformation And Analysis Of Transgenic Plants.

2.9.1 Transformation.

Nicotiana tabacum (Petite Havana SR1) plants were transformed using the leaf disk transformation method as described in Draper *et al* (1988). Young, nearly fully expanded leaves were removed from greenhouse grown plants and surface sterilised in 10% bleach for 15 minutes. All further manipulations were carried out with sterile equipment in a laminar flow hood. The leaves were then washed four times with sterile tap water before 1cm square disks were cut, avoiding the midrib and main veins, and inoculated by a 30 minute immersion in a suspension (overnight culture diluted 1/50 with liquid MSD4X2 (MS medium

containing 30 g/l sucrose, 1mg/l NAA and 0.1mg/l BAP)) of *Agrobacterium* containing the binary construct of interest. After shaking to remove excess *Agrobacterium*, the disks were placed on solid MSD4X2 plates and incubated at 25C in growth rooms for 2 days. The disks were then transferred to selective MSD4X2 plates (containing 400µg/ml augmentin and 100µg/ml kanamycin) and grown under the same conditions until antibiotic resistant shoots arose from the callus around the leaf tissue. Once large enough these shoots were aseptically removed and transferred to solid MSO medium (MS containing 30g/l sucrose) containing the same antibiotics. In this medium, the shoots produce roots, and the plantlets can then be transferred to soil.

2.9.2 DNA Miniprep For PCR.

1cm^2 of young leaf tissue was taken and ground quickly in 250µl extraction buffer (100mM Tris-HCl pH 8.0, 50mM EDTA, 500mM NaCl, 10mM 2-mercaptoethanol and 1.4%w/v SDS) and then incubated at 65°C for 10 minutes. After the addition of 65µl 5M potassium acetate the samples were incubated on ice for 5 minutes. After centrifugation at 13000rpm in a minifuge, the supernatant was taken and to it added 320µl propan-2-ol and 30µl 3M sodium acetate. After 10 minutes at -20°C and spinning for 5 minutes as before, the pellet was air dried and resuspended in 50µl of dH_2O.

2.9.3 RNA Miniprep For RT-PCR.

1cm^2 of young leaf tissue was taken and ground quickly in 400µl TLES buffer (100mM LiCl, 100mM Tris-HCl pH 8.0, 10mM EDTA and 1%w/v SDS). After the addition of 500µl phenol/chloroform and vortexing the samples were spun for 5 minutes at 13000rpm in a minifuge. 300µl of the aqueous fraction were taken and re-extracted with an equal volume of phenol/chloroform. 200µl of the aqueous layer was taken and 20µl 3M sodium acetate pH 5.2 and 500µl ethanol added. After vortexing and incubating for 30 minutes at -20°C, the samples were spun as above and resuspended in 50µl dH_2O. 50µl 4M LiCl was added and after incubation on ice for 30 minutes the RNA was pelleted by spinning as before, washed with 70%v/v ethanol and air dried before being resuspended in 20µl dH_2O.

2.9.4 Total Protein Extraction.

1-2g young leaf tissue was homogenised in an equal volume of scFv extraction buffer (100mM Tris-HCl pH7.7, 5mM EDTA, 50%v/v ethelyne glycol, 20mM Na_2SO_3 and 2mM phenylmethylsulphonyl fluoride). Insoluble material was removed by centrifugation at 13000g for 10 minutes at 4°C after which 20µl of 10% w/v polyethyleneimine per ml supernatant was added and after mixing the solution clarified by centrifugation as before. The supernatant constituted the total protein fraction.

2.9.5 Apoplastic Protein Extraction.

Extracellular fluids were removed by cutting leaves into approximately 2cm squares and vacuum infiltrating them with ice cold 50mM $CaCl_2$. The infiltrated leaf pieces were then placed into a 20ml syringe and centrifuged for 5 minutes at 2000x*g* at 4°C. The resulting solution constituted the apoplastic fraction.

2.10 Manipulation Of Phage Display Libraries.

2.10.1 fdtetDOG1 Phage Libraries.

After transformation of TG1 cells with the ligation mix and growth of colonies on agar plates containing 12.5 µg/ml tetracycline, single colonies were picked into small volumes of liquid media. After overnight growth, the cells were pelleted by centrifugation and the supernatant used as a source of phage particles.

2.10.2 Recombinant Phage Antibody System Library.

Libraries constructed in pCANTAB5E were manipulated exactly as described in the instruction manual, essentially as described below for the synthetic Nissim library.

2.10.3 Nissim Library.

2.10.3.1 Growth Of The Library.

50µl of the supplied glycerol stock (approximately $5X10^8$ clones) was used to inoculate a 50 ml culture in 2xTY (16g tryptone, 10g yeast extract, 5g NaCl per litre) containing 100µg/ml ampicillin and 1% glucose (2xTY-AG). After growth at 37°C until OD600=0.5, 40ml of the culture was taken to prepare a secondary stock of the library (sections 2.10.3.3 and 2.10.3.4) and the remaining 10ml infected with a 20:1 ratio (phage : cells) of M13-KO7 helper phage. After incubation for 30 minutes at

37°C, the cellswere spun for 10 minutes at 3300x*g*. The pellet was resuspended in 30ml of 2xTY containing 100µg/ml ampicillin and 25µg/ml kanamycin (2xTY-AK) and 270ml of prewarmed 2xTY-AK added. This culture was then grown at 30°C overnight.

2.10.3.2 Preparing Phage Particles.

The culture prepared in section 2.10.3.1 was spun at 10000x*g* for 10 minutes, and 1/5th volume of 20% PEG6000, 2.5M NaCl (PEG/NaCl) added to the supernatant. This was then mixed well and left at 4°C for 60 minutes. After centrifugation at 10000x*g* for 30 minutes, the pellet was resuspended in 40ml dH_2O and 8ml of PEG/NaCl added. After mixing, this was left at 4°C for 20 minutes. This mixture was then centrifuged for 30 minutes at 3300x*g* and the supernatant removed. The pellet was respun briefly and any remaining supernatant removed. The pellet was then resuspended in 2ml of PBS and spun in a minifuge at 13000rpm for 5 minutes. The supernatant was then filter sterilised and stored at 4°C. This procedure yields approximately 10^{13} phage.

2.10.3.3 Selection On Immunotubes.

A Nunc-immunotube was coated overnight at room temperature with 4ml of 10µg/ml antigen in PBS. The tube was then washed three times with PBS (simply poured in and out) and then filled to the brim with 2% skimmed milk powder in PBS (2%MPBS) and incubated at 37°C for 2 hours to block any spare sites on the tube. The tube was again washed 3 times with PBS. 10^{13} phage in 4ml 2%MPBS were then added to the tube and incubated rotating for 30 minutes at room temperature followed by 90 minutes stationary at room temperature. The unbound phage in the supernatant were then discarded. The tube was then washed 20 times with PBS containing 0.1% Tween 20 (PBST) and 20 times with PBS. Excess PBS was shaken from the tube and 1ml 100mM triethylamine added and incubated rotating for 10 minutes. The triethylamine was then poured into a tube containing 0.5ml 1M Tris.HCl pH 7.4 to neutralise it, and 200µl of Tris pipetted into the immunotube. These pooled eluted phage were then added to 9ml of exponentially growing TG1 cells and incubated at 37°C for 30 minutes. 4ml of TG1 was also added to the immunotube. The TG1 cells were then pooled and 100µl taken to plate out serial dilutions on TYE (15g agar, 8g NaCl, 10g tryptone, 5g yeast extract per litre) containing 100µg/ml ampicillin and 1% glucose (TYE-AG) in order to determine the number of phage eluted. The remaining bacteria were

then spun at 3300x*g* for 10 minutes, resuspended in 1ml of 2xTY and plated on 2 large petri dishes containing TYE-AG. These plates were then incubated overnight at 30°C.

2.10.3.4 Further Rounds Of Selection.

2ml of 2xTY was added to each of the large petri dish cultures and the cells loosened with a glass spreader. 50µl of the bacteria were used to inoculate a 50ml culture in 2xTY-AG which was grown at 37°C, and the remainder of the cells were frozen at -80°C. The bacteria were grown until they reached an OD600 of approximately 0.5, when they were then rescued as described in section 2.10.3.1.

2.11 Immunological Analysis.

2.11.1 Dot Blots.

2µl aliquots of antigen were dotted onto PVDF membrane which had previously been wetted with methanol and subsequently washed in distilled water, and the dots allowed to dry. After incubation in 3% skimmed milk powder, 0.1% Tween 20 in TBS (50mM Tris.HCl, 150mM NaCl pH7.6) (TBS-MT) shaking for 60 minutes, primary antibody was added at an appropriate dilution in 10% skimmed milk powder in TBS (TBS-M). After incubating with shaking for 90 minutes, the membrane was washed with three changes of 0.1% Tween 20 in TBS (TBS-T) and alkaline phosphatase conjugated secondary antibody added in TBS-M. After a 60 minute incubation, the membrane was washed as before and then rinsed in phosphatase reaction buffer (100mM Tris-HCl pH9.5, 1mM $MgCl_2$) before being placed into the same buffer containing 0.5 mg ml^{-1} BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 0.3mg ml^{-1} NBT (nitro blue tetrazolium) to develop. When a sufficient reaction had occured, it was stopped by rinsing the membrane thoroughly in water before air drying.

2.11.2 Western Blotting.

2.11.2.1 SDS-PAGE Gels.

In general, 11% SDS-acrylamide resolving gels were cast in Biorad minigel kits followed by a 5% SDS-acrylamide stacking gel. Samples were prepared for electrophoresis by the addition of an equal volume of 2X denaturing loading buffer (125mM Tris HCl pH 6.75, 20% v/v glycerol, 4% w/v SDS, 10% v/v 2-mercaptoethanol and 0.002% w/v bromophenol blue) and boiling for 5 minutes. Gels were run at 125V until sufficient migration had occured. Gels were either

transferred to nitrocellulose as described below or stained in 0.1% w/v Coomassie blue in 10% v/v acetic acid and 50% v/v methanol. After staining, gels were destained in the same solution lacking Coomassie blue until the protein bands were clearly visible.

2.11.2.2 Transfer To Nitrocellulose.

A semi-dry blotting apparatus was set up in the order given with pieces of 3MM and nitrocellulose cut to the size of the gel, starting at the anode:

i- 3MM soaked in anode buffer 1 (300mM Tris-HCl pH 10.4, 10% v/v methanol)

ii- 3MM soaked in anode buffer 2 (25mM Tris-HCl pH 10.4, 10% v/v methanol)

iii- nitrocellulose

iv- SDS-PAGE gel

v- 3MM soaked in cathode buffer (25mM Tris-HCl pH 9.4, 40mM aminohexanoic acid, 20% v/v methanol).

This apparatus was run at 125mA for 60 minutes and then the apparatus disassembled and the nitrocellulose checked for transfer of protein by incubation in 0.1% w/v Ponceau red. After rinsing in water to show the protein bands, the stain was washed off with TBS-T. The membrane was then probed with antibodies as described in section 2.9.1.

2.11.3. ELISA.

Nunc Immunoplates were coated overnight at room temperature with 10μgml-1 antigen in PBS. After rinsing the wells with PBS, primary antibody (or phage antibody) in PBS-M was added and incubated at room temperature for 90 minutes. After three rinses with PBS-T, HRP conjugated secondary antibody (or HRP conjugated anti M13 antibody) was added and incubated at room temperature for 60 minutes. After three washes in PBS-T, substrate solution ($200\mu gml^{-1}$ ABTS (2',2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium) in 50mM citric acid pH4.0 and 0.175% v/v of 30% H_2O_2) was added and a green colour developed if there was any binding of antibody to the antigen. These readings were measured for their absorbance at 405nm in a microtitre plate reader.

Chapter 3 : Overexpression And Purification Of Pectate Lyase C.

3.1 Introduction.

As described in the main introduction, the purpose of this work was to raise antibodies to an enzymatic pathogenicity factor in order to try to inactivate this factor by binding to, or otherwise affecting, the active site of this enzyme. The pathogen of choice was *Erwinia carotovora* subsp *carotovora*, as this pathogen causes its disease symptoms by secretion of a battery of enzymes. In order to be able to raise an immune response against an *Erwinia carotovora* subsp *carotovora* (Ecc) extracellular enzyme, it was first necessary to purify one of these enzymes. The enzyme of choice was pectate lyase C (PelC) from Ecc strain SCRI193 (Plastow *et al.*,1986). It was decided to use PelC as it was one of the two extracellular forms of pectate lyase secreted by Ecc and these extracellular forms have been shown to be important factors in the pathogenicity of *Erwinia* species by disruption of the secretion pathway and also by the creation of mutations in the genes encoding these enzymes (Kotoujansky 1987). A clone of this gene was obtained from Prof. George Salmond, University of Warwick. As part of his group's work, all four pectate lyase genes had been cloned from this strain (pel A,B,C and D). The gene encoding the PelC enzyme was isolated by Prof. Salmond's group from a chromosomal library of Ecc DNA, and identified initially by the copper acetate agar plate assay. This initial clone was then subcloned into pUC19 (Yanisch-Perron *et al.*,1985) to give the plasmid pJS6197, carrying a 1.8kb insert of Ecc DNA (Hinton *et al.*,1989). This clone was used to purify the pelC enzyme.

Initial experiments were carried out, including Western blot analysis, activity assays and attempted purification, but the results from these experiments led us to conclude that in this plasmid, the *pelC* gene was not being expressed at a high enough level to allow us to purify enough of the enzyme to use in inoculation programs and subsequent analysis. In *E. coli* it has also been noted that the expression of the extracellular forms of *Erwinia* spp pectate lyases results in the accumulation of these enzymes in the periplasm (Collmer *et al.*,1985; Keen *et al.*,1984; Lei *et al.*,1985; Reverchon *et al.*,1985). As insufficient quantities of pectate lyase were being produced from the original clone it was decided to make use of an *E. coli* expression system. The vectors used in these systems contain a powerful, inducible

promoter under whose control the gene of interest can be cloned to create a transcriptional fusion. There are a wide range of vectors available for high level expression of foreign proteins in *E. coli*, including several in which a translational fusion is created with a peptide that facilitates purification and can be cleaved off after this has been performed. Already in use in the laboratory however was the T7 based expression system, centred around the plasmid pT7-7 (Tabor and Richardson 1985). As this had already been used with some success in the laboratory, and the fact that several straightforward purification protocols for pectate lyases have been described (Ward and DeBoer 1987; Keen and Tamaki, 1986), this was the expression system chosen. The gene of interest is cloned into the pT7-7 vector to create a translational fusion with the ATG start site following the T7 gene 10 promoter (Tabor and Richardson 1985). This promoter is not used by any *E. coli* RNA polymerases and transcription only occurs in the presence of T7 RNA polymerase, a polymerase not normally present in *E. coli*. The T7 RNA polymerase is encoded by the T7 gene1, and is highly selective for its own T7 promoters, none of which are normally found in *E. coli*. Hence the gene of interest is not transcribed unless a source of T7 RNA polymerase is provided in the cell. In the system used here, there are two possible ways of producing T7 RNA polymerase. The first of these is to use the *E. coli* strain BL21:DE3. This is a strain that has been modified by the integration into the bacterial chromosome of the T7 RNA polymerase gene (T7 gene1) under the control of the IPTG inducible lacUV5 promoter (Studier and Moffatt 1986). Thus by addition of IPTG to the bacterial medium at the appropriate stage of growth, T7 RNA polymerase is produced which causes the high level transcription of the target gene required to accumulate large amounts of protein. The system utilised here is slightly different in that the source of the T7 RNA polymerase is a plasmid borne T7 gene 1 under the control of the lambda P_L promoter (Tabor and Richardson 1985). This plasmid also contains the temperature sensitive lambda repressor cI^{857} (Roberts and Devoret 1983). At low growth temperatures, this repressor acts in the normal manner, repressing transcription from the PL promoter. This mutated repressor is inactive at higher temperatures however, allowing expression of the T7 RNA polymerase from the PL promoter. Thus the RNA polymerase is produced at elevated temperatures, allowing the high level transcription of the target gene. This plasmid containing the polymerase gene, pGP1-2 (Tabor and Richardson 1985), is maintained with a

different antibiotic resistance to that of the pT7-7 plasmid to allow their co-propagation within the same cell.

In order to maximise the titre of antibodies raised against a given protein within a preparation, it is necessary to have the antigen as pure as possible. Any contaminating protein will obviously elicit an immune response, maybe stronger than the desired response, depending on the characteristics of the two proteins. There are many conventional ways of purifying proteins according to their various characteristics. These include size, isoelectric point, hydrophobic interactions and affinity for various substrates. The method chosen here involves purification on the basis of molecular charge. In this ion-exchange system, proteins are bound to an oppositely charged chromatographic matrix by reversible electrostatic interactions. The bound proteins are then eluted off the matrix by either increasing the ionic strength or varying the pH of the elution buffer. The high pI of pelC (10.3 (Hinton *et al.*,1989)) allows its purification on the basis of its charge by binding to, and subsequent elution from, a weakly acidic cation exchanger composed of carboxymethyl groups in the matrix. In this case, the protein was bound to the column in a buffer of low ionic strength, and then eluted by washing through with a buffer of higher ionic strength. As is often the case with protein purification protocols, concentration of the final protein was necessary. Many techniques are available for the concentration of protein samples, such as chemical precipitation (eg ammonium sulphate), lyophilisation and dialysis. There are many disadvantages associated with each of these methods, including the unwanted concentration of buffer salts and time consuming methodology. The method of choice involved ultrafiltration through a membrane with a defined pore size. Pre-assembled Centricon microconcentrator units (Amicon) were used. Upon centrifugation, any molecules with a molecular weight smaller than the pore size will pass through the membrane into a lower reservoir. This leaves the larger molecules, including the protein of interest, in the upper reservoir in a smaller volume. These units can also be used to de-salt samples, or to change the buffer, by successive concentration and reconstitution of the retentate.

This chapter describes the overexpression of Ecc pelC in a T7 based system in *E. coli* and its subsequent purification by cation exchange and ultrafiltration. Also shown is the subcellular localisation of the enzyme when it is overexpressed, and evidence for its activity.

3.2 Results.

3.2.1 Confirmation Of Pectate Lyase Activity.

Initially it was necessary to determine that the *pelC* clone, pJS6197, exhibited pectate lyase activity. This was confirmed by an assay measuring the digestion of polygalacturonic acid (PGA) that is distributed throughout an agarose gel. After a suitable incubation for this digestion to occur, staining with Ruthenium Red leaves a red precipitate where intact PGA is present in the matrix. Thus the presence of clear halos around the site of sample application shows the presence of pectate lyase activity, which degrades the substrate. For this experiment, a simple sonicate prepared from *E. coli* containing either pUC19 as a control or pJS6197 was applied to the gel to demonstrate the presence of this enzyme activity (Fig 3.1). There is considerable clearing in the areas surrounding the site of application of the pJS6197 sonicate, but no clearing around the site of application of the pUC19 sonicate, showing that this halo effect is due to the expression of pectate lyase from the insert DNA in pJS6197.

3.2.2 PCR Amplification Of The *pelC* Gene.

From the nucleotide sequence of the *pelC* gene (Hinton *et al.*,1989), PCR primers were designed so as to allow the amplification of the entire coding region of the *pelC* gene. Included in these primers were restriction enzyme sites to allow easy cloning of the PCR product as a translational fusion with the ATG start site in pT7-7. The *Hin*dIII site already present at the 3′ end of the gene was the restriction site included in the 3′ primer (PELC3′), and an *Nde*I site was introduced in the 5′ primer (PELC5′). The sequences of these two primers are shown below, with the restriction sites underlined. (The extra sequence at the 5′ end of PELC5′ was introduced in order to allow easy cloning of this PCR product into another expression vector in the event of this one being unsuccessful.)

PELC5′ : 5′ CCGAGCTCG<u>CATATG</u>AAATACCTACTACC 3′

PELC3′ : 5′ CC<u>AAGCTT</u>GTTTGCAGG 3′

These primers were used in a standard PCR reaction using pJS6197 plasmid DNA as template. The product of this reaction was a DNA fragment of the

size expected, 1.2kb (Fig 3.2). It should be noted that the positions where these primers annealed to the *pelC* gene allowed the inclusion of the natural signal sequence in the new constructs, as it was anticipated that the usual periplasmic targeting of this enzyme in *E.coli* could be of some use. This could be important because a major problem in protein purification is often contamination of the protein of interest with other proteins, but as less proteins are present in the periplasm compared to the cytosol, it should be easier to purify a protein from this fraction.

3.2.3 Cloning Of *pelC* Into pT7-7.

The PCR product yielded by amplification with the PELC3' and PELC5' primers was gel purified and digested with *Hin*dIII and *Nde*I. This was ligated to pT7-7 which had been digested with the same enzymes and gel purified. This gave us an in-frame *pelC* gene, including the portion of the gene encoding the signal peptide. The reading frame of the product is shown below, with the T7 gene 10 ribosome binding site underlined and the sequence derived from the PCR product in bold type.

AAGAAGGAGATATACAT **ATG AAA TAC CTA**
rbs Met Lys Tyr Leu

This construct, pT7-7pelC, was then transformed into *E. coli* strain K38 already harbouring the plasmid pGP1-2, which encodes the T7 RNA polymerase. These transformants were grown at 30°C to prevent any transcription of the T7 gene 1 which occurs upon growth at 37°C (Studier and Moffatt, 1986).

3.2.4 Induction Of PelC Expression.

Liquid cultures of K38(pGP1-2 / pT7-7pelC) were grown and induced by elevation of the growth temperature from 30°C to 37°C during mid-log phase (OD600 approx. 0.8) for 30min, and then return to 30°C for a further 2 hours. Aliquots of both empty vector (pT7-7) and pT7-7pelC were analysed in both induced and uninduced cultures by SDS-PAGE (Fig3.3A). Western blot analysis was carried out on an identical gel using an anti pelC ascites fluid that was a gift from Sanofi, France (Fig 3.3B). As can be seen from the SDS-PAGE analysis, there was a massive increase in a protein of size 42kDa

(arrowed) in the induced pT7-7pelC cultures as compared to the pT7-7 cultures (Fig 3.3A). This size is consistent with the expected size of the pelC protein. This band was confirmed to be the pelC protein by the Western analysis (Fig 3.3B). It is also clear from this analysis that there is also a considerable amount of pectate lyase present in the uninduced pT7-7pelC culture. This is due to a well-documented 'leakiness' in the control of T7 RNA polymerase production (Tabor and Richardson, 1985; Studier and Moffatt, 1986). To combat this in order to allow the overexpression of otherwise lethal proteins, some groups have resorted to including a terminator for *E. coli* RNA polymerase into the plasmid pGP1-2 between the PL promoter and the T7 gene 1, in order that expression of the T7 gene 1 depends on transcriptional readthrough (Tabor and Richardson, 1985). This drastically reduces the level of expression of the target gene, but allows it to be expressed at a low level, whereas before it would not have been possible to express it at all. Also noticeable on the SDS-PAGE analysis (Fig 3.3A) are highly induced proteins of approximately 48kDa and 55kDa, but as these are also present in the induced pT7-7 culture, they are assumed to be artifacts of shifting cultures to an elevated growth temperature.
Also analysed on this gel was protein extracted from cultures containing cells harbouring the original plasmid containing the *pelC* gene, pJS6197. As discussed in the introduction to this chapter, the expression levels were low from this plasmid, and this is confirmed by the low level of signal seen on this Western blot (Fig 3.3B). The pectate lyase detected on this blot has been retained within the confines of the cell, as the samples were prepared from pelleted cells, and not from the culture supernatant. This was as expected, as described in the introduction to this chapter, but further analysis of the subcellular location of the enzyme was undertaken in order to determine whether the enzyme had passed into the periplasm and existed in a soluble form, or if, as described for many other proteins, had been sequestered into insoluble deposits (inclusion bodies).

3.2.5 Subcellular Location Of Overexpressed Pectate Lyase.

The expression of various *Erwinia* spp pectate lyases in *E. coli* has led to the accumulation of enzyme in the periplasm, indicating that the first step in secretion, the passage across the inner membrane has taken place, but the second step, passage across the outer membrane into the medium, has not. As discussed in the previous section, the protein has been retained within

the cell, but the precise location of the protein is unknown. In order to determine whether passage across the inner membrane had occured, a periplasmic fraction was prepared as described. Fig 3.4 shows SDS-PAGE and Western blot analysis of this fraction, along with the insoluble fraction, and total soluble protein. A fraction containing the soluble protein lacking the periplasmic fraction could not be obtained due to the 'destructive' nature of the method employed to obtain the periplasmic fraction. As can be seen in this figure, there is immunologically detectable pectate lyase in all of these fractions. This can largely be explained by the inability to obtain all the periplasmic fraction. The method employed relies on the disruption of the outer membrane by chloroform and extraction of the periplasmic contents, but there is obviously not total extraction. This explains the presence of more pelC in the total soluble protein fraction than in the periplasmic fraction. In the insoluble fraction, there was also detectable pelC, and this can be explained by the formation of inclusion bodies. These are often formed when overexpression of a protein is attempted. It is usually the case that inclusion bodies are cytoplasmic, though they may also be found in the periplasm. They occur because of a build up of a partially folded (or 'molten globule') state of the protein due to the rate limiting folding step being that of folding the intermediate state into the fully folded state. As the proteins in the partially folded state do not have their native conformation, there are often tendencies to aggregate, as hydrophobic residues found normally on the interior of the protein may be on an exposed face. Once a certain level of aggregation has occurred, these deposits become insoluble - so called inclusion bodies. These proteins have usually aggregated to such an extent that they can no longer just continue along the folding pathway, but have to be returned to a totally unfolded state in order to become properly folded (Mitraki and King, 1989). As stated, periplasmic inclusion bodies may also be formed. These are formed in the same way as cytoplasmic inclusion bodies, due to translocation across the inner membrane requiring that the protein be in a partially unfolded state. As can be seen in Fig 3.4, there is only a small amount of pectate lyase present in these insoluble deposits, even though the amount of culture these deposits were isolated from was greater than that of the total cell protein. In other overexpression experiments (eg Strandberg and Enfors, 1991), the amount of overexpressed protein present in insoluble deposits constitutes a major proportion of that protein, and large efforts have been exerted in an attempt to resolubilise these deposits to

form native protein (eg Marston, 1987; Lin and Cheng, 1991). It may be due to the periplasmic targeting of the pectate lyase here, that there has not been extensive inclusion body formation. Indeed, some expression vectors now come with the option of a periplasmic targeting leader sequence for this reason (eg the pET system from Novagen offers a choice of leader sequences).

As expected, the periplasmic fraction contained a large proportion of the pectate lyase, and this was in a soluble, active form (Fig 3.5). It can be seen here that compared to a control of induced pT7-7 periplasmic fraction, there is a large amount of pectate lyase activity associated with this fraction. It is important in this work to obtain native, active protein, as raising antibodies against the denatured protein may not give rise to antibodies which recognise the active site of the enzyme. In this work the aim is to raise antibodies recognising this region, so it is desirable to use native protein as an inoculum.

3.2.6 Purification Of Overexpressed Pectate Lyase.

As discussed in the introduction to this chapter, there are several published purification protocols for Erwinia spp pectate lyases. The protocol chosen here is that of Keen and Tamaki (1986), which involves the purification on the basis of molecular charge. The high isoelectric point of pelC, 10.3, makes this form of purification an attractive choice. At a pH of 8.0, as used in this protocol, the pelC molecules have a net positive charge. This allows them to interact with the negatively charged carboxymethyl groups in the matrix used while most other proteins do not bind and wash straight through. It is useful here that few proteins have a basic nature and so mostly have a net negative charge at this pH. To elute the bound protein from the matrix, the salt concentration of the washing buffer was increased, and the positively charged sodium ions compete off the pectate lyase molecules from the carboxymethyl groups. As the periplasmic fraction contained active, soluble pectate lyase, it was decided to use this cell fraction to apply to a column of this nature. Although the total soluble protein fraction contained more pectate lyase, it was decided not to use this fraction as it had many more species of contaminating proteins (Fig 3.4). This was more likely to present a problem in the purification than was the slightly lower protein levels found in the periplasmic fraction. The periplasmic fraction was thus used in the purification, and the pectate lyase eluted from the column in a single peak

(Fig 3.6). It can be seen from this graph that activity of the fractions corresponds to the protein levels in the fractions (as determined by absorbance at 280nm). Various fractions containing pectate lyase activity were analysed by Western blotting (Fig 3.7). As there were many fractions exhibiting pectate lyase activity, the total volume in which the protein was present was high (about 12ml). As described in the introduction to this chapter, many methods for the concentration of protein samples are available, but ultrafiltration was the method of choice here, due to the ease of use and it allows the buffer the protein is in to be changed. The resultant protein that we obtained after these purification and concentration protocols was homogeneous as determined by SDS-PAGE, and was obtained at a concentration of approximately 3mg of protein per litre of induced culture. Fig 3.8 shows SDS-PAGE analysis of protein at each step, demonstrating the purification of the overexpressed protein into a homogeneous preparation.

3.3 SUMMARY

In this chapter, the *pelC* gene from *Erwinia carotovora* subsp *carotovora* has been cloned into the pT7-7 expression vector, and large amounts of pelC protein produced by induction of this system. It has been shown that the signal sequence present in this preprotein allows targeting of a large proportion of the protein to the periplasm of *E. coli*, as seen by cell fraction studies. This periplasmic fraction was then subjected to cation exchange chromatography, making use of the basic nature of the enzyme, and then ultrafiltration through a porous membrane in order to purify this overexpressed protein away from any contaminating proteins. This produced largely pure pelC, as determined by SDS-PAGE, with a yield of 3mg pure pelC per litre of induced culture. This protein can now be used to mount an immune response to raise antibodies of interest.

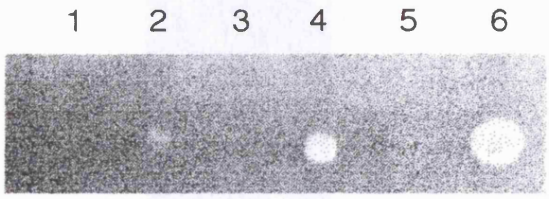


Fig 3.1. Activity Of Pectate Lyase From Clone pJS6197.
1,3,5 - 1, 2 and 5μl of XL1 (pUC19) sonicate.
2,4,6 - 1,2 and 5μl of XL1 (pJS6197) sonicate,
dotted onto a PGA-Agarose gel stained with ruthenium red to determine enzyme activity as seen by zones of clearing.

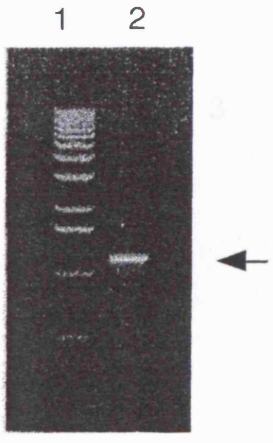


Fig 3.2. PCR Amplification Of *pelC* Gene.
Lane 1 - molecular weight markers.
Lane 2 - amplified *pelC* (1.2kb-arrowed).

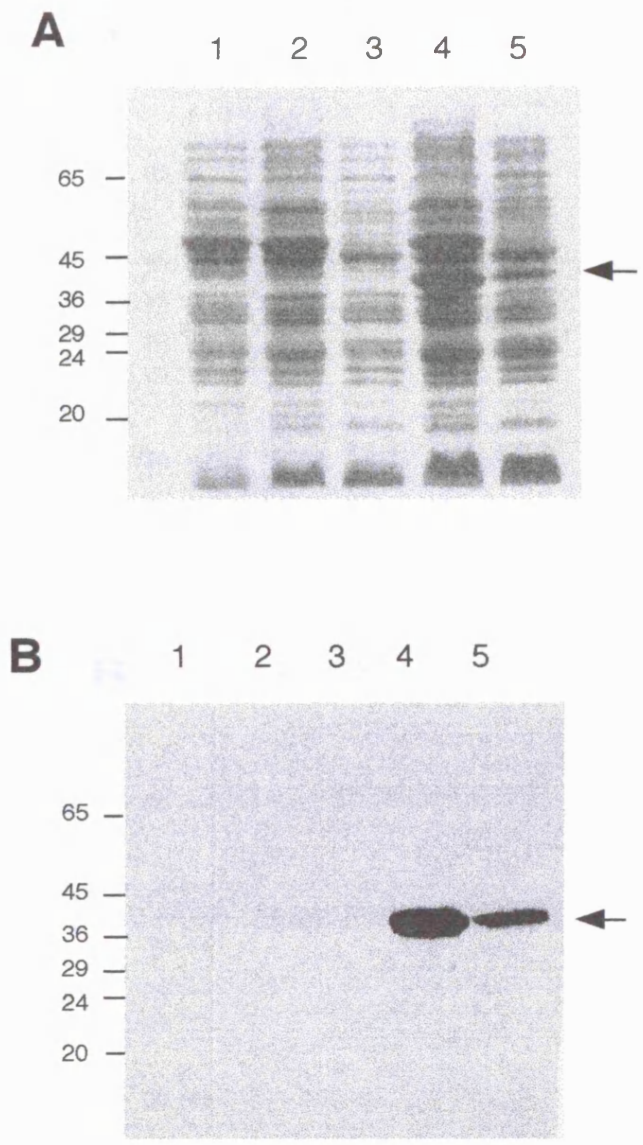


Fig 3.3. SDS-PAGE Analysis Of pT7-7pelC Cultures.
A : SDS-PAGE and B : Western Blot Analysis of overexpressed PelC protein :
Lane 1 - XL1 (pJS6197) total protein.
Lane 2 - K38 (pGP1-2 / pT7-7) induced total protein.
Lane 3 - K38 (pGP1-2 / pT7-7) uninduced total protein.
Lane 4 - K38 (pGP1-2 / pT7-7pelC) induced total protein.
Lane 5 - K38 (pGP1-2 / pT7-7pelC) uninduced total protein.
Molecular weight markers shown in kDa. Pectate Lyase (42kDa) arrowed.

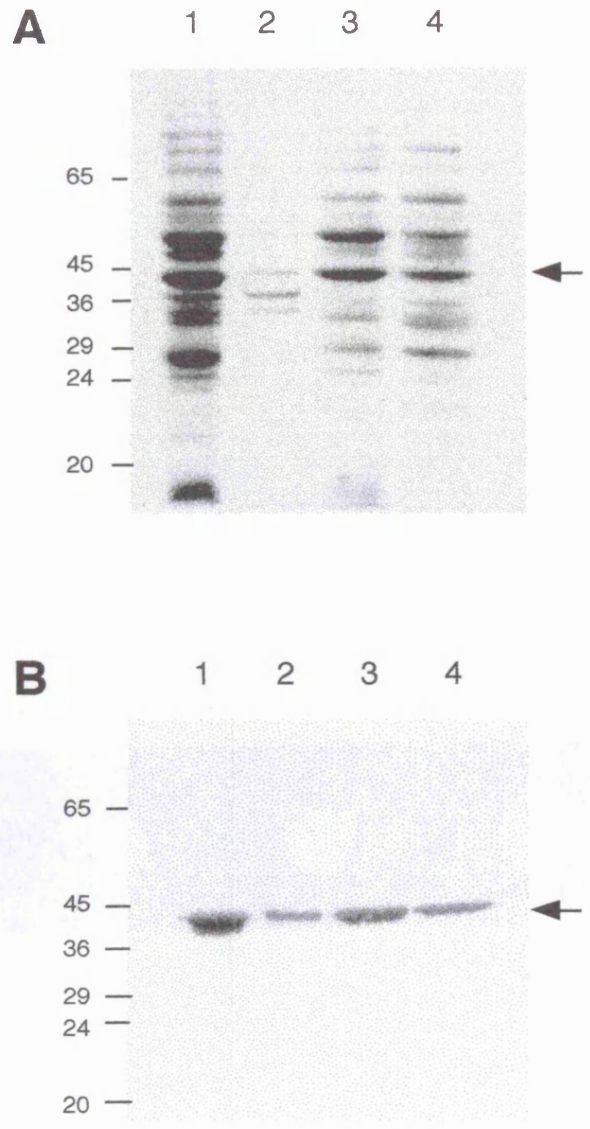


Fig 3.4. SDS-PAGE Analysis Of Subcellular Location Of PelC Protein From XL1 (pGP1-2 / pT7-7pelC) cultures.
A : SDS-PAGE and B : Western Blot Analysis of overexpressed PelC protein extracts
Lane 1 - 100μl total protein.
Lane 2 - 500μl insoluble fraction.
Lane 3 - 125μl soluble fraction.
Lane 4 - 250μl periplasmic fraction.
Molecular weight markers shown in kDa.
Pectate Lyase (42kDa) arrowed.

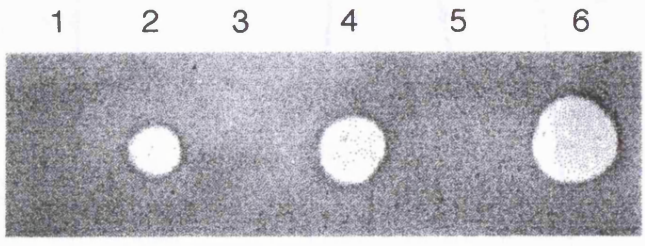


Fig 3.5. Activity Of Overexpressed Pel C On PGA-Agarose Gel.

1,3,5 - 1,2 and 5μl induced pT7-7 periplasmic fraction.

2,4,6 - 1,2 and 5μl induced pT7-7pelC periplasmic fraction

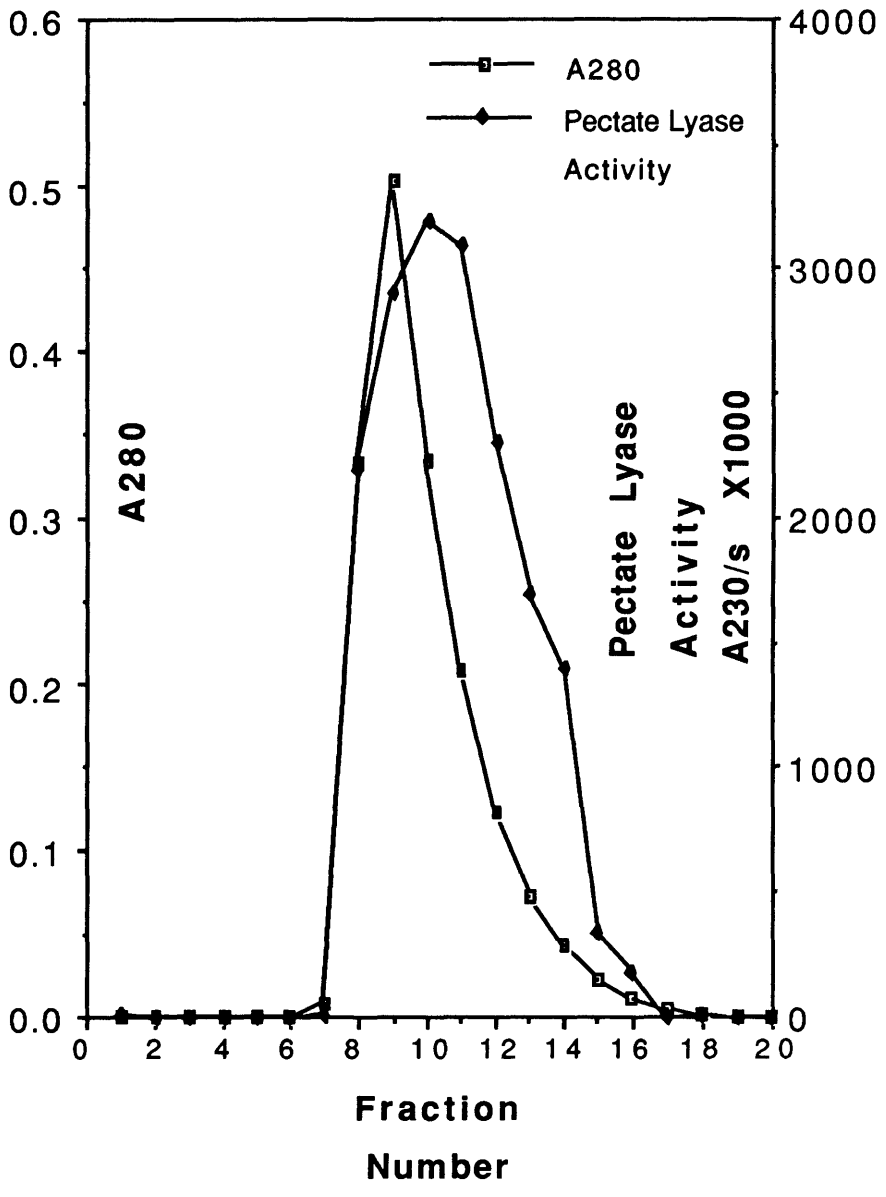


Fig 3.6. Elution Profile Of Pectate Lyase From Cation Exchange Column.

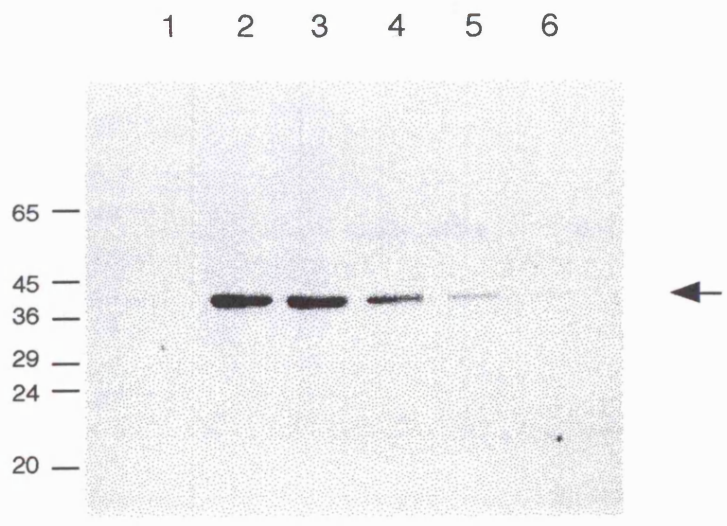


Fig 3.7. Western Blot analysis Of Eluted Column Fractions. 20μl of fractions 6,8,10,12,14 and 16 (lanes 1-6) probed with an anti-pelC antibody. Molecular weight markers shown in kDa. Pectate Lyase (42kDa) arrowed.

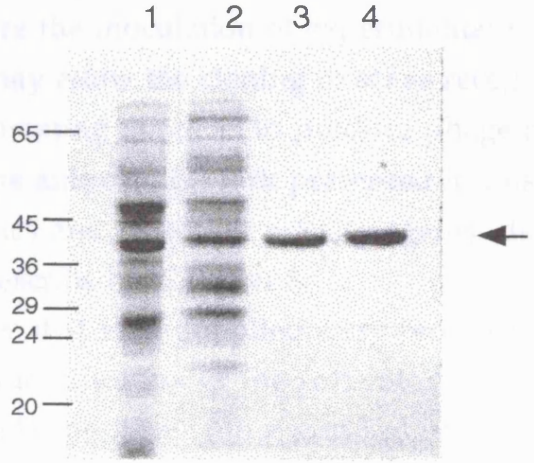


Fig 3.8. SDS-PAGE Analysis Of Steps In PelC Purification.

Lane 1 - total cell protein (100μl cells).
Lane 2 - periplasmic fraction (250μl cells).
Lane 3 - column fraction 10 (20μl).
Lane 4 - concentrated pectate lyase.
Molecular weight markers shown in kDa.
Pectate Lyase (42kDa) arrowed.

Chapter 4 : Cloning And Expression Of scFvs From anti-Pectate Lyase Hybridoma Lines.

4.1 Introduction.

As described in the main introduction, the aim of this project was to clone and express antibody fragments recognising pectate lyase C purified from *Erwinia carotovora* subsp *carotovora*. The purification of this enzyme in bulk is described in Chapter 3. To aid this work, we were supplied with five hybridoma cell lines by Prof. A. Kelman, University of Wisconsin-Madison that were shown to recognise a range of pectate lyase preparations. As this gift was made before the inoculation of experimental mice began, it was decided that they may allow the cloning of scFvs recognising pectate lyase from these lines, obviating the need to produce phage display libraries from inoculated mice. The aim of this work presented in this chapter was to clone scFvs from these lines and to test for activity against the purified pectate lyase prepared as described in Chapter 3.
The hybridoma lines that were supplied were produced for the purpose of simple serological identification of the soft-rotting *Erwinia* species. The lines were made by inoculating mice with a purified preparation of an endopectate lyase, and the subsequent fusion of the spleen cells to NS-1 myeloma cells. A number of lines were screened and found to recognise a number of strains of soft-rotting *Erwinias* and indeed other pectate lyase producing bacteria (Klopmeyer and Kelman, 1988). Five of these lines were supplied to us.

4.1.1 Cloning scFvs.

As described earlier, scFvs consist of just the two variable regions of the antibody linked together with a flexible linker peptide to allow the two domains to fold and associate as they would have done in the parental antibody, thus giving an antigen combining site. This linker peptide usually consists of residues with small side chains in order to reduce any steric hindrance that may occur. The most commonly used linker is $(G_3S)_4$ (Huston *et al.*,1988). The reasons that so much research effort was directed towards scFvs are many fold. Firstly, an scFv is the smallest derivative of a whole antibody that contains a complete antigen binding site. This is of great importance in many applications,

where the size of an IgG molecule (150kD) precludes its use, for example in penetrating tissues for radio imaging or radiotherapy (Sutherland *et al.*,1987). Small fragments (eg scFvs - 30kD) are also cleared faster from the serum and from tissues (Covell *et al.*,1986), and although this could compromise their use as targeting agents, it aids the clearance of toxic drugs from the circulation. Small fragments are also of use in research, for example in high resolution X-ray crystallographic studies of antigen binding sites where the size of the molecule and the flexibility allowed by the hinge region of an entire antibody prevents accurate results (Boulot *et al.*,1990). scFvs are also the product of a single polypeptide meaning that it is encoded by a single gene. These genes can easily be introduced into bacterial (Pluckthun 1991) and mammalian cells (Ridder *et al.*,1995) for expression at high levels. Also scFvs isolated from bacterial systems are active (Pluckthun 1991), as they require no post-translational modifications, whereas whole antibodies do. Expression in *E. coli* also allows a battery of gene manipulation techniques to be used to further study and modify antibody genes. Manipulations may also be carried out which provide the scFv with an effector function, such as linking it to a toxic protein, or providing it with an enzymatic activity.

As mentioned, the polymerase chain reaction can be utilised to clone antibody genes. This has only been possible however due to the great similarity in sequence of all antibody variable regions. Thus sets of partially degenerate 'universal' oligonucleotide primers have been designed to allow the cloning of a wide range of scFvs (Orlandi *et al.*,1989; Ward *et al.*,1989; Clackson *et al.*,1991), and these primers have been appended with restriction enzyme sites to allow the cloning of these amplified fragments into vectors (eg pHen1, fdtetDog1(Hoogenboom *et al.*,1991) and the pASK series of vectors (Skerra *et al.*,1991)). The procedure for cloning an scFv is shown in Fig 4.1. RNA from an antibody producing population of cells (in this case the hybridoma lines that we have) is isolated and subjected to a reverse transcription reaction to form cDNA. The oligonucleotides used to prime this reaction are designed to be specific for immunoglobulin sequences, and one primer set is used for each of the heavy and light chain sequences. These primers straddle the junctions between the variable and constant regions of γ heavy chains and κ light chains (Clackson *et al.*,1991). These reverse transcription reactions leave two pools of cDNA which can then be amplified by PCR. Again the reactions for the heavy and light chains are carried out using specific primers which have been designed to amplify a wide range of immunoglobulin families (Ward *et al.*,1989;

Clackson *et al.*,1991). The products of these primary PCR reactions, a light chain of approximately 330bp and a heavy chain of approximately 370bp are then purified away from contaminating primers. In order to form a full length scFv, these two chains must be linked together using a flexible peptide, to allow the chains to fold and interact correctly to create an antigen binding site. The linker peptide used here consists of 15 residues with small side chains such that steric hindrance is kept to a minimum to allow maximum mobility, as has been shown using NMR spectroscopy (Freund *et al.*,1993). The peptide sequence is $(GGGGS)_3$ (Huston *et al.*,1988). The DNA encoding this sequence is amplified by PCR in such a way that the 5' end overlaps with the sequence at the 3' end of the previously amplified heavy chain, and the 3' end with the 5' end of the light chain. This allows the whole scFv to be assembled in a single PCR reaction using equimolar amounts of each of the three components: heavy chain DNA, light chain DNA and linker DNA. This reaction leaves a DNA fragment of 750-800bp which encodes a complete scFv. To amplify this product to provide enough insert for cloning, a PCR reaction is carried out using the primers used for the primary amplification of the separate chains, but only using the 'outside' primer for each pair in order to amplify the full length scFv. These 'outside' primers have been designed to contain restriction enzyme sites that are rare in immunoglobulin variable region genes (Hoogenboom *et al.*,1991), to allow the cloning of the scFv into a phage vector.

4.1.2 Cloning scFvs From Hybridoma Lines.

The steps described above have been used to clone scFvs from preexisting hybridoma lines of known and desirable specificity. This involved the use of RNA from the hybridoma line as a starting material. The cloning of scFvs from hybridoma lines has been of use when, for example, the use of the whole antibody in treatment was not desirable due to either size constraints or because of the presence of the effector functions in the constant region. It has also been suggested as a method for 'rescuing' unstable hybridoma lines by cloning the variable regions which may otherwise be lost, for example in human-mouse hybridoma lines (Winter and Milstein 1991). Also advantages of producing scFvs from hybridoma lines is the fact that expression in bacteria gives access to gene technology, and the fast growth of *E. coli* and its comparatively simple large scale fermentation makes large scale antibody production more convenient. Despite these advantages, the fact remains that in order to achieve the production of an scFv with predetermined specificity there is the necessity

of first producing a hybridoma line.

4.1.3 Phage Display Technology.

Recent advances in gene technology has led to the generation of a new range of vectors allowing selection of clones on the basis of function. These are based around bacteriophage vectors. In order to understand this technology, a brief description of the phage involved follows. In contrast to the lytic bacteriophage that complete their life cycle by lysing the host cell, filamentous bacteriophage (eg fd, M13) do not kill their host, and new phage particles are secreted without damage to the bacterial cell. The secreted phage consist of a single, circular, single stranded DNA molecule enclosed in a tubular protein coat. This coat consists of a cylinder made up of approximately 2700 copies of a small helical protein, the product of gene VIII. The two ends of the viral filament are capped by two structures formed by four minor coat proteins present at 4-5 copies per particle. These are the products of gene VII and IX on one end and VI and III at the other end. It was initially discovered that small peptides could be fused to pIII by including their DNA sequence in the parental gene III. This was found not to affect the growth, infection or propagation of the phage (Smith 1985), and that selection of the peptides by binding to a corresponding antibody over several rounds of infection and selection allowed the isolation of very rare phage (Smith 1985). As this phage particle contains a copy of its own DNA, the peptide sequence could easily be deduced by sequencing the single stranded DNA. Surprisingly, it was also found that larger proteins could be displayed on the phage, including folded antibody fragments.

4.1.3.1 Phage Display Of Antibody Fragments.

Initially, in order to advance this technology, scFvs were cloned from hybridoma lines into phage display vectors. These phage vectors contained the pIII fusion and other proteins of the phage on a single replicon, thus providing all phage functions within a single piece of DNA. Further vectors, called phagemid vectors have also been developed. These vectors contain the pIII fusion on a plasmid containing a phage origin of replication. Phagemids can be packaged into phage particles by rescue with helper phage such as M13KO7 that provides all phage proteins including pIII but is poorly packaged due to a defective origin of replication (Vieira and Messing 1987). The use of a helper phage that itself provides pIII reduces the incorporation of the fusion pIII into the phage particle due to competition, and such phage have been estimated to

have on average less than a single copy of the fusion protein per particle (Lowman *et al.*,1991). Other helper phage have been developed that do not provide pIII (eg M13ΔgIII). The resulting phage are thus multivalent with respect to the fusion protein (Griffiths *et al.*,1993). The valency is reduced however by the fact that the pIII fusions are often proteolysed, thus giving a population of phage with a varying number of fusion proteins (Winter *et al.*,1994).

At the time that this work was initiated, many of the vectors described above had not been developed, and one of the most widely used phage vectors was fdtetDOG1, a vector constructed for the purpose of cloning scFvs using the primer sets previously described (Ward *et al.*,1989; Clackson *et al.*,1991). This vector contains the entire fd bacteriophage genome, along with restriction sites compatible with those introduced at the ends of the scFv DNA to allow a translational fusion of an scFv to be made with the gene 3 protein for phage display of the cloned scFv. This vector also contains a gene encoding tetracycline resistance in order to allow for selection in bacteria. A map of this vector is shown in Fig 4.2.

In order to ensure that the display of the scFv on the surface of the phage was working, a positive control was required. A plasmid was supplied by Prof. G. Winter, MRC Unit For Protein Engineering, University Of Cambridge, that contained a previously cloned and characterised scFv (D1.3) that recognises hen egg lysozyme (HEL) (Amit *et al.*,1986). As this gene was contained within a pUC19 backbone, it was necessary that this scFv was also cloned into the phage vector fdtetDOG1.

Described in this chapter is the cloning of the positive control scFv, D1.3, into the phage vector and the cloning of scFvs from the five hybridoma cell lines supplied by Prof. Kelman.

4.2 Results.

4.2.1 Cloning D1.3 scFv Into fdtetDOG1.

4.2.1.1 Amplification Of D1.3 scFv.

As the D1.3 scFv in the state supplied had been cloned into pUC19, it was first necessary to amplify the scFv with the PCR primers designed to add *Apa*L1 and *Not*1 restriction sites to the 5' and 3' ends respectively. This

enables the cloning of the scFv into fdtetDOG1 which contains these sites as the means of creating a transcriptional fusion with gene 3. 10 ng of scFvD1.3mycTag plasmid DNA was used as template in a standard PCR reaction using Jk1NOT10 and VH1BACKAPA10 primers (Appendix I). (Jk1NOT10 was used instead of the mix of four primers Vk4FORNOT, as this was the primer which matched the D1.3 scFv sequence exactly). The product of this reaction was run through an agarose gel (Fig 4.3A) and the 750bp band (arrowed) excised. The DNA in this agarose slice was recovered by electroelution.

4.2.1.2 Cloning Into fdtetDOG1.

The scFv encoding DNA fragment recovered was digested in standard conditions, first with *Apa*L1 and following phenol/chloroform extraction and ethanol precipitation with *Not*1. Following phenol chloroform extraction and ethanol precipitation, this fragment was ligated with similarly digested fdtetDOG1 vector to create the plasmid fdD1.3. This ligation mix was then electroporated into TG1 cells and the transformants screened for presence of the D1.3 scFv insert by PCR using the same primers used for the amplification step (Jk1NOT10 and VH1BACKAPA10). One of these positive clones was chosen for activity of the displayed scFv by ELISA.

4.2.1.3 Confirmation Of D1.3 scFv Activity.

The activity of the fdD1.3 clone was determined by ELISA. An ELISA plate was coated with hen egg lysozyme (HEL), and BSA and PELC to act as a negative controls. This plate was probed and developed following the protocol supplied with the Pharmacia detection module using both empty vector phage fd (supernatant from TG1 infected with fdtetDOG1) and the recombinant phage fdD1.3. A positive signal was observed with the HEL / fdD1.3 combination, but the others remained at background level, indicating the activity of the phage displayed D1.3 scFv (Fig 4.3B). These phage can now be used as a positive control in future ELISAs.

4.2.2 Cloning And Phage Display Of scFvs From Anti-Pectate Lyase Hybridoma Lines

4.2.2.1 Detection Of Pectate Lyase With These Antibodies.

Along with the anti pectate lyase hybridoma lines were sent concentrated culture supernantants of the five lines (4-5, 4-10, 5-9, 5-12 and 7-4) and these

were used in an ELISA to confirm that these antibodies did indeed recognise the pectate lyase prepared earlier. BSA was used as a negative control (Fig 4.4A). As can be seen from this data, all but one of the antibodies recognise the pectate lyase preparation with high specificity. Antibody 4-10 shows a low binding activity for pectate lyase along with a high absorbance value for the negative control antigen, BSA. This indicates that non-specific binding of this antibody is taking place and this may reduce the usefulness of the antibody in this work. The other differences in values for binding pectate lyase cannot be deemed significant, as the concentration of the antibodies may vary greatly between the samples.

4.2.2.2 Growth Of Hybridoma Lines And Confirmation Of Antibody Activity.

The five hybridoma lines were grown as described, and subcultured at regular intervals to give the desired cell density. The supernatant from these cultures were recovered by centrifugation and used in an immunoassay to confirm that the antibodies are still being produced. It was essential to check the continued production of the antibodies as it is a relatively common phenomenon for hybridoma lines to stop secreting one or both of the antibody chains, often caused by the loss of the gene encoding them (Chuck and Palsson 1992). Obviously if this were the case then it would be impossible to recover those genes. The supernatants were used in an ELISA to detect binding activity against pectate lyase and BSA as a negative control (Fig 4.4B). It can be seen from this data that lines 4-5, 5-9, 5-12 and 7-4 are all producing antibodies that recognise pectate lyase, but line 4-10 again shows a decrease in absorbance compared to the other lines. It does appear to be producing some specific antibodies, and the decrease in the absorbance value compared to the other lines may be due solely to a lower concentration of antibodies secreted into the culture medium. This data shows that the scFvs from these lines may be isolated, and that they should bind pectate lyase, with the exception of line 4-10.

4.2.2.3 Isolation Of RNA From Antibody Producing Hybridoma Lines.

Total RNA was prepared from approximately 10^6 cells from each of the four remaining lines. The only line which yielded any RNA detectable on an

agarose gel was line 7-4. This is probably due to the poor health of the cells. This is likely to be caused by the fact that the hybridoma lines had been stored at -80°C (and not under liquid nitrogen) for approximately 5 years before being supplied to us. It is recommended to maintain the health of hybridoma lines that they be raised from cryopreservation, grown and returned to cryopreservation every year to ensure the 'viability' of the cells stored (Harlow and Lane 1988). Although antibodies were being produced, the health of all the lines deteriorated with many more dead cells being present, and this will have decreased the number of viable, healthy cells to a level below which RNA could be isolated using the method of choice. Line 7-4 was the healthiest of the lines, and this resulted in the ability to purify RNA from this line only. Several more attempts were made to grow and isolate RNA from the other lines, but met with no success. Even upon returning to the original lines supplied, no good growth could be promoted. Thus line 7-4 was the only line with which this attempt to isolate scFvs could continue.

4.2.2.4 First Strand cDNA Production And Primary PCR.

Two first strand cDNA reactions were set up, one for the heavy chain using primer CH1FOR and one for the light chain using primer CkFOR (Appendix I). 2.5µg of 7-4 RNA was used in each reaction. 1µl of light chain cDNA was taken and used directly in a 100µl primary PCR reaction containing primers Vk2BACK and Vk4FORNOT (Appendix I). A similar reaction was set up for the heavy chain cDNA using primers VH1FOR2 and VH1BACKAPA10 (Appendix I). An annealing temperature of 55C was used for each of these reactions. 25µl of each of these reactions were run on an agarose gel to confirm the amplification of the heavy and light gene fragments (Fig 4.5A). A heavy chain product of approximately 370bp and a light chain product of 330bp can be seen as would be expected. The remainders of these primary PCR reactions were run out and these products eluted from the gel and diluted to 50ngml^{-1} in order to proceed with the assembly reaction.

4.2.2.5 Amplification Of The Peptide Linker Encoding Fragment.

As described previously, a peptide linker is essential in allowing the two chains to associate properly in an scFv conformation. The DNA encoding

this linker was amplified from the plasmid scFvD1.3mycTag (from which the D1.3 scFv had been amplified). 10ng of template plasmid was used in a 100µl reaction using primers MOLINKFOR and MOLINKBACK (these primers are complementary to Vk2BACK and VH1FOR2 respectively) (Appendix I). 10µl of this reaction was run through a high percentage agarose gel to reveal a product of 93bp as expected (Fig 4.5B). The remainder of this reaction was also electrophoresed and the fragment isolated from the gel and diluted to 25ngml^{-1}.

4.2.2.6 Assembly And Amplification Of scFv 7-4.

In order to link together the three chains involved in the construction of an scFv, an assembly reaction is carried out initially using equimolar amounts of the chains and no primers. This results in the formation of a number of products, including the full length scFv. 250ng of each of the heavy and light chains prepared in section 4.2.2.4 were used along with 50ng of linker DNA in a 25µl assembly reaction. Two such reactions were set up. This reaction consists of 7 cycles of 1 minute denaturing (94C) and 2.5 minutes combined annealing and extension (72C). Vent polymerase (a proofreading polymerase) was used in this assembly reaction as it exhibits a 5 to 15 fold higher fidelity than *Taq* polymerase and also provides blunt ends (as opposed to the 3' A overhangs given by *Taq*) to ensure that the correct sequence was maintained across the linker junctions. One of the two reactions was run through an agarose gel to monitor the assembly (Fig 4.5C). As can be seen, there is a smear of products with a few distinct bands. These include the individual heavy and light chains, and the full length assembled scFv (arrowed) at approximately 750bp. The smear of products is due to the high concentration of DNA in the reaction and the fact that there are several different combinations of products that can be formed. The other assembly reaction was used as template for the amplification reaction that needs to be performed to bulk up the scFv in order to obtain enough for cloning. 1µl was used in a normal 100µl PCR reaction containing primers VH1BACKAPA10 and Vk4FORNOT. Four such reactions were set up and 30 cycles of 1 minute denaturing (94C) and 2.5 minute combined annealing and extension (72C) performed. The reactions were then pooled and 20µl run out on an agarose gel (Fig 4.5D). As can be seen, there are also multiple products of this reaction. This is due to the fact that there are so many different

combinations of 'primers' that can be used. For example, any linker fragment that was not used up in the assembly reaction could act as a primer with either of the light or heavy chain primers to produce a different size product. The major product however is the full length 750bp scFv (arrowed), and this has changed from being a minor product after the assembly reaction (Fig 4.5C) to being the major product after amplification of this range of products (Fig 4.5D).

4.2.2.7 Cloning scFv 7-4 Into fdtetDOG1.

The remainder of the assembled and amplified reaction was run through an agarose gel and the major product of 750bp excised and purified from the gel by electroelution. The resulting fragment was then ethanol precipitated and resuspended in water at approximately $50ngml^{-1}$. This fragment was then digested as described before (section 4.2.1.2) with *Apa*L1 and *Not*1 and ligated to similarly digested fdtetDOG1 to create plasmid fd7-4. The resulting ligation mix was then transformed into *E.coli* TG1 cells by electroporation and transformants screened for the presence of the 7-4 scFv by PCR analysis using primers VH1BACKAPA10 and Vk4FORNOT. The presence of a 750bp band in three of the transformants obtained indicated that these colonies contained the fd7-4 plasmid.

4.2.2.8 Determination Of fd7-4 Activity.

ELISA analysis was carried out in order to determine whether the cloned 7-4 scFv retained the binding activity of the parental monoclonal antibody. An ELISA plate was coated with HEL and BSA to act as negative controls and pectate lyase to detect any binding activity. This plate was then probed with empty vector fd, fdD1.3 as a positive control and fd7-4. After development using the standard method, activity was seen in the HEL / fdD1.3 combination but with no others (Fig 4.6). This was the case with all of the three PCR positive clones. Even upon concentration of the phage in the culture supernatants ten-fold by PEG precipitation, no detection of pectate lyase could be made with fd7-4. In order to determine whether any PCR based errors could be attributed to causing this lack of recognition, the nucleotide sequence of the 7-4 scFv was determined.

4.2.2.9 Sequence Analysis Of fd7-4.

The nucleotide sequence of scFv 7-4 along with the expected translation product is shown in Fig 4.7. All the three scFvs cloned showed this same sequence. This sequence shows that there are no errors such as deletions, insertions or frameshift mutations that may cause inactivity of the scFv. Upon searching the Kabat database of antibody sequences with this sequence (Martin 1996), the heavy and light chains were shown to be variable region genes, with the heavy chain being classed as an IgGIIA chain and the light chain as an IgGkIV chain. The complementarity determining regions were also analysed (Martin 1996) to check that they occured in recognised positions with the expected conserved residues preceding and following them. The CDRs were found to be normal, and to occur in conserved regions as expected. The CDRs, along with the linker peptide are highlighted in Fig 4.7B. The residue length and Chothia classes of the CDRs were determined:

CDR	Length	Class
L1	10	I
L2	7	I
L3	9	I
H1	10	III
H2	16	I
H3	9	-

Upon searching the EMBL sequence database with the heavy and light chain sequences seperately, high homology matches were found with murine immunoglobulin sequences, with a maximum identity of 87% found with the heavy chain and 92% with the light chain. From this analysis it would appear that the heavy and light chains from the hybridoma line 7-4 have been cloned and assembled into an scFv, but this remains inactive.

4.3 Summary.

The work in this chapter describes the cloning of an scFv from one of the cell lines that were a gift from Prof A. Kelman. The other lines failed to yield any RNA on purification due to the poor health of the cells. The one line

that did yield RNA, line 7-4, was amplified and cloned into scFv conformation, yet failed to recognise the antigen, pectate lyase. The sequence of this scFv was determined and shown to be immunoglobulin variable regions with recognised positions and conformations for the complementarity determining regions. There may be several possible reasons for the inactivity of scFv 7-4. When this work was carried out it was generally accepted that the cloning of an scFv from a hybridoma line would lead to the production of an active single chain antibody. Since then however, there have been reports suggesting why this may not occur. It is possible that the primers used to amplify the heavy and light chains have introduced amino acid sequences that would not normally be found in the regions in which they anneal. This may cause a change in the framework regions which may in turn cause the scFv to be inactive. This is particularly true in the region following CDR H3, where the primer VH1FOR2 encodes for sequence up to 1bp from the 3' end of this CDR. Although the amino acids immediately after CDRH3 (WGQG) correspond to those expected (WGXG) (those residues underlined are always present), the glutamine residue may not be the actual residue encoded by the original 7-4 sequence. This may cause the structure supporting CDR3 to be altered, thus altering the conformation of CDR3, which has been shown to be important in antigen specificity.

Another possible reason for scFv 7-4 not recognising pectate lyase is the fact that the myeloma line used in the production of these hybridoma lines (myeloma line NS-1) has been shown to produce its own kappa light chain that can be secreted as part of a hybrid immunoglobulin molecule (ie one containing a mixture of specific heavy chains with either specific or NS-1 kappa chains). It is quite possible that the light chain that has been cloned in this work is indeed the NS-1 light chain. If this is the case, then the scFv 7-4 that has been produced contains only the 7-4 heavy chain, linked to the inactive NS-1 kappa chain. Although the heavy chain has been shown to encode much of the specificity in engineered antibodies (Ward *et al.*,1989), it is possible that in this case, the heavy chain alone is not sufficient to recognise pectate lyase. The similarity between the NS-1 light chain and other, specific light chains has been shown to be very high, such that techniques such as hplc have had to be used in order to seperate hybrid molecules from specific molecules (Abe and Inouye 1993). The nucleotide sequence of the NS-1 kappa chain has not been deposited in the Kabat or

EMBL databases however, so the sequence shown cannot be compared to it. It has also been reported that the related myeloma line SP2/0 also produces an aberrant kappa chain at high levels (Duan and Pomerantz 1994). These authors describe the construction of scFvs from hybridoma lines being disrupted by this, and the development of a PCR screen of scFvs using the CDR sequences of the aberrant chain as template. They also describe the production of an SP2/0 kappa chain specific ribozyme which has been packaged into a retroviral expression system. The suggestion is that this ribozyme could be transduced into hybridoma lines produced using myeloma SP2/0 and expressed in order to eliminate the endogenous kappa transcript (Duan and Pomerantz 1994).

It is also possible that the scFv that has been cloned is not derived from the 7-4 hybridoma line at all, despite all the precautions that were taken, such as spatially separating pre and post amplification reactions so as to reduce that chance of contamination, and also working in a sterile laminar flow hood for the pre amplification reactions. Negative control reactions were also carried out at every step, merely omitting the template to ensure that the reaction mixes were not contaminated. It is possible despite these precautions that the scFv was cloned from another source of antibody genes that were present in the laboratory. This is thought to be unlikely, but is still a possibility.

It is thought to be likely that the light chain cloned in this work was not in fact that from the hybridoma line 7-4, but the kappa chain that this line will express as a result of being the product of a fusion with the myeloma cell line NS-1. This would explain the inactivity of the scFv, as only the specific heavy chain would be present, and this alone may not be sufficient to recognise pectate lyase. It has recently been recommended that in the cloning of an scFv from a hybridoma line, it is important to produce a large number of clones (10^5), and to then select those scFvs from this population by panning for activity (Antibodies On Display, Stratagene, 1995). This would then reduce the chance of isolating an scFv that contains an abberant chain encoded by the myeloma line used in the production of that hybridoma line.

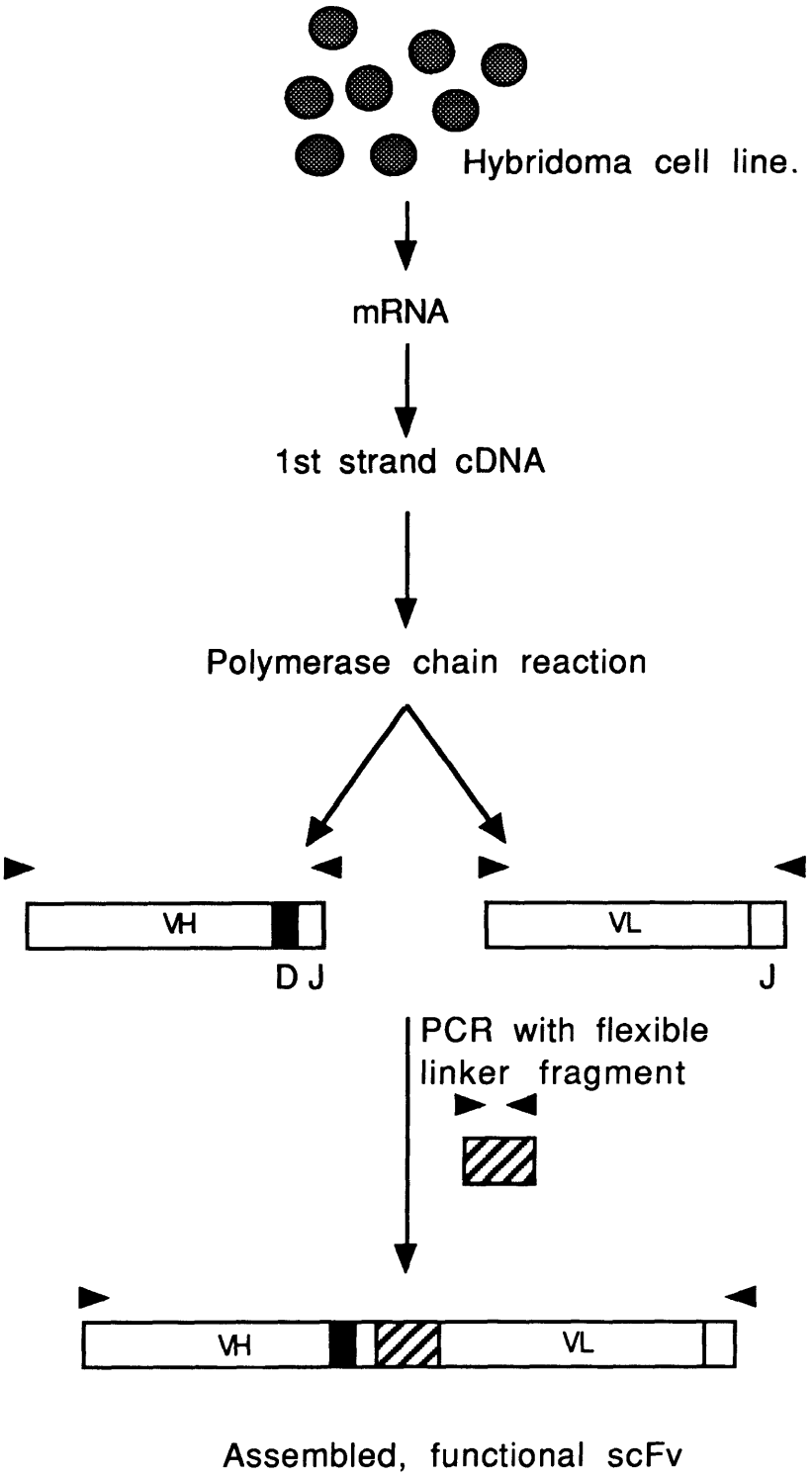


Fig 4.1. Steps Taken In Cloning An scFv From A Hybridoma Line.
VH - heavy chain variable region; VL light chain variable region; D - D segment; J - joining segment.

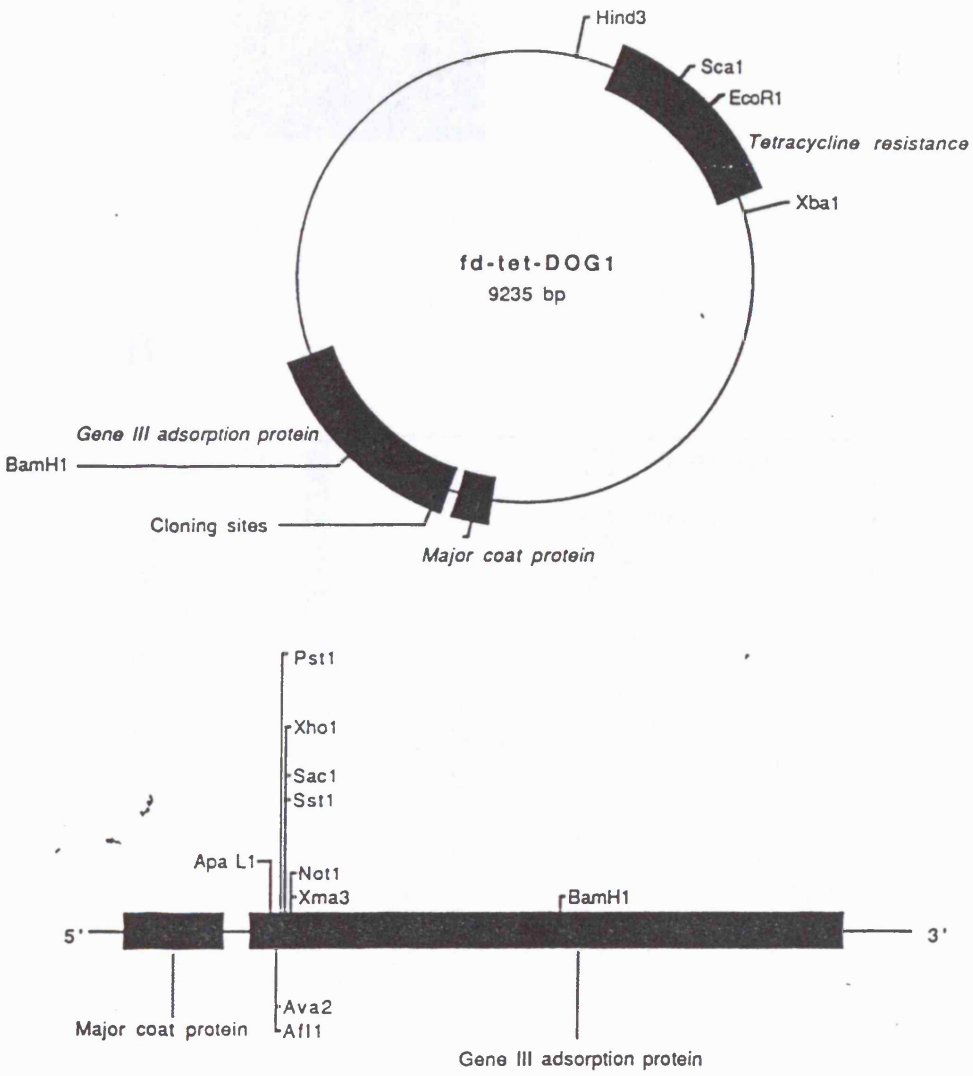
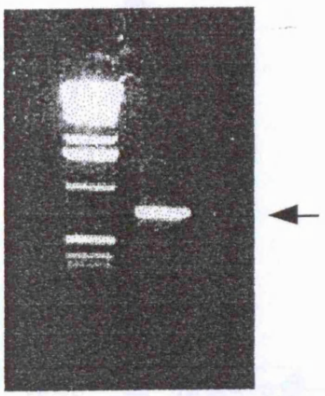


Fig 4.2. Vector fdtetDOG1.

A



B

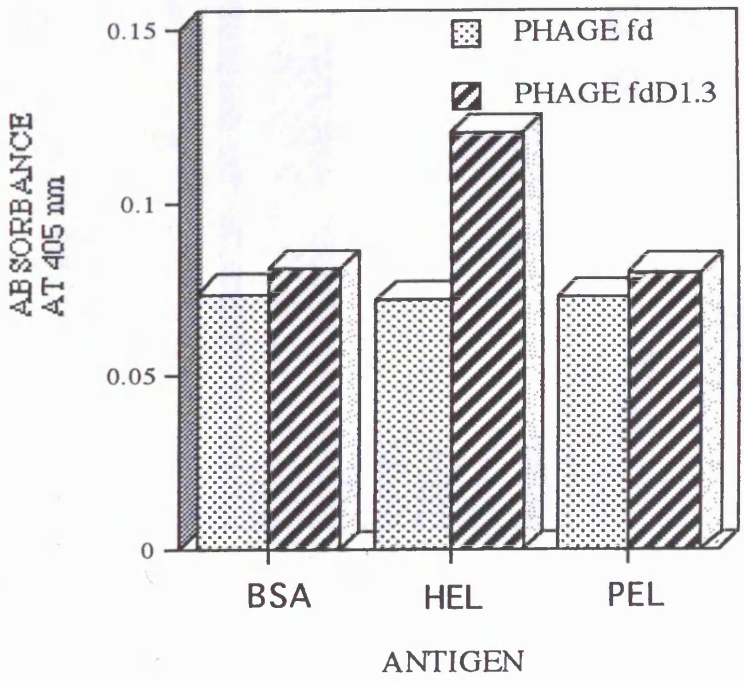


Fig 4.3. Cloning And Phage Display Of scFv D1.3.

A : Lane 1 - molecular weight markers.
Lane 2 - amplified D1.3 scFv (approx 750bp) arrowed.
B : Absorbance values from ELISA with phage fd and fdD1.3 on BSA (bovine serum albumin), HEL (hen egg lysozyme) and PEL (pectate lyase).

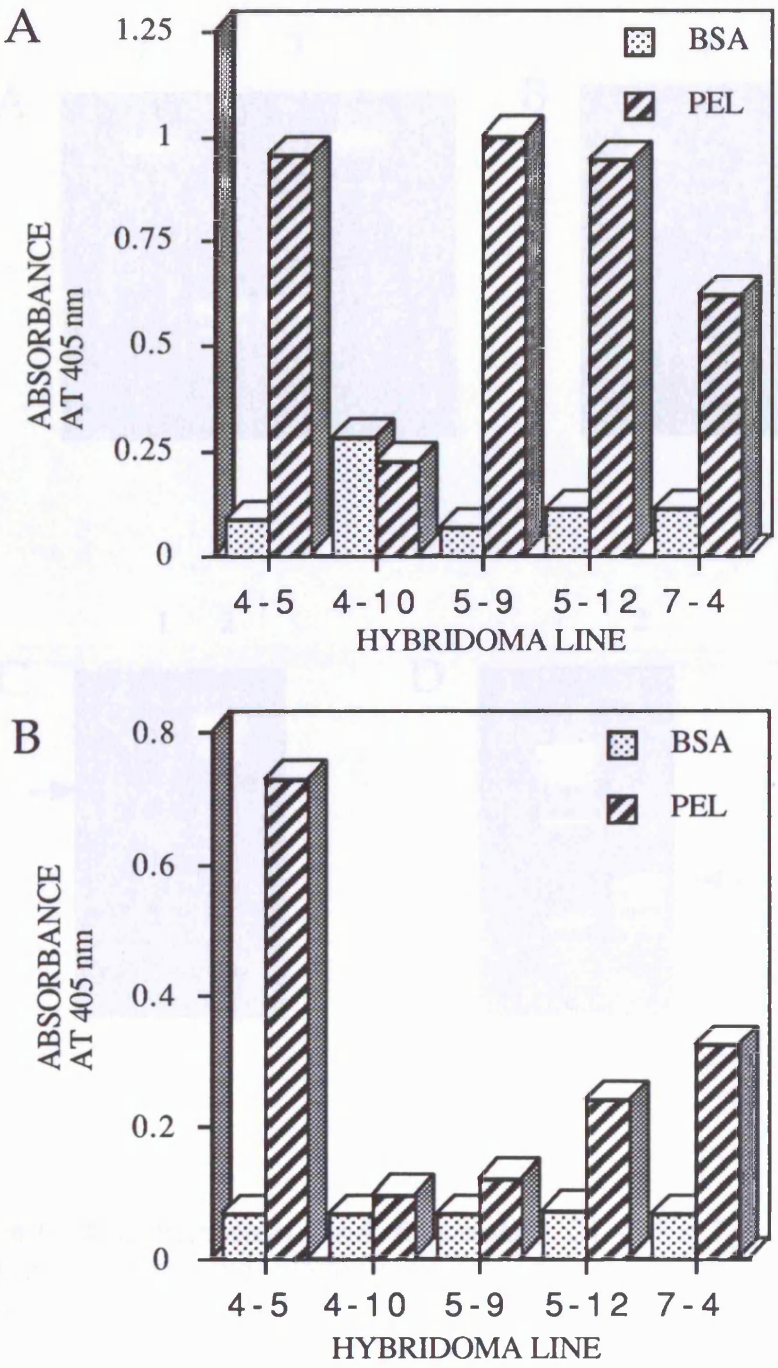


Fig 4.4. Activity Of Hybridoma Lines Raised Against Pectate Lyase.

A : Absorbance values from ELISA using concentrated antibodies against pectate lyase supplied by Prof Kelman.

B : Absorbance values from ELISA using hybridoma culture supernatant against BSA (bovine serum albumin) and PEL (pectate lyase).

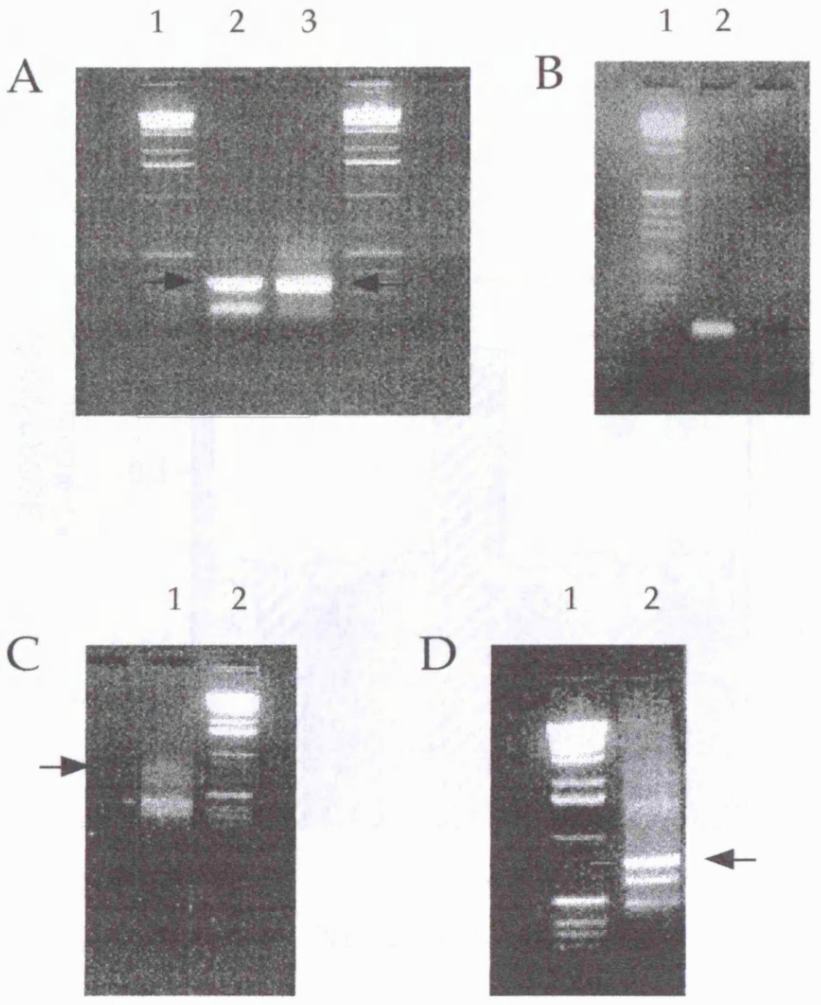


Fig 4.5. Amplification And Assembly Of scFv 7-4.
A : Lane 1 - molecular weight markers. Lane 2 - amplified heavy chain (approx 370bp - arrowed). Lane 3 - amplified light chain (approx 330bp - arrowed).
B : Lane 1 - molecular weight markers. Lane 2 - amplified peptide linker (93bp - arrowed).
C : Lane 1 - assembled scFv 7-4 (approx 750bp - arrowed). Lane 2 - molecular weight markers.
D : Lane 1 - molecular weight markers. Lane 2 - amplified assembled scFv 7-4 (approx 750bp - arrowed).

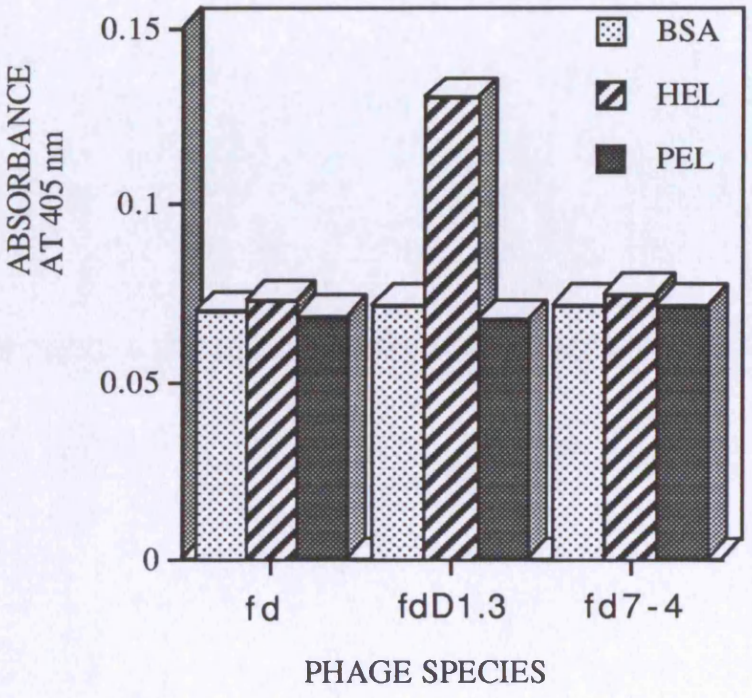


Fig 4.6. Activity Of scFv7-4 Against Different Antigens. Absorbance values from ELISA using BSA (bovine serum albumin), HEL (hen egg lysozyme) and PEL (pectate lyase) with phage species fd, fdD1.3 and fd7-4.

A

```
  1  CAGGTGGAGCTGCAGGAGTCAGGACCTGAGTTGGTGAAACCTGGGGCCTC
 51  AGTGGAGATTTCCTGCAAGGGTACAGGATACACATTCACTGACTACAACA
101  TGCACTGGGTGAAACAGAGCCCTGGAAAGAGCCTTGAGTGGATTGGAAAT
151  TTTTATCCTTACAATGGTGCTACTGGCTACGACCGGAAATTCAAGAGCAA
201  GGCCACATTGACTGTAGACTATTCTTCCAGTATAGCATACATGGAACTCC
251  GCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGTGCAAGAAACTAT
301  AAGTACGACGGCTTTGCTTACTGGGGCCAAGGGACCACGGTCACCGTCTC
351  CTCAGGTGGAGGCGGTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCGG
401  ACATTGAGCTCACCCAGTGTCCAGCAATCATGTATGCATCTCCAGGGGAG
451  AAGGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGCAGTG
501  GTACCAGCAGAAGTCAGGCTCCTCCCCCAAAAGATGGATTTATGACACAT
551  CCAGACTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGGG
601  ACCTCTTACTCTCTCACAATCAGCAGCATGGAGGCTGAAGATGCTGCCAC
651  TTATTACTGCCAGCAGTGGAGTAGTAACCCACACACGTTCGGAGGGGGGA
701  CCAAGCTGGAGCTGAAACGGGCG
```

B

```
                                   H1                                H2
QVELQESGPELVKPGASVEISCKGTGYTFTDYNMHWVKQSPGKSLEWIGNFYPYNGAT

                                                          H3
GYDRKFKSKATLTVDYSSSIAYMELRSLTSEDSAVYYCARNYKYDGFAYWGQGTTVTV

                                                       L1
SSGGGGSGGGGSGGGGSDIELTQCPAIMYASPGEKVTMTCSASSSVSYMQWYQQ

                 L2                                              L3
KSGSSPKRWIYDTSRLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNP

HTFGGGTKLELKRA
```

Fig 4.7. Analysis of scFv 7-4 Sequence.
A : Nucleotide Sequence and
B : Expected Translation Product Of scFv7-4.
Complementarity determining regions are underlined; linker peptide shown in bold type.

Chapter 5 : Construction And Screening Of Phage Display Libraries.

5.1 Introduction.

As attempts to isolate scFvs from pre-existing hybridoma lines secreting antibodies recognising pectate lyase were unsuccessful, an alternative approach was the construction of phage display libraries from mice immunised with the pectate lyase purified previously (Chapter 3). It was thought that this approach may yield a different range of scFvs that may have some affect on the activity of the pectate lyase used as an inoculum. For this work it was important to use active enzyme as the inoculum (as prepared in chapter 3). This is because the protein will be in a native state, and hence any antibodies raised against this protein would also recognise the enzyme *in vivo*. If this was not the case, then it would be unlikely that any antibodies that are isolated would recognise the active site, and would thus not be able to affect the activity of the enzyme. It is also possible that an antibody that does bind to the active site of the pectate lyase purified would also bind to the active sites of other pectate lyase isozymes, and also possibly pectate lyases from other species. If this antibody were also to inactivate PelC, then there would be a likelyhood that it would also have this effect on other pectate lyases.

Alongside the attempts to isolate scFvs recognising pectate lyase were similar attempts to isolate scFvs that recognised, and also possibly affected the enzyme activity of the enzyme polygalacturonase from *Botrytis cinerea*. As described in the introduction, *Botrytis cinerea* is an important fungal plant pathogen, and effects its pathogenicity by the secretion of enzymes including polygalacturonase. Polygalacturonase is the first enzyme secreted by this, and other species of fungal pathogen when grown on isolated cell walls (Johnston and Williamson 1992). *Botrytis cinerea* attacks a wide range of species, particularly soft fruit (McNicol *et al.*,1985), and there is considerable variation in the production of polygalacturonases between fungal isolates. This variation reflects the broad host range of this pathogen (Johnston and Williamson 1992). In order that attempts could be made to isolate scFvs recognising this important fungal pathogenicity factor, polygalacturonase purified from *Botrytis cinerea* was supplied by Dr Jon Ride, University of Birmingham.

5.1.1 Phage Display Libraries.

As described previously, the cloning of scFvs from hybridoma lines still requires the cloning of that hybridoma line, a time consuming and labour intensive exercise. In order to circumvent these problems, advances were made in the construction of phage display libraries. The aim was to mimic features of immune selection discussed earlier, such as affinity maturation. In order to mimic the primary immune response, cDNA produced from the spleen of immunised mice was used as a starting material. This, when amplified using the universal primers described earlier (Chapter 4), gave a pool of many different heavy and light chains whose transcripts were expressed in the B cells of the immunised mice. These were again linked together with the peptide linker in a random manner to give a whole range of scFvs whose affinities for various antigens should reflect the immune response of the immunised mice. This pool of scFvs can then be cloned into phage or phagemid vectors to create a library of scFvs from the B cells of the immunised animal. These scFvs are displayed in a functional form on the tip of the phage. After growing up a large culture of bacteria producing these phage and isolation of the phage from the culture supernatant, they can be panned against the antigen of interest which has been immobilised. After removing the phage which do not bind by washing, the remaining phage can be eluted and reintroduced into *E. coli* (Fig 5.1). By carrying out several rounds of this 'panning', phage displaying scFvs which bind to the antigen can be isolated (Clackson *et al.*,1991). Even with an enrichment of antigen binders of only 50 per round of panning, after four rounds of panning the enrichment factor rises to around 10^7. This technique was initially demonstrated by the production of scFvs from mice immunised with the hapten 2-phenyloxazol-5-one (phOx) (Clackson et al.,1991). From this library, several scFvs which bound to the hapten were isolated. The initial library was studied and it was found that it was diverse and contained most of the VH and Vk subgroups. After selection with antigen, scFvs that bound to the hapten were found to be encoded by V genes similar to those found in hybridoma lines secreting antibodies recognising the hapten, but in promiscuous combinations. This meant that the same V gene was found in combination with several different partners. From these promiscuous chains, hierarchical libraries were produced by the combination of the promiscuous chain with entire repertoires of the other chain, and these led to the isolation of scFvs which also showed strong binding (Clackson *et al.*,1991). The use of immunised mice as the source of material for this work was preferable due to

the fact that immunisation leads to not only an increase in the number of cells making an immune response, but also in the levels of immunoglobulin mRNA. It has been estimated that a resting B cell contains around 100 copies of Ig mRNA whereas a hybridoma cell (and also presumably an active, antibody secreting plasma cell) contains approximately 30000 copies. Thus libraries derived from this material is greatly enriched in V genes encoding a portion of an antigen binding site (Hawkins and Winter 1992). However, in random combinatorial libraries as described above, the VH and VL pairings are made at random, thus destroying the V gene pairings found in the immune lymphocyte. Nevertheless, libraries derived from immune lymphocytes contain antigen binding fragments at low frequencies (often less than 1:5000 (Caton and Kaprowski 1990, Persson *et al.*,1991), at best 1:500 (Mullinax *et al.*,1990)), and the power of selection from phage display systems allows the isolation of these binding scFvs.

5.1.2 scFvs Isolated From Phage Display Libraries.

Many different antibodies have been isolated from individual phage display libraries with specificities against many different types of antigens. An added advantage of phage display libraries is the ability to isolate scFvs that could never have been made by conventional hybridoma technology. These include anti-self scFvs such as those against endoplasmic reticulum proteins such as protein disulphide isomerase (Clark and Freedman 1994). These antibodies cannot be made in hybridomas because they would simply bind to this antigen and hence never be secreted. Another major advance in the development of phage display technology has been the creation of human scFv phage display libraries.

5.1.3 Human Phage Display Libraries.

Previously, in order to use antibodies derived from mouse hybridomas in therapeutics, it was desirable to alter them. This is due to the human immune system seeing the mouse antibody as foreign, and thus seeking to destroy them. Different attempts were made to reduce this effect, such as the transfer of the mouse variable regions onto the human constant regions to form a partially chimeric antibody. This was partially successful, but due to the remaining mouse sequence, recognition as foreign still occurred (Adair 1992). More successful were attempts to introduce the murine CDRs into a background of the human framework regions, thus producing a fully humanised antibody.

Even this was not totally successful, as the removal of the murine framework regions can alter the conformations adopted by the CDRs, thus altering the binding characteristics of the antibody (Adair 1992). After the development of mouse scFv phage display libraries, it was discovered that such libraries could be made of human scFvs (Marks *et al.*,1991a). By the same methods as those used previously, 'universal' primers were used to amplify human variable regions, assemble these into functional scFvs and screen with antigen. This has been of great interest to the therapeutics industry, with the realisation that human antibodies can now be made whereas previously, human-mouse hybridoma lines had been produced but were inherently unstable. Again, antiself antibodies were able to be made, and also scFvs against disease causing agents. For example, recombinant antibodies have been isolated from immunised patients with binding activities against a range of viral antigens such as HIV gp120 (Barbas *et al.*,1992a), respiratory synctial virus (RSV) (Barbas *et al.*,1992b) and hepatitis B virus (Zebedee *et al.*,1992). Indeed, the antibody fragments recognising HIV and RSV were capable of neutralising virus infection (Barbas *et al.*,1992a, Barbas *et al.*,1992b). These results demonstrate the great power of cloning scFvs from human phage display libraries. This work also reflects on the efficacy of isolating scFvs that interfere with molecules in order to affect their normal activity, an effect that is the aim of the work presented in this chapter.

5.1.4 By-Passing Immunisation.

As the display of scFvs on the surface of phage, and their selection by panning against antigen mimics the primary immune response in animals, given a large and diverse enough naive (ie unimmunised) library, it should be possible to isolate scFvs with binding specificity to any given antigen. This should be possible because of the large number of individual variable chains pairing up randomly to create a diverse range of unpredictable binding specificities. Such a library was made using human peripheral lymphocytes as a source of V genes that were isolated using family specific primers (Marks *et al.*,1991b). These VH and VL repertoires were combined at random in order to destroy the original combinations. From this library, scFvs with many different binding specificities were isolated, including those against BSA, turkey egg lysozyme and the hapten phOx (Marks *et al.*,1991a). Also isolated were antibodies against human self antigens such as tumour necrosis factor α, carcinoembryogenic antigen, mucin and CD4 (Griffiths *et al.*,1993). Although many anti-self specificities were

isolated from such a library, it is impossible to prove that one or another of the antibody chains were derived from B cells with self specificity, and it was also found that most of the chains were somatically mutated, suggesting that antigen driven selection had taken place before cloning was carried out. This led to the idea that a totally synthetic human scFv library would be of great use.

5.1.5 Synthetic Libraries.

In order to create a diverse synthetic library, all 49 germ line human VH gene segments were cloned, and were manipulated in such a way as to give a random H3 loop of between 4-12 residues (Nissim *et al.*,1994). This heavy chain repertoire was combined with a single human lambda light chain. After selection of scFvs against a wide range of antigens, including haptens (eg phOx and FITC), foreign antigens (eg maltose binding protein, ovalbumin) and self antigens (eg thyroglobin and human elongation factor-1α), it was found that scFvs with a binding activity to all antigens tested could be isolated (Nissim *et al.*,1994). This 'single pot' library has been made widely available and has become a popular tool for the isolation of scFvs, as it removes all the technical problems associated with the creation of a new phage display library for each new antigen. This type of library may also allow new epitopes to be discovered, to which monoclonal antibodies have not been raised in the past due to a lack of immunogenicity (Nissim *et al.*,1994). It is possible however that scFvs isolated in this fashion may have low affinity, due to there not being any maturation of the chains, as would normally occur in an immune response, but this is counterbalanced by the fact that an antibody has been raised at all against any particular epitope.

Although these advances in the cloning of scFvs have been made in recent years, at the time this work was initiated (early 1993), the choice of approaches, and vectors available to create libraries was much narrower. Most work involved the use of the pHen1 (phagemid) and fdtetDOG1 (phage) (Ward *et al.*,1989) vectors from the laboratory of Greg Winter. It was decided initially to attempt to construct a library in the phage vector fdtetDOG1, as there was already some experience of using this vector, and the corresponding oligonucleotide primers had already been synthesised (Chapter 4). This vector contains all the genes necessary for the growth and propagation of recombinant phage particles, along with the tetracycline resistance gene to allow for selection. Once recombinant phage particles

have been constructed, they can easily be grown at a large scale and isolated from the culture supernatant. These phage can then be panned on solid phase antigen to select those that bind to the antigen, and the non-binders washed away. Repetition of this procedure following the reinfection of *E.coli* results in continually more stringent selection for binding scFvs.

5.2 Results.

5.2.1 Construction Of A Phage Display Library In fdtetDOG1.

As large amounts of pectate lyase had been purified, but only a relatively small quantity of polygalacturonase had been supplied, it was decided that initial attempts to construct libraries from pre-immune mice would be conducted with pectate lyase only.

5.2.1.1 Immunisation Of Experimental Mice.

10 female BALB/c mice were inoculated with pectate lyase as described on days 0, 4, 7 and 11. After these initial inoculations to mount an immune response, a test bleed was taken on day 14. These bleeds were tested for pelC binding activity on dot-blots. There were differing levels of pelC binding activity in the different mice, but all of the mice exhibited some response. The three mice exhibiting the best response from the initial test bleeds were then boosted with another inoculation after a two week rest period, and then were sacrificed 3 days later and the spleen removed and blood collected.

5.2.1.2 Production Of scFvs.

A scheme of the steps taken to construct an scFv library are similar to those used to isolate scFvs from hybridoma lines (Fig 4.1). RNA was isolated from the spleen following isolation of the lymphocytes from the rest of the tissue using the method described. 700ng of this total RNA was then subjected to reverse transcription reactions using oligonucleotides CH1FOR for the heavy chain reaction and CκFOR for the light chain reaction. Primary PCR reactions were then performed, using (after optimisation by altering the quantity of cDNA added until a reaction resulted in a strong product with low background) 1μl of light chain cDNA per 100μl reaction using primers Vκ2BACK and Vκ4FORNOT, and 2μl of heavy chain cDNA in conjunction

with primers VH1FOR2 and VH1BACKAPA10. These reactions were electrophoresed through an agarose gel, and yielded a heavy chain of approximately 370bp and a light chain of approximately 330bp (Fig 5.2A). These PCR products were isolated from the gel and diluted to 50ngml^{-1}. These chains were then used in an assembly reaction as described previously with the linker fragment produced in chapter 4 (section 4.2.2.5). Again, one of the two reactions was run out on a gel to monitor the assembly (Fig 5.2B). The assembled scFv product is arrowed. A portion of the other corresponding reaction was used in an amplification reaction using primers Vκ4FORNOT and VH1BACKAPA10, and again the minor product of the assembly reaction becomes the major product of the amplification reaction (arrowed) (Fig 5.2C). This band represents the randomly assembled heavy and light chains that initially existed in the spleen of the mice that were used.

5.2.1.3 Cloning scFv Library Into fdtetDOG1 And Screening Of Activity.

The assembled and amplified library of scFvs was eluted from an agarose gel and cloned into fdtetDOG1 as described previously (section 4.2.1.2). After electroporation into TG1 cells, 274 colonies were obtained. This library size was considerably smaller than libraries described previously (eg Clackson *et al.*,1991). There are several possible reasons for this. Due to the need to incubate restriction digests involving PCR products for a long period of time to ensure efficient cutting (several sources suggest overnight incubations) it is possible that an exonuclease activity had destroyed the overhangs left by the restriction enzymes, thus reducing the efficiency of ligation. The large size of the vector (9.2 kb) reduces the transformation frequency from the levels that are quoted for frequencies with supercoiled small vectors (which transform with a high frequency), and this will reduce the size of a library made in a large vector. After this work had been completed, another possible factor contributing to the small library size was revealed. It was discovered that the transformation frequencies of plasmids containing the tetracycline resistance gene for selection were greatly decreased upon electroporation (Steele *et al.*,1994). This work was conducted with the plasmid pBR322, which carries both the tetracycline and ampicillin resistance genes to allow a comparison of electroporation frequencies using

these different antibiotics for selection. It was found that transformation frequency was reduced by two orders of magnitude when using tetracycline instead of ampicillin. As the protein involved in tetracycline resistance is found on the inner cytoplasmic membrane and acts by either decreasing transport of the antibiotic into the cell or enhancing its export, the effect of electroporation in partially solubilising the membranes may cause damage to this receptor, thus reducing its effectiveness (Steele *et al.*,1994). Upon screening these colonies, only 31 contained inserts of the correct size (approximately 750bp) as determined by PCR screening using primers Vκ4FORNOT and VH1BACKAPA10. This could also reflect the presence of an exonuclease activity, with the overhangs produced on the digested vector being destroyed allowing the self-ligation of the vector. These positive clones were then grown up and the phage particles recovered from the culture supernatant. These phage samples were then tested for their recognition of pectate lyase in an ELISA using BSA as a negative control. None of the phage gave a signal higher than background for pectate lyase, even upon concentration by PEG precipitation. It was thus determined that the library made from mice immunised with pectate lyase did not contain any scFvs that recognised pectate lyase.

5.2.1.4 Further Attempts At Constructing Libraries In fdtetDOG1.

Two subsequent attempts were made in the construction of an anti pectate lyase library in this vector, including the use of restriction enzymes from different manufacturers to try to circumvent the possible problem with exonucleases, but met with no more success than the first attempt. The primary cause of the lack of pectate lyase binding scFvs was considered to be the small size of the libraries made. As discussed in the main introduction, libraries constructed from immune lymphocytes contain antigen binding sites at low frequencies, considered to be at best 1:500 (Mullinax *et al.*,1990) but more normally 1:5000 (Caton and Kaprowski 1990; Persson *et al.*,1991). It is therefore not surprising that no binding phage were detected in libraries whose combined members numbered less than 200. At the time this work was carried out, the technology involved in the construction of phage display scFv libraries was still in its infancy, with very few published reports of scFvs isolated from them (eg Clackson *et al.*,1991; Marks *et al.*,1991), and most of these reports originated from the laboratory of Greg Winter, whose group pioneered this work. Indeed, visits to the laboratory of Greg Winter

were made to seek advice and discuss problems with the researchers involved in this work, but this did not result in any further success. Whatever the reasons for the small library size however, it is unrealistic to expect to be able to isolate an scFv of interest from such small libraries.

5.2.2 Use Of The Recombinant Phage Antibody System (RPAS).

It was around this time that a great deal of interest had been generated in the construction of phage display libraries, and a modular kit was launched by Pharmacia Biotech, the RPAS kit. This kit was supplied in three parts, the first part dealing with the amplification of scFvs, the second with their expression as gene 3 fusions on the tips of phage, and the third part concerned the detection of phage that bound the antigen of interest. The marketing of this kit was such that it was seen as a foolproof method for those who had encountered problems making libraries in other systems, with the kit containing most components, detailed protocols and the offer of considerable technical back-up. The vector around which this system is based is pCANTAB5E (Fig 5.3), a phagemid vector that carries the amber stop codon between the scFv and the gene 3 sequences, thus allowing expression of the scFv on the tip of a phage in a suppressor strain such as TG1, and the production of soluble antibodies in a non-suppressor strain such as HB2151. The major difference between this vector and the phage vector used previously is the fact that it contains no other phage genes apart from gene 3, and the remaining phage functions must be provided by superinfection with a helper phage such as M13K07. As no success had been met with library construction in fdtetDOG1, the RPAS kit was purchased and library construction carried out exactly as per the supplied instructions.

5.2.2.1 Amplification Of scFvs Using RPAS.

Mice were inoculated and boosted as before with pectate lyase (section 5.2.1.1) and the spleens from the best three mice removed. mRNA was extracted by first preparing total RNA using a Pharmacia RNA Extraction Kit, followed by selection of mRNA with a Pharmacia QuickPrep mRNA Purification Kit. 500ng of spleen mRNA was used for separate first strand cDNA synthesis reactions using the pre-primed first strand kit supplied with the kit. These complete reactions were then used as templates for primary amplification reactions, using the supplied light primer mix and the combination of the two supplied heavy primers. The products of these

reactions were then run through an agarose gel and purified using the supplied spin columns. After quantitation of the concentration of each PCR product by running through a gel alongside supplied concentration markers, an assembly reaction was carried out. 50ng of each chain was used in this reaction along with the linker-primer mix supplied, and the assembly reaction continued for 7 cycles (94C 1min, 63C 4min). After this assembly reaction was complete, the second amplification was carried out by addition to the entire assembly reaction of an amplification mix containing the RS primer mix, which consists of the outside primers appended with the restriction sites used in this system (*Sfi*1 and *Not*1). After a further 30 cycles (94°C 1min, 55°C 2min, 72°C 2min), the products were purified using the supplied spin columns. After quantitation of the assembled scFv by comparison to the supplied concentration standards, the scFvs were digested as directed, initially with *Sfi*1 followed by *Not*1, and again purified using the supplied spin columns. This digested library of scFvs was then ligated to the predigested pCANTAB5E supplied with the kit, and the ligation mix transformed by electroporation into TG1 cells.

5.2.2.2 Screening Of The RPAS Library.

After transformation into TG1 and plating out, the library was seen to contain only 5000 members. Despite this small size, panning with immobilised pectate lyase was carried out according to the supplied protocols. After application of 10^{11} phage to the first round of panning, 10^5 phage were eluted. After reinfection of *E. coli* and superinfection with helper phage, these phage were subjected to a second round of panning. In this second round, of 10^{11} phage applied, $3X10^4$ phage were eluted. Upon a third round of panning, 800 phage were eluted. These clones were then grown individually in microtiter plates, superinfected with helper phage to allow the formation of functional phage particles and tested for binding to pectate lyase by ELISA. None of the phage gave signals above background, indicating that none of the clones recognised pectate lyase. To confirm that the clones did indeed contain scFv genes, a selection of clones were subjected to PCR screeing using primers VH1BACKAPA10 and Vκ4FORNOT. Although these are not the exact primers that were used in the construction of the library (whose sequence would not be revealed by

the manufacturers), they should amplify most of the genes present and indeed the majority of the clones did yield PCR products consistent with the presence of scFvs.
A further attempt was made to construct a library using this system, but this was also unsucessful. Since this work was carried out, several changes have been made to this system, including the publication of a newsletter concerning its use (Antibodies On Display, Stratagene). The first issue of this newsletter (Nov. 1995) was published some two years after the kit was first launched, and contained sections detailing the improvements made to the kit and many more troubleshooting guides for use of the kit. This highlights the fact that at the time of use, the kit was very much at an experimental stage, and feedback gained from its end users resulted in many changes being made. Indeed, during the course of using this kit, two sets of light chain primers had to be returned to the company due to their inability to amplify a product from cDNA, whereas the primers that had been used previously (Vk4FORNOT and Vk2BACK) did yield a product. Despite these problems, there have been reports made of successful use of the kit (Meng *et al.*,1995; Meulemans *et al.*,1994). It is interesting to note, however, that in the first of these examples (Meng *et al.*,1995), two attempts had to be made in order to isolate scFvs that recognised the rattlesnake toxin crotoxin. The first attempt, which followed the kit instructions, resulted in a library of 10^6 clones which contained no crotoxin binders. A second library was then made, with an additional step being added, the panning of isolated spleen B cells on crotoxin so as to increase the proportion of individual chains that would recognise crotoxin. From a library of $1.5X10^6$ clones, several crotoxin specific scFvs were isolated. It can be seen from these results that even with a good immune response such as that reported, no scFvs recognising the antigen of interest were isolated by following the kit instructions. It is possible that this is an isolated case due to the antigen, but shows that a good immune response will not necessarily result in isolation of scFvs. Despite this, the most likely cause for the inability to isolate scFvs from the library made from the RPAS kit is the small number of clones obtained.

5.2.3 Screening Of A Synthetic Human scFv Library.

Due to the many technical problems associated with the construction of phage display libraries, and the fact that a new library had to be made for each antigen, a large research effort was exerted by other groups towards the

construction of synthetic libraries which contain a large number of different clones from which a wide range of antigen binding activities can be selected. Due to the obvious advantages associated with the production of human antibodies *in vitro* for medical research, a human synthetic library was constructed (Nissim *et al.*,1994). As discussed in the main introduction, much of the binding activity of an antibody is derived from its heavy chain, and particularly CDR3 of the heavy chain. For this reason the library was constructed by cloning the germ-line genes from all 49 rearranged VH genes, and manipulating them by PCR to introduce a random CDR3 of between 4 and 12 residues (Tomlinson *et al.*,1992). These heavy chains were then combined in scFv conformation with a single Vλ light chain. This resulted in a library of greater than 10^8 clones which was shown to contain scFvs that recognised a range of antigens, including haptens, foreign and self-antigens (Nissim *et al.*,1994). This library was eventually made available to other groups, so it was decided that this may present a method to isolate scFvs recognising both pectate lyase and polygalacturonase.

5.2.3.1 Growth And Panning Of The Synthetic Human Library.

The synthetic human scFv library was supplied to us in the form of a glycerol stock, along with a positive control phage which had been isolated from this library that recognised maltose binding protein (MBP). This phage could be grown and used in an ELISA to ensure that all the infection and growth stages had proceded properly. The library was grown as described in the supplied protocols, infected with helper phage and grown to yield a population of library phage. The phage were harvested from the culture supernatant and concentrated 100-fold by PEG precipitation. The phage were then used in a panning step on immunotubes coated overnight with pectate lyase (PEL) or polygalacturonase (PG). The unbound phage were then washed from the tube, and the bound phage eluted. *E. coli* TG1 cells were then infected with these eluted phage and plated out overnight. This step was included to reduce the chance of losing clones due to their slow growing nature. In liquid culture this would result in the clones being swamped by the others giving rise to an inability to rescue them. The colonies from these plates were then scraped into liquid media, grown for a short time and the whole infection, growth and panning procedure

repeated a total of five times. In each panning step, a total of approximately 10^{13} phage were applied to each tube. The numbers of phage eluted at each stage is shown in Table 5.1.

Pan No.	PG.	PEL.
1	$9X10^6$	$6X10^6$
2	$4X10^6$	$3X10^6$
3	$3X10^6$	$1.5X10^6$
4	$7.5X10^7$	$9X10^6$
5	$1.2X10^9$	$4.5X10^6$

Table 5.1. Number of phage eluted from successive panning steps performed against immobilised pectate lyase (PEL) and polygalacturonase. (PG).

It can be seen from the number of phage eluted from the pan against pectate lyase (Table 5.1) that there were approximately the same amount of phage eluted from each step of the panning. It would be expected that as the panning proceeded, there would be an enrichment for scFvs binding pectate lyase, and hence an increase in the number of phage bound to (and thus eluted from) the pectate lyase. This was however the case for the panning against polygalacturonase (Table 5.1). As can be seen, there was an approximately 20 fold increase in the number of phage eluted from the latter steps of panning. This would suggest that there is an enrichment for polygalacturonase binding scFvs.

From the phage eluted from pan 5, 95 colonies were picked from each of the PEL and PG populations into microtiter plates, along with one well containing the anti-MBP phage. These cultures were grown and infected in order to produce functional phage, and ELISAs carried out on plates coated with PEL or PG respectively. From the PG ELISA, four wells showed absorbance values considerably above the background value (Fig 5.4). The further analysis of these four polygalacturonase binding clones will be discussed later.

From the ELISA performed with phage eluted from the panning with PEL,

no values above background were seen, except that for the positive control. A further 190 colonies were grown and used in an ELISA, but these did not yield any binding phage either. In order to determine whether there were any steps which could be to blame for this lack of positive clones, ELISAs were performed with the phage eluted from the later panning steps, and also the concentrated phage used for these panning steps. These 'polyclonal' phage contain many different phage species, but any values in ELISA above background will indicate the presence in that population of antigen binding phage. It can be seen from this data (Fig 5.5) that for the PG phage, there is an increase in absorbance values in each step, with the values for the pre-pan phage being considerably higher than the eluted phage due to the fact that these phage have been concentrated. For the PEL phage, it can be seen that there is no increase in the absorbance values (Fig 5.5), indicating that at none of the steps has there been any PEL recognising phage.
Because of this failure to obtain any PEL recognising scFvs, the whole panning procedure was repeated for PEL, but still no binding phage were isolated. The reasons for this inability to isolate scFvs recognising this antigen are unknown, as scFvs recognising a wide range of antigens have been isolated from this library (Nissim *et al.*,1994), and it has been expected that scFvs recognising any given epitope may be isolated. It has been noticed however, that upon panning a precursor of this library, in which the VH chains have been constructed with a random CDR3 of 5 or 8 residues, that scFvs recognising haptens have been easily isolated, but scFvs recognising protein antigens have been rare, indeed with only one of four protein antigens screened providing a single, binding scFv (Hoogenboom *et al.*,1992). This was thought to be due to either an avidity effect or due to binding site morphology. A significant fraction of the phage population will display multiple copies of the fusion protein, and due to a higher coating density of hapten as compared to protein on solid phase this avidity effect may result in the easier isolation of hapten binders. It was also considered possible though that the choice of the light chain and HCDR3 length may have pre-disposed the structures of the repertoire to hapten and not protein binding, although the choice of CDR3 length (5 or 8 residues) should have allowed the formation of either flat or pocket like binding sites (Hoogenboom *et al.*,1992). The library screened with pectate lyase however should have had sufficient diversity in the binding site to allow isolation of scFvs, as scFvs

recognising a wide range of proteins have been isolated (Nissim *et al.*,1994). It is also possible that, despite an immune response being raised in immunised mice, the pectate lyase prep was not rich in distinctive epitopes. This would mean that in the pre-immune libraries constructed, there would be a low level of antigen binding chains, and the random association of these chains would destroy the low frequency binders. This may also explain the inability to isolate an scFv recognising pectate lyase from the human synthetic library. If there are no epitopes present to which any reasonable affinity antibodies will bind, then obviously no scFvs will be isolated.

5.2.3.2 Further Analysis Of The PG Binding Clones.

To ensure before any other analysis was carried out that the scFvs isolated still recognised PG, and were specific for this protein, an ELISA was carried out using HEL, BSA and PEL as negative controls using new phage preparations (Fig 5.6). As can be seen from this data, clones B2, C1 and F12 all recognise PG well, with no absorbance values above background for the negative control antigens. Clone E3 no longer appeared to recognise PG however, and the slight increase over the background level is due to the PG prep having a slight contaminating peroxidase activity, so slowly 'developing' the peroxidase substrate. This clone was then re-grown from the original glycerol stock made from the culture when binding was initially detected. The phage harvested from this culture also failed to recognise PG. It is possible that some deletion or rearrangement has occured in this clone, so it was discarded, leaving three PG recognising scFvs. As the quantity of polygalacturonase remaining was limiting, and the production of more enzyme was not feasible in the time available, it was decided that only one of the scFvs would be used in the further analysis of its function, as this would allow all the appropriate experiments to be carried out to completion. The scFv C1 was chosen as the scFv to continue working on, as it exhibited the highest binding of PG in the cultures grown up to provide phage for these experiments.

5.2.3.3 Sequence Analysis Of scFv C1.

In order to check that scFv C1 was indeed an scFv, and to determine the type of heavy chain and the sequence of the CDR3, the nucleotide sequence of the heavy chain was determined. The full sequence of the C1 heavy chain

and its expected translation product is shown in Figure 5.7. From analysis of this sequence, it can be deduced that the heavy chain component of scFv C1 is the VH1 class DP-5 heavy chain. The CDRs of this heavy chain are class 1 for H1 and unclassified for H2 (Tomlinson *et al.*,1992). The engineered CDR H3 is of 8 residues (LVMYDIVN), which is within the range of CDR lengths contained within this library.

5.2.3.4 Binding Of C1 To Other Polygalacturonases.

If scFv C1 was to be effective in reducing plant disease symptoms upon infection with *Botrytis cinerea*, it would also be useful if it bound to polygalacturonases produced by other fungal pathogens. There has been shown to be some conservation of peptide sequence among polygalacturonases, and most polygalacturonases studied to date have contained three distinct conserved domains (Kitamoto *et al.*,1993). Despite the presence of these three domains, the overall level of homology is low between plant, fungal and bacterial polygalacturonases. *Cryphonectria parasitica* is the fungal causal agent of chestnut blight, a canker disease which destroyed the American chestnut as a commercial species half a century ago. It was found that *C. parasitica* also produces polygalacturonase both in culture and in blight cankers. For this reason PG was purified from this species and its enzymatic activity characterised (Gao and Shain 1994). A gift of a sample of this polygalacturonase was made by Dr Shain, University of Kentucky, in order that we may determine whether or not scFv C1 binds to this protein as well as to the PG isolated from *Botrytis cinerea*. ELISAs were carried out using both *B. cinerea* and *C. parasistica* PG coated wells probed with either C1 or the anti-maltose binding protein scFv AMBP (Fig 5.8). As can be seen from this data, there is no apparent binding of C1 to *C. parasitica* PG, whereas there is good binding to the *B. cinerea* PG, as was expected. Thus C1 does not bind to this different PG, and must bind to a site which is not conserved between the two proteins.

5.2.3.5 Effect Of C1 On PG Activity.

To determine whether the binding of C1 to polygalacturonase had any effect on enzymatic activity, experiments were carried out using mixtures of different phage species with an optimised quantity of polygalacturonase and then measuring the release of reducing sugars following digestion of sodium polypectate. The results of this experiment are shown in Figure 5.9.

It can be seen that there is quite distinct polygalacturonase activity (measured by release of reducing sugars) upon addition of PG to the reaction mix as compared to the addition of water. The addition of C1 to the mix results in no release of reducing sugars, even in the presence of PG. Although this may appear at first glance an interesting result, it should be noted that the addition of control AMBP phage to the mix including PG also results in no PG activity. Thus the effect of C1 on PG activity is not a specific one. It is difficult to say what is causing this reduction in PG activity upon the addition of these two different phage species, as the phage had been resuspended in water so as not to include any contaminants which may have affected the assay. It is possible that the sheer amount of protein added in the form of phage may have resulted in some sort of reduction of activity of the enzyme, or even the availability of substrate. One way to determine whether this was the case would be to produce soluble scFv and see if there was any reduction in this effect on PG activity. Before this was carried out however, it was decided to attempt to determine the type of epitope that scFv C1 bound to on the polygalacturonase molecule.

5.2.3.6 Location Of C1 Binding Site.

As many fungal proteins, including secreted enzymes, have been found to be glycoproteins, it was decided that it would be interesting to know whether C1 bound to the protein backbone of PG or to any carbohydrate moïties which may be present on the protein. In order to determine this, it was necessary to strip these carbohydrate domains from the protein backbone and perform a comparison of binding of C1 to the protein backbone and to the whole glycoprotein. To achieve this, the PG was first coated onto an ELISA plate and then one set of wells stripped of carbohydrate by the action of periodate, and one set of wells incubated without periodate, and then a standard ELISA carried out (Fig 5.10). It can be seen from this data that there is a dramatic eight-fold reduction in C1 binding upon treatment with periodate. This indicates that C1 binds to a carbohydrate epitope on the protein, and any remaining binding is thought to be due to incomplete removal of carbohydrate during the incubation with periodate. To check that this reduction in binding was not due to some sort of inhibition caused by periodate, wells coated with maltose binding protein (MBP), which was bought as a recombinant protein and so will contain no carbohydrate domains, were treated in an identical manner prior

to probing with AMBP phage (Fig 5.10). This resulted in a slight reduction in binding of AMBP (one and a half fold), but this is nowhere near the eight-fold reduction seen upon treatment of PG. These results demonstrate that the site of binding of scFv C1 is on a carbohydrate side chain of the polygalacturonase. As this was the case, it was decided that it would be unlikely that scFv C1 would have any effect on the activity of the enzyme.

5.3 Summary.

The work described in this chapter involves the cloning of scFvs which recognise pathogenicity factors using a phage display library approach. Initial attempts to construct phage display libraries against pectate lyase in the phage vector fdtetDOG1 were not successful due to the reasons described in the text. Also unsuccessful were attempts to use the Pharmacia Recombinant Phage Antibody System (RPAS). At the time that this work was attempted, there were few examples of success being met using this system, and subsequent improvements have been made to the kit, including a change in the sequence of the light chain primers. At the time that this kit was in use, it was still in its infancy, and the sheer small size of the library constructed made it unlikely that an interesting scFv would be isolated.

Success was met however in the screening of a preconstructed, synthetic human phage display library. No scFvs were isolated that bound to the pectate lyase that was prepared in Chapter 3, possibly because of a lack of available epitopes, but scFvs were isolated that bound to polygalacturonase isolated from the fungal pathogen *Botrytis cinerea*. Initially four scFvs were isolated, but one of these did not display any binding sctivity on re-screening. As there was a lack of available polygalacturonase at this stage, it was decided that a detailed analysis of the characteristics of just one of the scFvs would be made. The nucleotide sequence of the heavy chain of scFv C1 was determined and shown to be derived from the VH1 DP-5 heavy chain (Tomlinson *et al.*,1992), with an eight residue CDR H3. scFv C1 did not bind to another fungal polygalacturonase that was a gift, that from *Cryphonectria parasitica*. There was no conclusive data concerning the effect on the activity of *Botrytis* polygalacturonase, as the control scFv AMBP showed the same inhibition as C1, presumably an artefact of the sheer amounts of protein (in the form of phage) being added to the reaction

mix. Upon investigation of the type of epitope bound by scFv C1, it was found to bind to a carbohydrate epitope, as shown in a dramatic reduction in binding upon treatment of the polygalacturonase with periodate, which removes carbohydrate groups. This also suggests that C1 would have no effect on the activity of PG, as binding to the carbohydrate is unlikely to affect either the active site of the enzyme, or introduce some conformational change which may alter activity.

The work described in this chapter demonstrates the efficacy of using a synthetic scFv phage display library in isolating scFvs against different antigens.

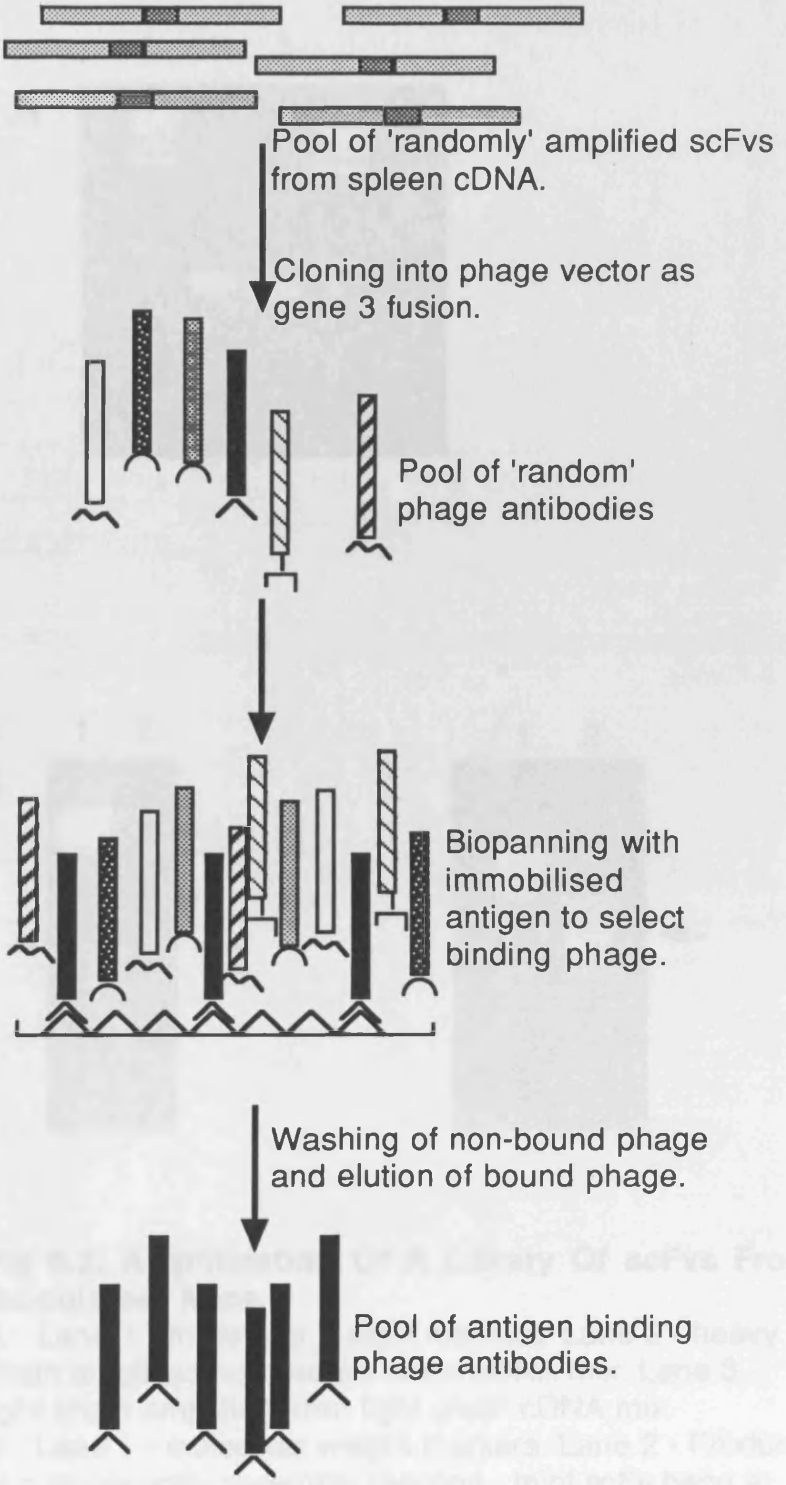


Fig 5.1. Schematic Diagram Showing Panning Of An scFv Population To Enrich For Phage Binding The Antigen Of Interest

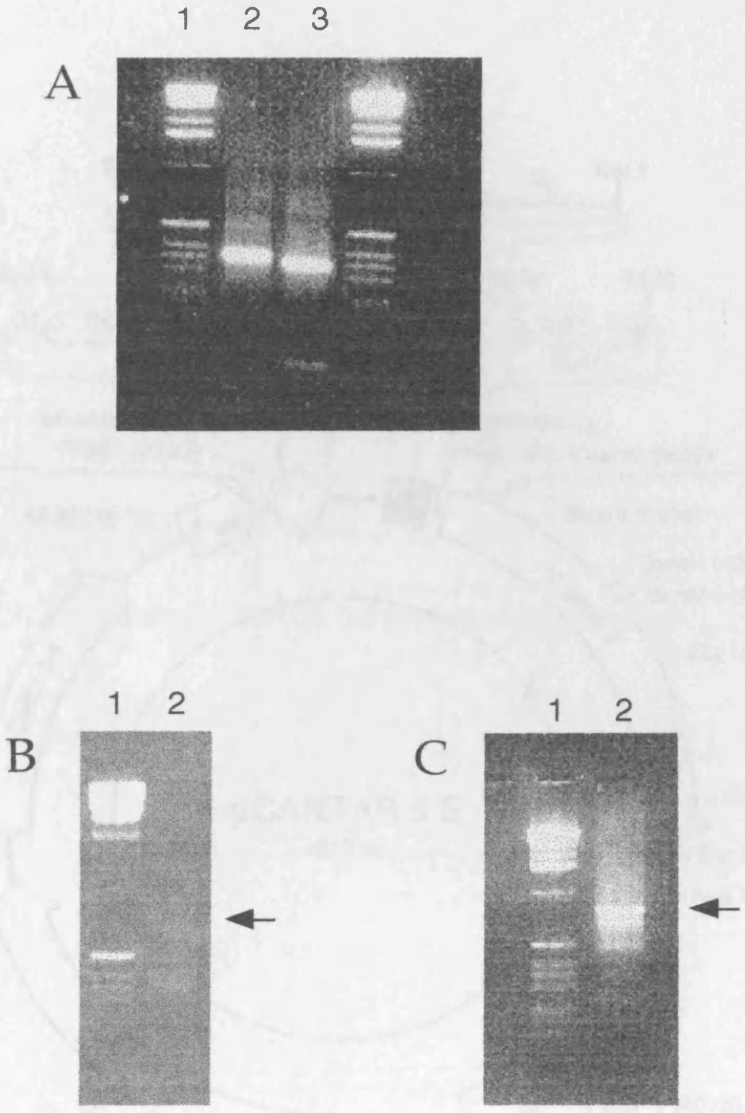


Fig 5.2. Amplification Of A Library Of scFvs From Inoculated Mice.
A : Lane 1 - molecular weight markers. Lane 2 - heavy chain amplified from heavy chain cDNA mix. Lane 3 - light chain amplified from light chain cDNA mix.
B : Lane 1 - molecular weight markers. Lane 2 - Product of a single scFv assembly reaction - faint scFv band at approx. 750 bp arrowed.
C : Lane 1 - molecular weight markers. Lane 2 - scFv library after amplification of product shown in B. scFv product arrowed.

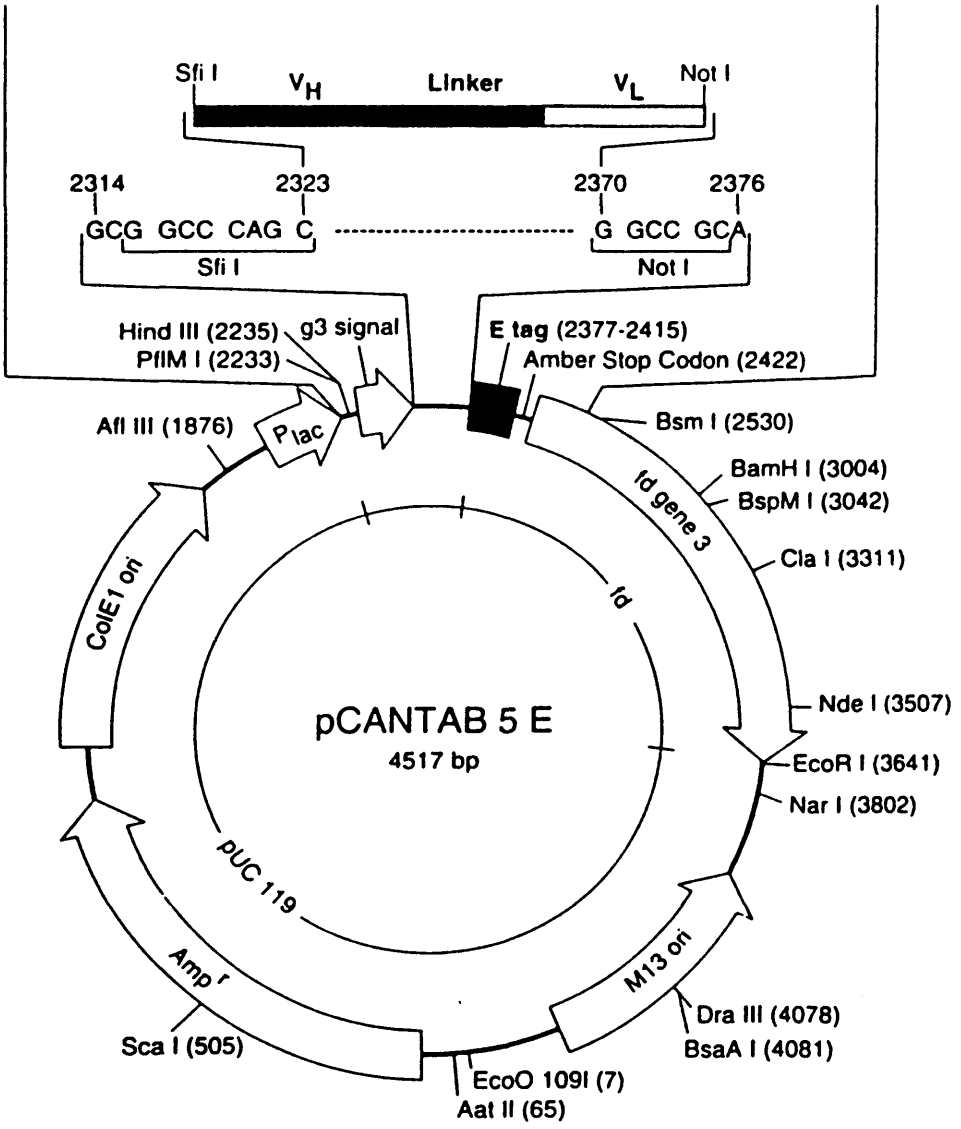


Fig 5.3. Vector pCANTAB5E As Used in Recombinant Phage Antibody System Kit.

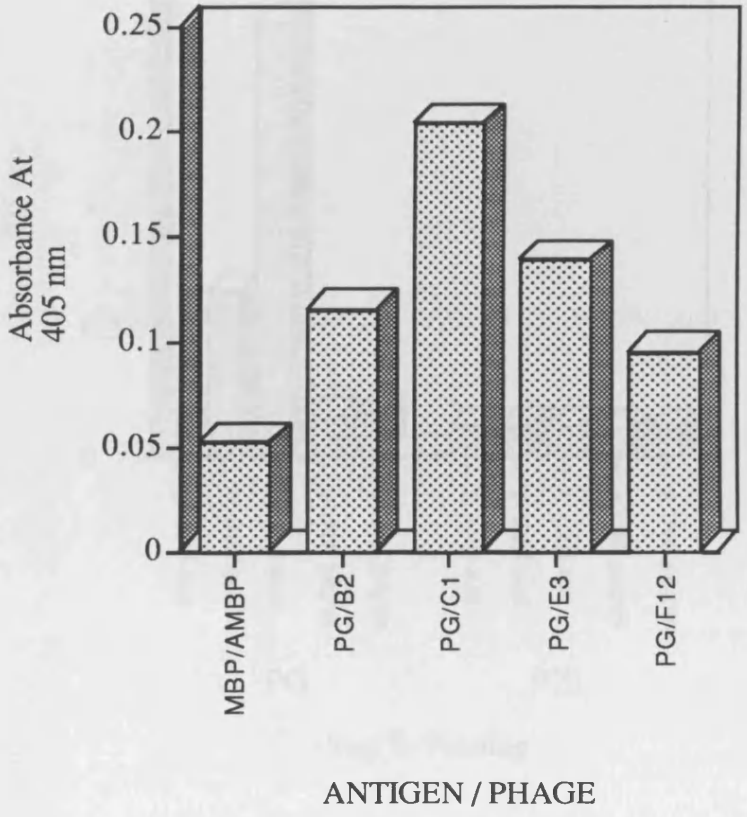


Fig 5.4. Recognition Of Polygalacturonase By Phage Isolated From Human scFv Library Screened With Polygalacturonase. Absorbance values from ELISA using maltose binding protein (MBP) and AMBP (control phage antibody) and 4 clones recognising polygalacturonase (PG) - B2, C1, E3 and F12.

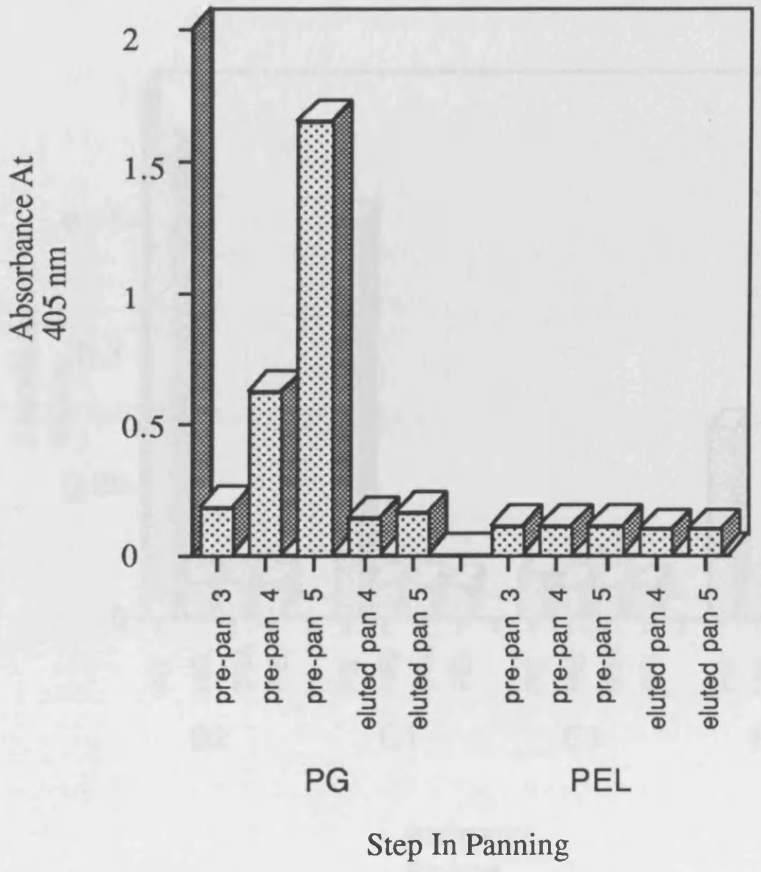


Fig 5.5 Activity Of Phage Populations From Libraries Panned Against PG and PEL At Different Stages Of Panning. Absorbance values from ELISA using phage populations prepared for subsequent panning steps (pre-pan) and phage eluted from these steps (post-pan) respectively on either polygalacturonase (PG) or pectate lyase (PEL).

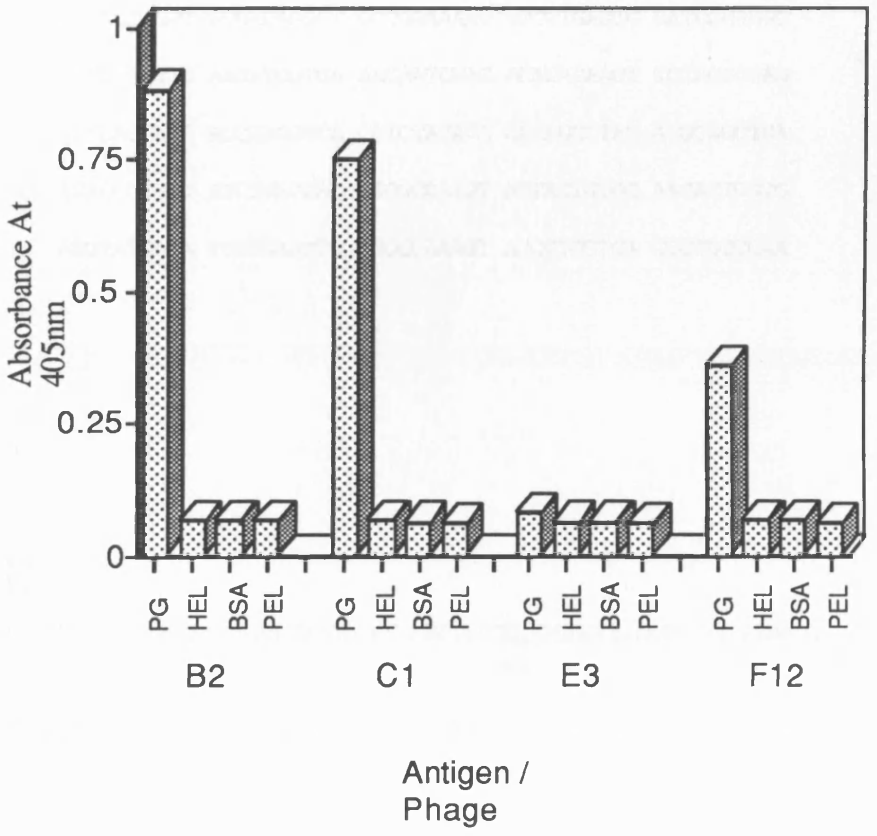


Fig 5.6. Specificity Of Phage Isolated From Library Panned Against Polygalacturonase. Absorbance values from ELISA using phage species B2, C1, E3 and F12 on polygalacturonase (PG), hen egg lysozyme (HEL), bovine serum albumin (BSA) or pectate lyase (PEL).

A

```
  1  CAGGTGCAGC TGGTGCAGTC TGGGGCTGAG GTGAAAAAAC CTGGGGCCTC
 51  AGTGAAGGTC TCCTGCAAGG TTTCCGGATA CACCCTCACT GAATTATCCA
101  TGCACTGGGT GCGACAGGCT CCTGGAAAAG GGCTTGAGTG GATGGGAGGT
151  TTTGATCCTG AAGATGGTGA AACAATCTAC GCACAGAAGT TCCAGGGCAG
201  AATCACCATG ACCGAGGACA CATCTACAGA CACAGCCTAC ATGGAGCTGA
251  ACAGCCTGAG ATCTGAGGAC ACGGCCGTGT ATTACTGTGC AAGACTGGTG
301  ATGTATGATA TTGTGAATTG GGGCCAAAGT ACCCTGGTCA CCGTGTCCAA
351  A
```

B

QVQLVQSGAEVKKPGASVKVSCKVSGYTLTELSMHWVRQAPGKGLEWMGG
H1

FDPEDGETIYAQKFQGRITMTEDTSTDTAYMELNSLRSEDTAVYYCARLV
H2

MYDIVNWGQSTLVTVSK
H3

Fig 5.7. Analysis of scFv C1 Sequence.
A: Nucleotide Sequence and
B: Expected Translation Product Of C1 Heavy Chain.
CDRs H1, H2 and H3 are shown underlined.

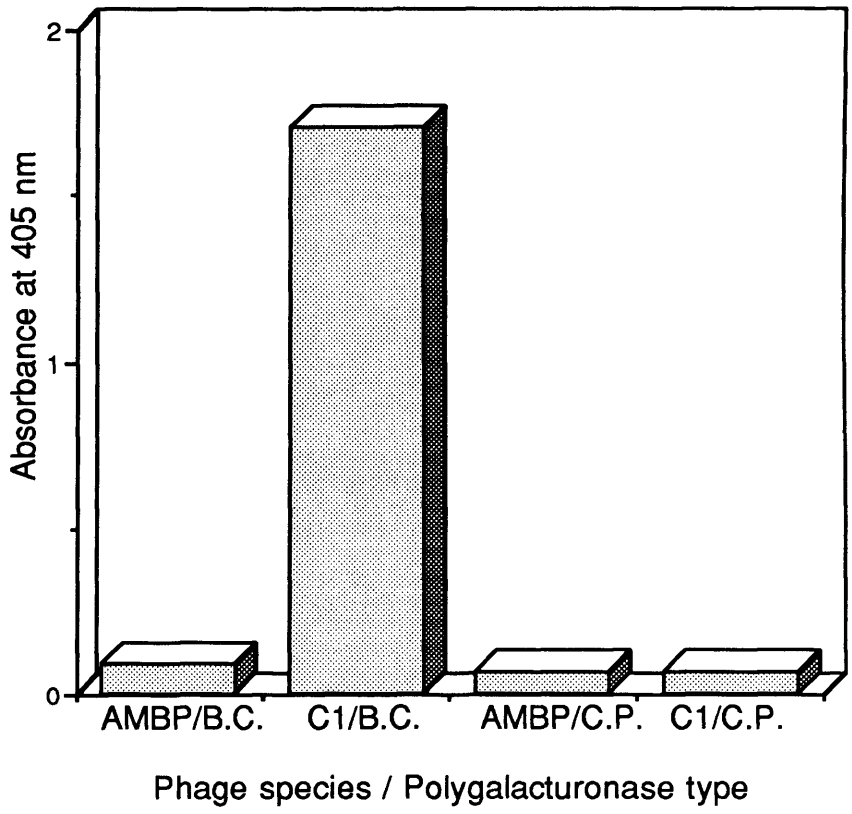


Fig 5.8. Activity Of scFvC1 Against Polygalacturonase From Botrytis cinerea and Cryphonectria parasitica.
Absorbance values from ELISA using phage species AMBP (as a control) and C1 against polygalacturonase from *Botrytis cinerea* (Bc) and *Cryphonectria parasitica* (Cp).

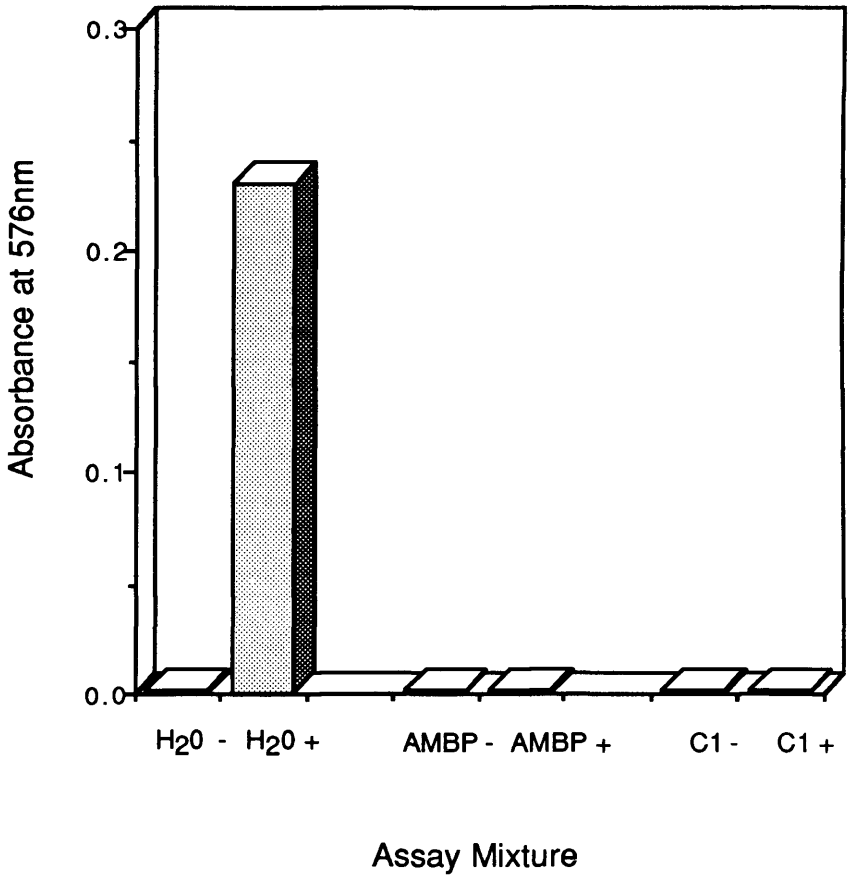


Fig 5.9 Effect Of Binding Of scFv C1 To Botrytis polygalacturonase On Enzyme Activity.
Absorbance values from reducing sugar assay after incubation with sodium polypectate following incubation of H_20, AMBP or C1 with (+) or without (-) *Botrytis cinerea* polygalacturonase.

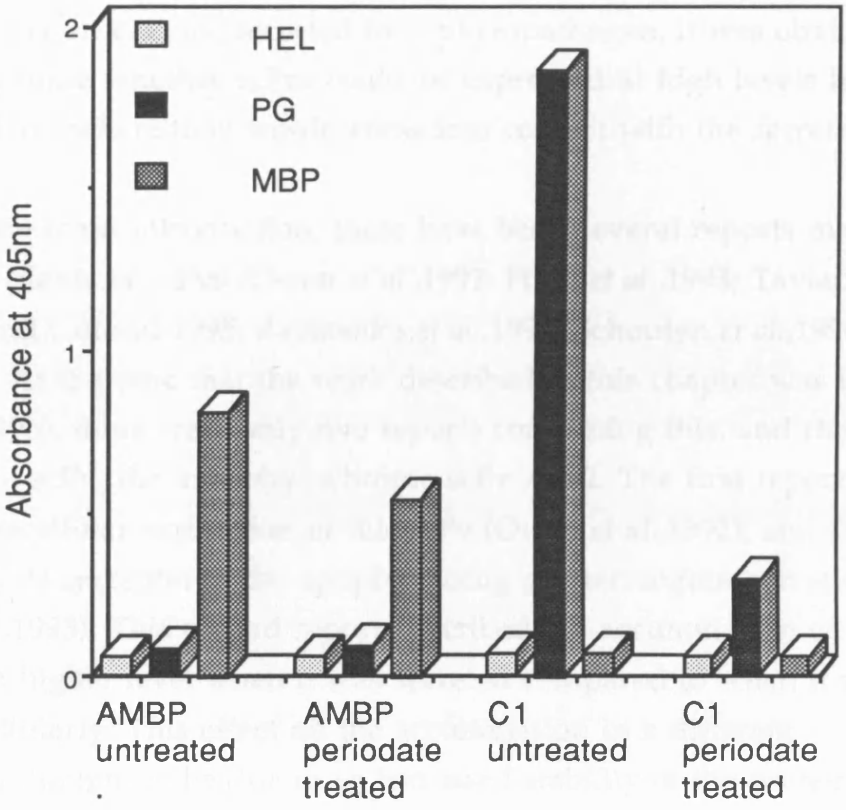


Fig 5.10. Determination Of Epitope Type Bound To By scFv C1.
Absorbance values from ELISA of AMBP or C1 on hen egg lysozyme (HEL), *Botrytis* polygalacturonase (PG) and maltose binding protein (MBP) either untreated or periodate treated to remove carbohydrate groups.

Chapter 6 : Expression And Secretion Of scFvs In Transgenic Tobacco.

6.1 Introduction.

As the aims of this project were to introduce scFvs into transgenic plants with the desire of inactivating an enzyme secreted by a phytopathogen, it was obviously important to determine whether scFvs could be expressed at high levels in the apoplast of the plant, where they would come into contact with the secreted enzymes.

As described in the main introduction, there have been several reports made of the expression in plants of scFvs (Owen *et al.*,1992; Firek *et al.*,1993; Tavladoraki *et al.*,1993; Fiedler and Conrad 1995; Artsaenko *et al.*,1995; Schouten *et al.*,1996; Rosso *et al.*,1996). At the time that the work described in this chapter was initiated however (early 1994), there were only two reports concerning this, and these involved the same scFv, the anti-phytochrome scFv AS32. The first report described the intracellular expression of this scFv (Owen *et al.*,1992), and the second concerned its secretion to the apoplast using a heterologous secretion signal (Firek *et al.*,1993). This second report described the accumulation of the scFv to a five-fold higher level when it was secreted compared to when it was expressed intracellularly. This effect on the accumulation in a different compartment was thought to be due to an increased stability of the protein in the apoplast compared to the cytosol, possibly due to interaction with its antigen, phytochrome, when expressed intracellularly. As this was the only report of secretion of an scFv, and it showed the scFv accumulated to a higher level, it was decided that it would be important to determine whether this effect was seen with other scFvs expressed in these compartments in transgenic tobacco.

As at the time no success had been achieved in the isolation of scFvs recognising pathogenicity factors from plant pathogens (Chapter 4), two further scFvs that were available were used in this work. The first of these was the scFv D1.3, which recognises hen egg lysozyme (Amit *et al.*,1986) and has been described previously (Section 4.1). The second scFv, BE3, was a gift from Dr J. Milner, University of Bath, and was isolated from a hybridoma cell line that produced antibodies recognising a helicase of the herpes simplex virus and was isolated in the hope that it would prove to reduce the activity of this enzyme, and so affect viral propagation.

As a comparison of these scFvs in either the cytosolic or apoplastic compartments was desired, the scFvs had to be cloned as a translational fusion with a secretion

signal. Previous success with the secretion of full length antibodies had been described using the signal sequences present in mouse immunoglobulin genes, but these were already present on the cloned genes and so required no further manipulation. As the scFvs used here had no signal sequence attached to them, it was decided to use the signal peptide from the tobacco pathogenicity-related protein 1a (PR1a). This signal sequence had already been used in the laboratory for the secretion of the scFv AS32 (Firek *et al.*,1993), and also of B-glucuronidase (Firek *et al.*,1994) and so was available for use. This signal sequence had been isolated by PCR and cloned into the vector pBluescript SK- to produce pSK-PR1a (Firek *et al.*,1993).

In order to detect the accumulation of scFv within the transgenic plants, it was necessary to have an antibody to use in immunological analysis. With the use of an antibody, it would be possible to detect scFv proteins in extracts made from the transgenic plants. In the work carried out previously on a single scFv, AS32, antibodies had been raised in rabbits to this protein after it had been overexpressed and purified (Owen *et al.*,1992). As the work described in this chapter was utilising different scFvs, it was decided that the most effective way of detecting the scFvs was to include a short peptide tag in the translated scFv. At the time this work was started, there were a few commercially available antibodies to peptide tags that had been used, but it was decided that a novel tag would be designed and used. The tag that was designed was based on the major coat protein of bacteriophage fd. This protein is present at approximately 2700 copies per phage particle, and hence most antibodies that have been raised against these filamentous phage recognise this protein, more specifically the N-terminal acidic region that faces outwards on the phage particle and hence is the most exposed part of the protein (Rasched and Oberer 1986). As there were several sources for antibodies against the bacteriophage fd in the laboratory, it was decided to use these 20 acidic region amino acids as a peptide tag.

The work described in this chapter involves the cloning of the DNA encoding the peptide tag into a translational fusion with the secretion signal along with restriction enzyme sites available for cloning scFvs into this vector, and the subsequent transfer of these genes into plant transformation vectors. The analysis of the transgenic plants produced is described for each of the two scFvs in each of the cell compartments (cytosol and apoplast).

6.2 Results.

6.2.1 Construction Of Plasmid pTAG1.

As described in the introduction, it was desirable to construct a vector that contained the PR1a signal sequence along with a peptide tag and restriction sites allowing the insertion of scFvs and the removal of different cassettes with or without the signal peptide to be cloned into the plant transformation vector pRok2 (which is derived from pBin19, Bevan 1984). As the plasmid pSK-PR1a containing the PR1a signal sequence was already available (Firek *et al.*,1993), this was used as the basis for constructing this vector pTAG1 (Fig 6.1).

6.2.1.1 Cloning Of The Peptide Tag.

PCR primers were designed from the published nucleotide sequence of bacteriophage fd (Beck *et al.*,1978) in order to amplify the acidic region of the major coat protein, the gene 8 protein (g8p). These primers were appended with the restriction sites shown below to allow the digestion of the PCR product with these enzymes to allow cloning into pSK-PR1a.

g8AC5' 5'CGTCTAGA**ATG**GCTGAGGGTGACGATCCCGC3'
*Xba*I

g8AC3' 5'CGCTGCAGTTCGGTCGCTGAGGCTTGCAGG3'
*Pst*I

It should be noted that g8AC5' contains a translational ATG start codon (shown in bold), as in constructs without the PR1a signal sequence, there would otherwise be no start codon present (Fig 6.1B).

These primers were used in a normal 100µl PCR reaction using 10ng of fdtetDOG1 double stranded DNA as template. The resulting product was run through an agarose gel and the g8AC product of 82bp eluted (Fig 6.1A). This DNA fragment was then ligated to the PCR cloning vector pGEM-T (Promega) which contains single 5' T overhangs to facilitate the cloning of PCR products (which contain 3' A overhangs if produced with Taq polymerase). This step was included to enhance the digestion of the product with the enzymes *Pst*I and *Xba*I, as some restriction enzymes (including *Xba*I) do not cut well when their sites are found at the end of linear DNA (Stratagene catalogue). The resulting ligation mix was transformed into XL1-Blue cells and transformants screened by PCR using the primers RSP and M13-20. A positive clone giving a product of approximately

320bp (indicating a single insert) was chosen for use (pGEM-T:g8AC). pGEM-T:g8AC DNA was then digested with *Xba*I and *Pst*I and the 76bp fragment isolated from an agarose gel. This was then ligated to similarly digested pSK-PR1a DNA to create pTAG1 (Fig 6.1B). This ligation mix was transformed into XL1-Blue cells and transformants screened by PCR using primers RSP and M13-20 and a clone giving a 300 bp fragment chosen (pTAG1).

6.2.1.2 Confirmation Of Nucleotide Sequence.

To ensure that the cloning had not resulted in a change of reading frame which would cause any scFvs cloned into this vector not to be expressed, the nucleotide sequence of the relevant part of pTAG1 was determined and shown to contain the correct insert in frame (Fig 6.1B).

6.2.1.3 Confirmation Of Activity Of Anti-TAG Antibodies.

To ensure that the antibodies that were to be used to analyse transgenic plants would recognise this peptide tag, whole cell extracts of IPTG-induced *E. coli* either untransformed or carrying pTAG1 were prepared and used in a Western blot probed with anti-M13 antibodies which were a gift from Dr A. Willis, University of Leicester. As can be seen (Fig 6.2B) the blot resulted in many bands developing after probing with these antibodies (lane 2). However, these bands were also present in the *E. coli* controls (lane 1), but two specific bands were seen (arrowed). The reason for the bands coming up in the negative control is that the antibodies were produced by inoculating mice with concentrated culture supernatant containing phage M13, and this prep contains many contaminating *E. coli* proteins to which antibodies will also be produced. As the main use of these antibodies is to be in plant extracts however, this background should not be a problem. The two bands that were specific to the *E.coli* (pTAG1) of approximately 16 and 18 kDa are consistent with the detection of the peptide tag, and the sizes correspond to the lack of a translation stop codon being present in the insert, so the translation of the RNA continues until it comes across a stop codon within the vector. As the insertion of the signal sequence into pBluescript resulted in a shift of reading frame, the stop codon utilised will not be that normally used for B-galactosidase expression, but will occur at a codon which would not normally be in frame (in this case nucleotide 474). The presence of two bands either reflects readthrough from this stop codon or proteolytic degradation of the product.

6.2.2 Cloning scFvs D1.3 And BE3 Into pTAG1.

In order to clone the two scFvs into pTAG1, two PCR primers were designed in order to amplify these scFvs from the phage vector fdtetDOG1. D1.3 had already been cloned into this vector (section 4.2.1), but the scFv BE3 had been supplied in pCANTAB3, (a precursor vector of pCANTAB5E, which was used to attempt the construction of a phage display library (section 5.2.2)). Thus it was necessary to first clone BE3 into fdtetDOG1.

6.2.2.1 Cloning BE3 Into fdtetDOG1.

scFv BE3 was amplified by PCR using primers VH1BACKAPA10 and Vk4FORNOT from 10ng of pCANTAB-BE3 template in a normal 100µl reaction. This gave a product of approximately 800bp which was appended by *Apa*L1 and *Not*1 restriction sites in order to allow cloning into fdtetDOG1. This fragment was purified from the gel and cloned into fdtetDOG1 as described previously (section 4.2.1) to yield fdBE3.

6.2.2.2 Cloning scFvs From fdtetDOG1 Into pTAG1.

As described earlier, PCR primers were designed to allow the amplification of scFvs from fdtetDOG1 to allow their cloning into pTAG1. These primers (NEWFORECO and BACKECO) were designed to anneal in the vector sequences, in order to allow any scFv in fdtetDOG1 to be amplified. These primers were appended with *Eco*RI restriction sites to allow cloning into the *Eco*RI site of pTAG1, which will give a translational fusion between the peptide tag and the scFv. The primer sequences are shown below. The plant translational stop codon (TAA) is shown in bold.

NEWFORECO 5'CGGAATTC**TTA**TGCTAAACAACTTTCAACAGTTTC3'
*Eco*RI

BACKECO 5'CGGAATTCCCTTTCTATTCTCACAGTGCAC3'
*Eco*RI

These primers were used in normal 100µl PCR reactions using 10ng of either fdD1.3 or fdBE3 DNA as template. 20µl of each of these reactions was run on an agarose gel (Fig 6.2A), and the only product seen in each case was the scFv at 800bp (arrowed). The remains of these reactions were electrophoresed and the 800bp fragments isolated from the gel. These fragments were then digested with

*Eco*RI and ligated to similarly digested pTAG1 to create pTAGD1.3 and pTAGBE3 respectively. These ligation mixes were then transformed into XL1-Blue cells and the transformants screened by PCR initially with primers RSP and M13-20 to show single inserts with a product of approximately 1kb, and then with RSP and NEWFORECO to check the correct orientation of the insert, as the EcoRI sites on each end could result in the fragment ligating in either orientation. Clones giving a product of approximately 900bp demonstrated the correct orientation. One clone for each of pTAGD1.3 and pTAGBE3 were then selected and the nucleotide sequences determined to show that the reading frame had not been destroyed by the cloning (Fig 6.1B).

6.2.2.3 Confirmation Of TAG Function.

In order to confirm that the presence of a translational fusion of an scFv with the TAG peptide did not affect its performance, one of these clones, pTAGD1.3 was taken and cultures induced by IPTG as before to induce expression from the B-galactosidase promoter and blotted with anti M13 antibody. As expected, a band at 37kDa appeared (lane 3) that was not present in the negative control (Fig 6.2B). As before, several bands were present, but the majority of these were also present in the negative control. The major 37kDa protein detected corresponds to the size of the translational product from the B-galactosidase start codon to the plant terminator codon introduced by the primer NEWFORECO. The other bands that came up were of approximately 41, 28 and 25kDa. The larger of these is probably due to partial readthrough of the plant terminator codon, and the smaller two are likely to be degradation products of the 37kDa protein. Thus, the presence of an scFv in fusion with the TAG peptide did not affect its detection.

6.2.3 Production Of Transgenic Plants.

In order to produce transgenic plants to determine the accumulation of the scFvs in the different cell compartments, it was first necessary to clone the two scFvs, both with and without the PR1a signal peptide into a plant transformation vector. A vector already in use in the laboratory was pROK2, which contains a single CaMV 35S promoter and nos terminator sequences. Between these two components are several restriction sites that facilitated the cloning of the different scFv derivatives. As can be seen from the map of pTAG1 (Fig 6.1B), the removal of the insert (including scFv) as a *Kpn*I fragment would result in the presence of the PR1a signal peptide, whereas removal of the insert as a *Kpn*I-*Xba*I fragment would result in the removal of the signal peptide. These two fragments were

prepared from both pTAGD1.3 and pTAGBE3 and ligated to similarly digested pROK2. These ligation mixes (D1.3INT, D1.3SEC, BE3INT and BE3SEC) were transformed into XL1-Blue cells and the presence of an insert, and in the case of D1.3SEC and BE3SEC the correct orientation of the insert, was confirmed by PCR. These four plasmids were then purified and transformed by electroporation into *Agrobacterium tumefaciens* strain LBA4404. *Nicotiana tabacum* SR1 was transformed with these four constructs by the leaf disk method described. Relatively few transgenic plants were recovered (a total of 27 plants from all the constructs), but due to a lack of time no more transgenic plants could be generated and analysed.

6.2.4 Analysis Of Transgenic Plants.

6.2.4.1 Confirmation Of Presence Of T-DNA.

In order to confirm that the plants generated were indeed transformed, DNA was extracted from each of them by the mini-prep method described, and subjected to PCR using primers specific for the scFv portion of the insert (Vκ4FORNOT and Vκ2BACK to amplify the light chain region). The majority of the plants gave a PCR product of the expected size, whereas untransformed plants gave no product.

6.2.4.2 Confirmation Of Expression Of The Transgenes.

RT-PCR was used to detect the presence of scFv transcript in the transgenic plants. RNA was prepared from the transgenic plants using the miniprep method described, and subjected to a reverse transcription reaction using NEWFORECO as the primer. After this reaction, a small aliquot was used as template in a PCR reaction using primers VH1FOR2 and VH1BACKAPA10 to amplify the heavy chain portion of the scFvs. A portion of these reactions were electrophoresed through an agarose gel (Fig 6.3). As can be seen, in the majority of the plants there is easily detectable transcript. Similar reactions were also carried out following reverse transcription reactions lacking reverse transcriptase. These reactions were carried out to ensure that any bands that were observed following PCR were not due to amplification of any contaminating genomic DNA. None of these reactions yielded a product following PCR, proving that the products seen (Fig 6.3) were due to amplification of reverse transcribed RNA. A Northern blot was not carried out because the purpose of this experiment was purely to confirm that the transgenes were being transcribed, and the level of this transcription was not of interest.

6.2.4.3 Detection Of scFvs In Total Extracts.

In order to detect scFv in the transgenic plants, soluble protein extracts were made and 10μg electrophoresed through an SDS-PAGE gel before being transferred to nitrocellulose. Upon probing these blots with anti-M13 antibody, no bands were seen to develop quickly. After leaving these blots to develop for longer, many bands appeared (Fig 6.4). These bands were just due to non-specific reactions with plant proteins however, as they were also present in the lanes containing untransformed SR1 protein extracts. Extracts from all of these plants were remade and the experiment repeated, but with no difference in the results. Thus it was impossible to detect scFv protein in total extracts.

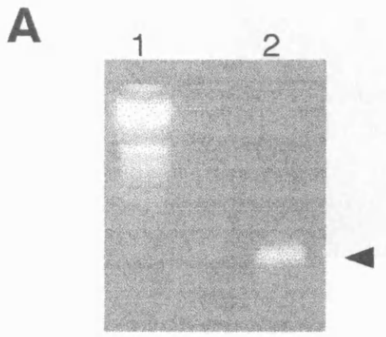
6.2.4.4 Detection Of scFvs In Extracellular Extracts.

As no scFv could be detected in the total extracts, apoplastic extracts were prepared from plants transformed with the secretory form of the scFvs. These extracts are likely to contain a higher concentration of scFv than the total extracts, as the same amount of scFv (presuming that the PR1a signal sequence results in the secretion of all the scFv, as was demonstrated by Firek *et al.*,1993) will be extracted in a much smaller volume. Thus it should be easier to detect scFvs in these extracts as compared to the total extracts. 10μg of apoplastic extracts from the plants containing the secretory scFv were blotted and probed with anti-M13 antibody as before. As can be seen (Fig 6.5), there is still no detectable scFv, and all the bands that have developed are present in the control lanes, and these bands have only come up as a result of the blot being left to develop for so long. Thus it must be concluded that there is no detectable scFv in the apoplastic extracts.

6.3 Summary.

The work described in this chapter concerns the transformation of tobacco with four scFv constructs, and the analysis of the resulting transgenic plants. The constructs that were used consisted of two different scFvs (D1.3 and BE3) either with (D1.3SEC, BE3SEC) or without (D1.3INT, BE3INT) the tobacco PR1a signal peptide, which has been shown to direct the secretion of another scFv to the apoplast (Firek *et al.*,1993). All of these constructs contained a TAG peptide, the acidic region of the M13 gene 8 protein (major coat protein) to which antibodies were available. The resulting transgenic plants were analysed and it was found that there was no detectable scFv protein present, even in the apoplastic fraction

of the plants in which the scFvs should have been secreted. The constructs made in the different steps of cloning the scFvs with the TAG sequence were all sequenced across the borders to ensure that the correct reading frame had not been disrupted, and the peptide TAG was shown to be detectable in extracts prepared from *E.coli*. The peptide sequence of the TAG was also checked to ensure that it contained no potential glycosylation sites that may otherwise have caused its epitopes to be masked *in planta*. Most of the plants analysed were shown to contain the T-DNA by PCR, and most of the plants demonstrated the presence of scFv transcript, as determined by RT-PCR analysis. Thus it appears that the absence of detectable scFv protein is due to some other factor.



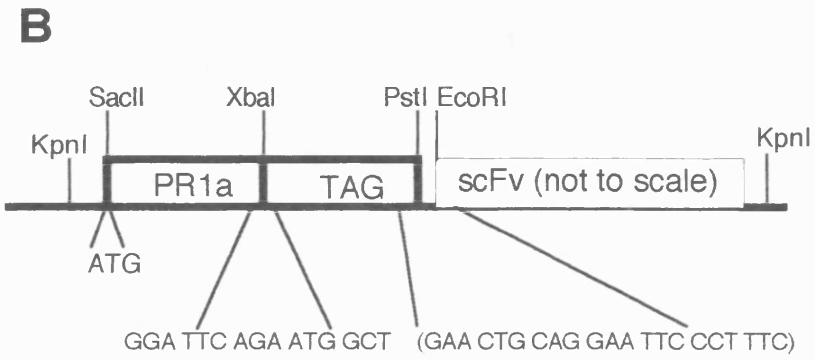


Fig 6.1. Construction Of pTAG1. **A** : Lane 1 Molecular weight markers. Lane 2 g8AC amplified product (82bp - arrowed). **B** : Map of pTAG1 showing sequences across borders. PR1a - tobacco PR1a signal sequence; TAG - g8AC sequence. scFv shown as fine lines (sequence across TAG-scFv border shown in parentheses)

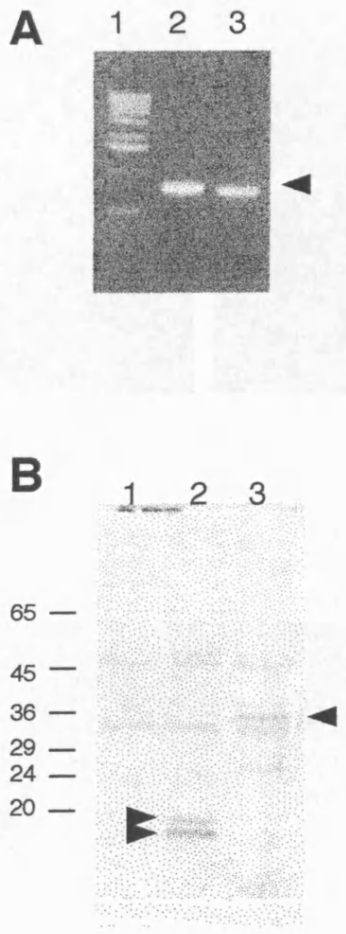


Fig 6.2. Construction Of pTAGD1.3 And pTAGBE3. **A** : Lane 1 molecular weight markers. Lane 2 D1.3Eco PCR product. Lane 3 BE3Eco. PCR product (both approx 800bp - arrowed). **B** : Western blot of induced XL1 carrying Lane 1 no plasmid; Lane 2 pTAG1 (16 and 18 kDa bands arrowed); Lane 3 pTAGD1.3 (37kDa band arrowed).

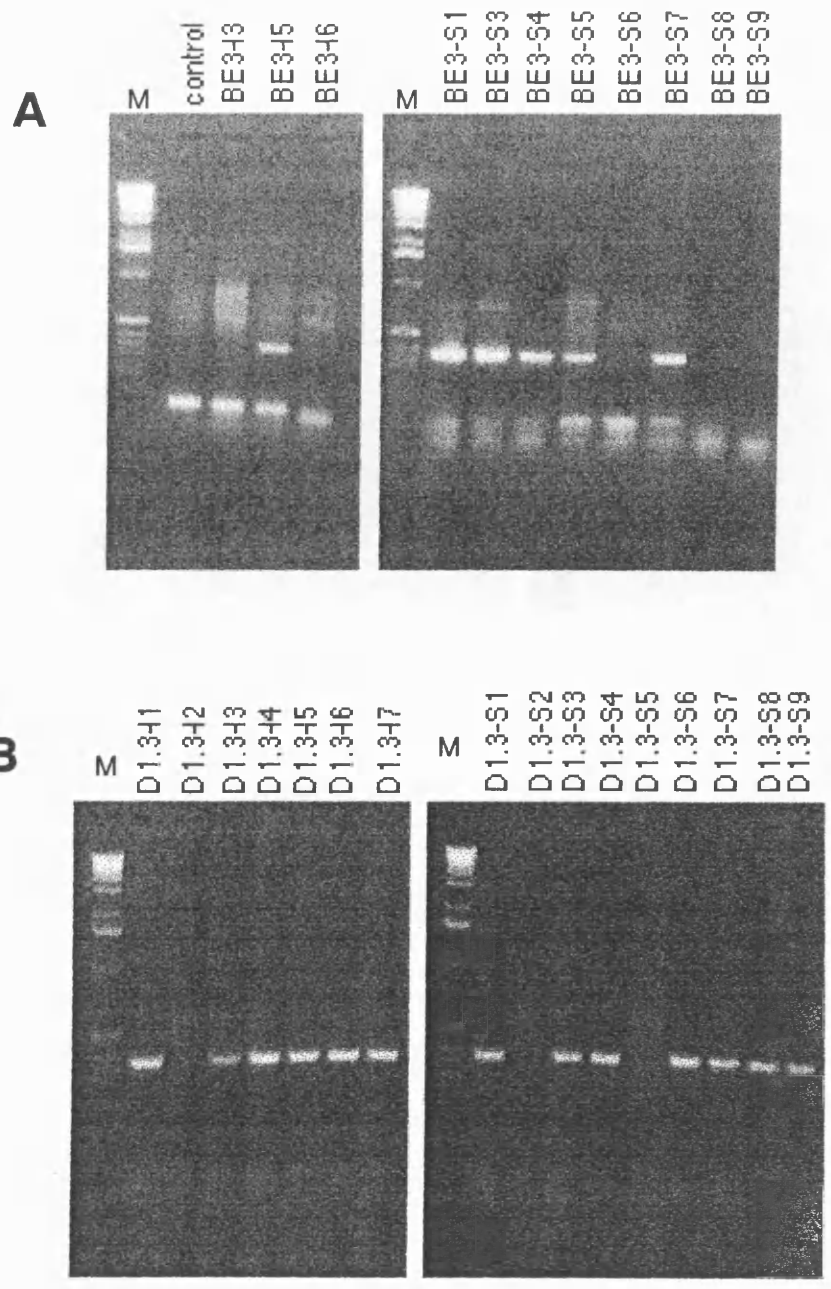


Fig 6.3. Confirmation Of Transcription Of Transgenes By RT-PCR.
A : BE3 plants. **B** : D1.3 plants. -I and -S suffixes refer to intracellular and secreted forms respectively.
M - molecular weight markers. control - reaction using SR1 RNA as template for RT-PCR.

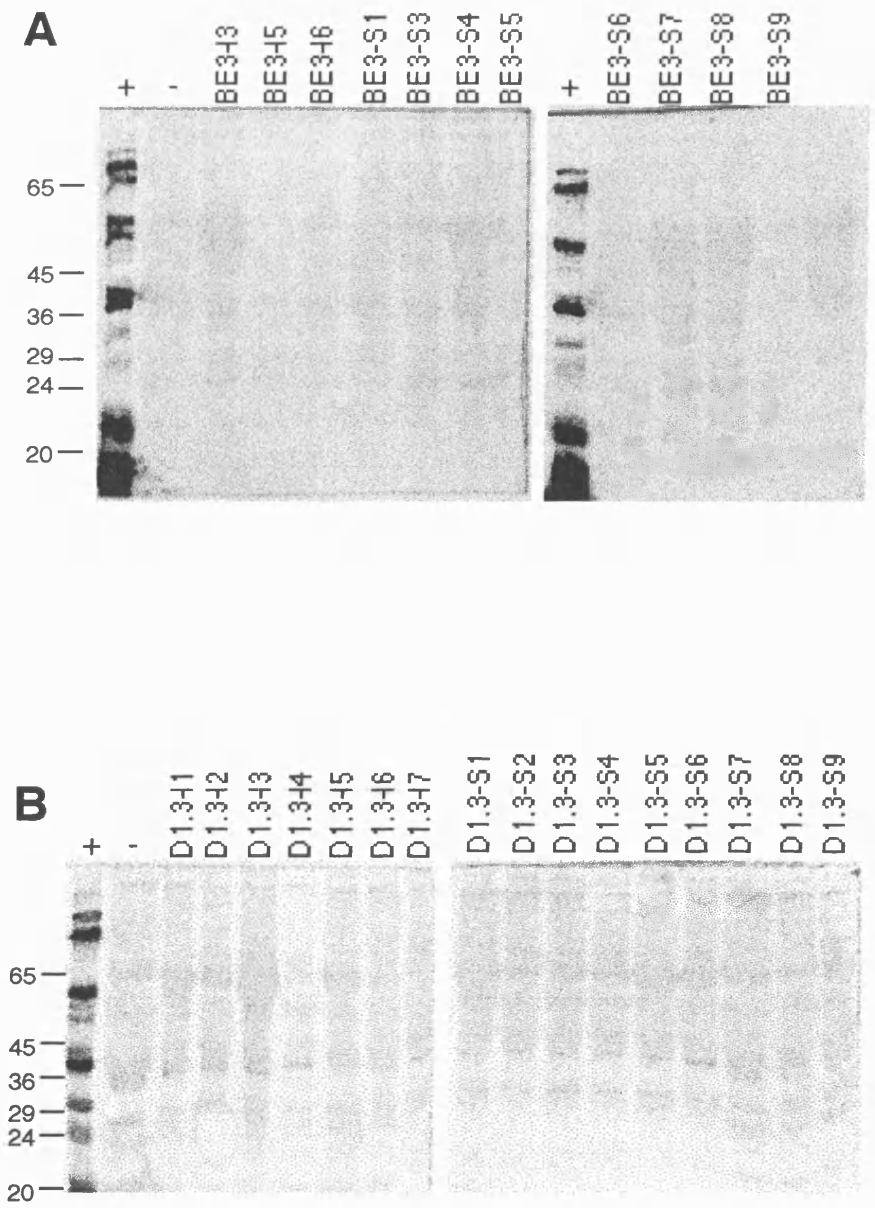


Fig 6.4. Detection Of scFv Protein In Total Protein Extracts. A : BE3 plants. **B** : D1.3 plants. -I and -S suffixes indicate intracellular and secreted forms respectively. + fd phage prep positive control. - SR1 total protein extract negative control

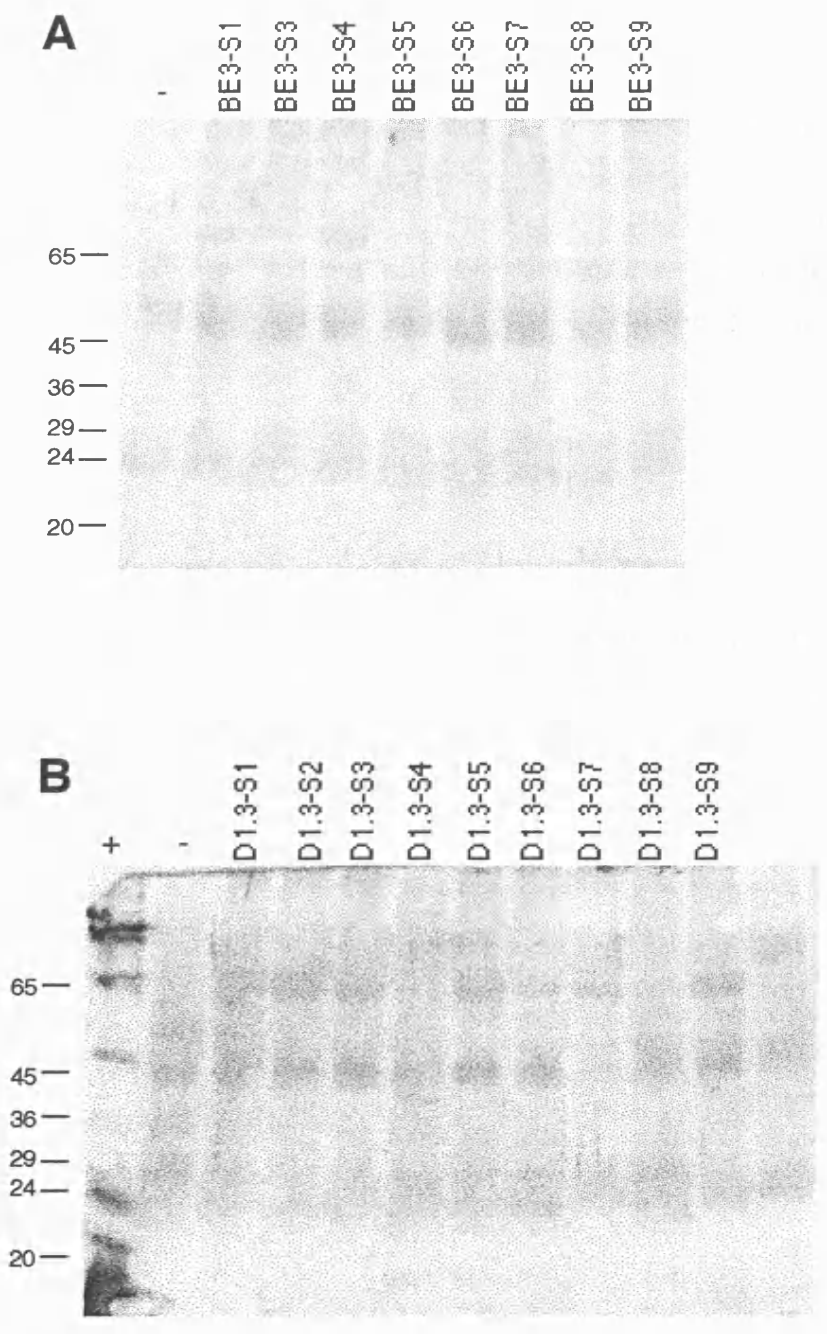


Fig 6.5. Detection Of scFv Protein In Apoplastic Fractions Prepared From Transgenic Plants Secreting scFv. A : BE3 plants.
B : D1.3 plants. + fd phage prep. - SR1 apoplastic fraction.

Chapter 7 : Discussion.

7.1 Aims.

The aims of the work described in this thesis were to isolate scFvs that recognised, and would hopefully have some effect on the activity of, enzymes that are important pathogenicity factors secreted by certain phytopathogens. These scFvs would then be expressed in transgenic plants to determine whether they had any effect on disease development or ingress of pathogen when these plants were challenged with the appropriate pathogen.

7.2 Summary Of Work Carried Out.

7.2.1 Isolation Of scFvs Recognising Pectate Lyase.

As the ultimate aim of this project was to generate transgenic plants expressing scFvs that recognised and possibly affected important pathogenicity factors, it was first necessary to isolate the genes encoding these scFvs. To this end, much work was concentrated on raising scFvs against the enzyme pectate lyase C from *Erwinia carotovora* subsp *carotovora*. As described in the main introduction, this is an extracellular pathogenicity factor of this important phytopathogen, and aids the ingress of the pathogen by disruption of the plant cell wall. As large quantities of this enzyme were required for the inoculation of experimental mice and subsequent analysis, this enzyme was overexpressed in the heterologous host *Escherichia coli*. As the secretion pathways differ in these bacteria, the enzyme was found localised in the periplasm, and was found to be active and soluble. As the overexpressed protein was found in the periplasm, this fraction was used to purify the enzyme, as this fraction contains many less contaminating proteins than the total cell fraction. The enzyme was purified by cation exchange chromatography, and was found to be pure enough for inoculation protocols and present in sufficient quantities for the uses described.

At this time, a gift of five hybridoma cell lines was made and it was decided that these would be used in an attempt to clone scFvs recognising pectate lyase C. Of these five lines, only one was healthy enough to grow up and isolate RNA in order to clone the corresponding scFv from the antibody, which had been shown to bind the pectate lyase purified. This scFv was cloned, expressed as a gene 3 fusion on the tip of a filamentous bacteriophage and found not to recognise

pectate lyase. There are several possible explanations for this, but the most likely reason for its inability to recognise pectate lyase is that the light chain that was cloned was actually the aberrant light chain expressed by the myeloma line used in the fusion to create the hybridoma line.
As the isolation of scFvs from hybridoma lines was not successful, experimental mice were inoculated and RNA isolated from the spleen to use in the construction of phage display libraries. Two different systems were used for this, but neither resulted in the isolation of any scFvs that recognised pectate lyase. This was most likely due to the severe technical difficulties encountered in the construction of these libraries resulting in a very small library size.
At this time, a synthetic human scFv library became available for screening, and this was used to screen for scFvs recognising pectate lyase. This approach was also unsuccessful, and it was considered possible that the pectate lyase that had been purified was not rich in distinct epitopes and hence no scFvs that would bind with a reasonable affinity were present in the library.

7.2.2 Isolation Of scFvs Recognising Polygalacturonase.

Upon screening this synthetic human library with polygalacturonase, an enzyme secreted by the phytopathogenic fungi *Botrytis cinerea*, scFvs were isolated that recognised this. One of these was studied in detail, and was found not to bind another PG isolated from *Cryphonectria parasitica*. It was, however, found to bind a carbohydrate epitope present on the protein. There was no evidence that this scFv may affect the activity of this enzyme, as would perhaps be expected if it did not bind to the protein core.

7.2.3 Expression Of scFvs In Transgenic Tobacco.

As the aim of this project had been to express scFvs in transgenic plants, and the targets of this study were enzymes that would be found in the extracellular milleau, it was important to determine whether scFvs could be expressed at reasonable levels in this compartment. Two scFvs were expressed in transgenic plants, but no scFv was found to accumulate in either the cytosol, or in the apoplast when a signal peptide was fused to the scFv. It has since been seen that the accumulation of scFvs to detectable levels is a relatively rare event, with reports of only one in four scFvs accumulating.

7.3 Future Work.

7.3.1 Generation Of Further scFvs.

The isolation of scFvs from the synthetic human scFv library screened in this study shows the possibility of using this system to generate further antibodies. The first piece of work that would be desirable to do would be to analyse the binding of the two other scFvs that recognise polygalacturonase. This would tell us whether they bound to different epitopes on the protein, or whether they were all only recognising the carbohydrate epitopes, as scFv C1 does. As discussed earlier, it is unlikely that if this was the case then the scFvs would have any effect on the activity of the enzyme. If these further scFvs were in fact found to bind to the protein core, then it would be interesting to repeat the experiments determining any effect on enzyme activity. As described in the relevant section, the sheer bulk of the phage displayed scFv may provide a problem, so the obvious approach would be to express the scFvs in a soluble form, by transfer of the plasmid into the non-supressor strain HB2151. If neither of these scFvs had any effect on the enzyme activity, it would obviously be desirable to screen more of the clones selected from this library. As a population of phage at each stage was kept as a glycerol stock, it would be an easy task to grow up more of the post pan 5 phage and perform further ELISAs to isolate further polygalacturonase binding clones. It would be expected that a large number of polygalacturonase binding clones could be obtained, as the initial four clones isolated in this study were found after screening only 95 clones. If after this work, it was apparent that most of the scFvs recognised the carbohydrate portion of the polygalacturonase, it would be possible to strip off the carbohydrate moieties, as was carried out to detemine the site of binding of scFv C1. This polygalacturonase (minus carbohydrate) could then be used to screen the library again, and it would be expected that several scFvs that bound to the protein would be isolated.

7.3.2 Expression Of scFvs In Transgenic Plants.

Since the time this work was started, it has become apparent that the expression of scFvs in transgenic plants is not a simple task, with no apparent reasons why some scFvs are seen to accumulate and others not. The most likely explanation is the difference in sequence between scFvs, although no rules have been proposed as to sequences which may or may not facilitate accumulation. It would still be interesting however to express the three scFvs that were isolated from the human synthetic scFv library and determine whether these would accumulate in

the apoplast of transgenic plants. Although the scFv C1 would not have an effect on the activity of the 'invading' polygalacturonase, it is possible that merely by binding to the protein, and hence increasing its molecular weight dramatically, that the movement of the enzyme through the cell wall would be retarded sufficiently to decrease the effect of the enzyme and thus afford the invaded plant more time in which to prepare an effective resistance response. It is likely however that if this was to be the case, then the scFv would have to accumulate to high levels, as the invasion by pathogens such as *Botrytis cinerea* takes place by a 'battering ram' approach in which a mass of enzymes are produced in order to break down cell walls.

There could, however, be a serious problem in the expression of scFvs from the synthetic human library in transgenic plants. If, as has been suggested, the sequence of the scFv determines whether or not an scFv will accumulate, the similarity in sequence of scFvs from this library may lead to problems. As all the scFvs contain the same light chain, and only 50 different heavy chains (each with an engineered CDR3 region), if the light chain present does not predispose the scFv to accumulation, then it is unlikely that any of the scFvs from this library would accumulate to detectable levels. It is also possible that the opposite may be the case, where many, or all, scFvs isolated from this library may accumulate in transgenic plants, again determined by the similarity of the sequences. At present however, there have been no reports of the expression of any scFvs from this library in transgenic plants, so it is not known whether these scFvs will accumulate or not.

Of all the interest that has been focussed on the production of antibodies, particularly scFvs, in transgenic plants, there have been relatively few reports of its success. Initial reports by Owen *et al.*,(1992) suggested that the intracellular expression of scFvs was possible, with accumulation of this scFv up to 0.1% total soluble protein (tsp). This success was confirmed by experiments by Tavladoraki *et al.*,(1993) with intracellular expression levels again up to 0.1% tsp. Since these reports however, there have been very few reports of successful intracellular expression of scFvs, with several reports describing no accumulation of scFv in the cytosol (Fiedler and Conrad 1995, Fecker *et al.*,1996 and Schouten *et al.*,1996). Bruyns *et al.*,(1996) report accumulation up to a maximum of 0.01% tsp. In light of these other results, it is perhaps not surprising that in experiments described in chapter 6, no scFv was detected in plants containing constructs lacking a signal sequence. The small number of plants analysed that were seen to be transcribing scFv mRNA (a total of only 7 plants) would obviously have contributed to this

lack of success, as some groups have had to screen up to 120 plants to isolate a plant expressing scFv to detectable levels (Owen *et al.*,1992). It has been suggested that scFv does not accumulate in the cytosol due to degradation by proteinases, as the addition of a six residue C-terminal extension in the form of an extended endoplasmic reticulum retention signal (Schouten *et al.*,1996) resulted in the accumulation of scFv up to 0.2% tsp, suggesting the role of exo-proteinases in the degradation of scFvs. It was also postulated that the hexapeptide possibly indirectly protected the scFv by its interaction with the cytosolic side of the ER salvage receptor. Having reviewed all of these results, it would appear that the intracellular accumulation of scFVs is rare, and targeting of scFvs to other compartments may provide more success.

After the first report of the expression of an scFv in plants (Owen *et al.*,1992), work was conducted to determine whether secretion to the apoplast may enhance accumulation of scFvs. It was found that this particular scFv (AS32) accumulated to 0.5% tsp upon secretion using the same signal peptide used in this thesis (the tobacco PR1a signal sequence) (Firek *et al.*,1993). This result suggested that other scFvs may also show this enhanced accumulation. This has been shown to be the case in some instances. The accumulation of an scFv which was undetectable when expressed cytosolically was shown to accumulate to 0.67% tsp when secreted (Fiedler and Conrad 1995). Another report demonstrates 'high' scFv levels upon secretion, but in this report the 'high' levels seen were to a maximum of 0.01% tsp compared to no detection of scFv upon intracellular expression (Fecker *et al.*,1996). Schouten *et al.*,(1996) also show the accumulation of a secreted scFv to 0.01% tsp when this scFv was undetectable intracellularly. It is clear however that secretion of an scFv to the apoplast will not necessarily result in its accumulation to a high level. It would appear that the scFvs used in this thesis fall amongst the catagory of scFvs which will not accumulate to reasonable levels even when secreted. The fact that some scFvs will accumulate and others will not suggests that despite the obvious sequence similarity between these genes, there may be some sequences that constrain the accumulation of that scFv, either by enhancing its proteolytic degradation or by some other means.

Several reports have been made of the accumulation of scFvs to more reasonable levels when they have been targeted to the endoplasmic reticulum. Merely the presence of an endoplasmic reticulum retention signal appears to enhance the accumulation of some scFvs as described above, and it appears that targeting of an scFv to the ER surpasses the scFv accumulated upon secretion, with reports of 1% tsp (Schouten *et al.*,1996) and a massive 4.8% tsp (Artsaenko *et al.*,1995). These

reports suggest that this accumulation is strictly due to its localisation within the ER and that it is consequently protected from proteolytic activity further down the secretory pathway, either intra- or extracellularly. Accumulation of another scFv to the same level cytosolically as when it is ER-targeted (0.01% tsp) (Bruyns *et al.*,1996) has shown that, despite these impressive reports, it is clear that this is not the case for all scFvs. Indeed it has been stated "In our work with scFv antibodies, for as yet unknown reasons, only about one in four accumulates to detectable levels. This is the case even if these scFvs are targeted to different subcellular targets, including the endoplasmic reticulum" (Whitelam and Cockburn 1996). It is apparent then, that there are no rules to be adhered to when attempting the expression of an scFv in transgenic plants.

7.4 Future Prospects.

7.4.1 Antibody Expression In Transgenic Plants.

Although the expression of antibodies in plants has been shown not to be a trivial task, it still holds great attraction. It is still the case that the expression of antibodies at high levels may provide an attractive alternative to other expression systems, especially if they may be easily purified, for example by expression as an oleosin fusion in seeds, which would allow the partitioning of the antibody molecule with the oleosin. The expression of antibodies in high quantities in storage organs such as potato tubers may also provide a suitable system.

There is also still interest in the expression of antibody derivatives in plants as either an experimental tool or as a means to improve crop performance. As an alternative to antisense technology, the expression of scFvs recognising a host protein may allow a broader range of effect, due to recognition of closely related molecules than would be afforded by antisense technology which relies solely on recognition at the nucleic acid level. Also, the use of antisense necessitates the cloning of the particular gene of interest, which may not be necessary if antibodies raised against the same protein from another species were used to create an scFv for expression. An additional advantage, which was the aim of this project, was to use antibodies which recognise foreign molecules, an approach which clearly cannot be contemplated using antisense. Despite these advantages, it is clear that the use of antisense is far more widespread than the use of antibodies in modulating a cellular activity, due to the far simpler and more reliable technology involved.

7.4.2 Alternative Strategies.

As the problems involved in the expression of antibodies in transgenic plants have become better known, other strategies have been developed as a means for the modulation of cellular activities. One of these approaches is similar to the phage display system used in this work, but instead of scFvs being displayed the functional 'unit' is a random peptide. By the creation of a phage library which displays a series of random peptides, and by panning in the same way as for an scFv phage display library, peptides which recognise the protein of interest can be isolated. It is envisaged that there may be isolated by this method peptides which may have an effect similar to that desired at the start of this project (ie blocking essential pathogenicity factors). The range of target molecules is as wide as that using an antibody approach, but similar problems may arise upon expression of these peptides in transgenic plants. The sheer small size of these peptides makes it unlikely that they will be stable upon expression, so strategies such as fusing them to 'carrier' proteins may have to be used to achieve the desired effect. Despite these possible downfalls, it is likely that the expression of peptides, as well as the expression of antibody fragments, in transgenic plants will become a more routine facet of plant molecular biology, both for pure science and applied applications.

Appendix I : Oligonucleotide Sequences.

RSPL	5' GGAAACAGCTATGACCATG 3'
M13-20	5' GTAAAACGACGGCCAGT 3'
CH1FOR - equimolar mix of :	
MOCG12FOR	5' CTCAATTTTCTTGTCCACCTTGGTGC 3'
MOCG3FOR	5' CTCGATTCTCTTGATCAACTCAGTCT 3'
MOCMFOR	5' TGGAATGGGCACATGCAGATCTCT 3'
CKFOR	5' CTCATTCCTGTTGAAGCTCTTGAC3'
VH1FOR2	5' TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC 3'
VH1BACKAPA10	5'CATGACCACAGTGCACAGGTSMARC TGCAGSAGTCWGG 3'
VK4FORNOT - equimolar mix of :	
JK1NOT10	5' GAGTCATTCTGCGGCCGCCCGTTTGA TTTCCAGCTTGGTGCC 3'
JK2NOT10	5' GAGTCATTCTGCGGCCGCCCGTTTTA TTTCCAGCTTGGTCCC 3'
JK4NOT10	5' GAGTCATTCTGCGGCCGCCCGTTTTA TTTCCAACTTTGTCCC 3'
JK5NOT10	5' GAGTCATTCTGCGGCCGCCCGTTTCA GCTCCAGCTTGGTCCC 3'
VK2BACK	5' GACATTGAGCTCACCCAGTCTCCA 3'
MO-LINK-BACK	5' GGGACCACGGTCACCGTCTCCTCA 3'
MO-LINK-BACK	5' TGGAGACTGGGTGAGCTCAATGTC 3'

Degeneracy code : S = C or G; M = A or C; R = A or G; W = A or T.

References.

Abe,N and Inouye,K. 1993. Purification of monoclonal antibodies with light chain heterogeneity produced by mouse hybridomas raised with NS-1 myelomas - application of hydrophobic interaction high-performance liquid chromatography. Journal Of Biochemical And Biophysical Methods **27**:215-227.

Abel,PP, Nelson,RS, De,B, Hoffmann,N, Rogers,SG, Fraley,RT, and Beachy,RN. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic-virus coat protein gene. Science **232**:738-743.

Adair,JR. 1992. Engineered antibodies for therapy. Immunological reviews **130**:5-40.

Alexander,D, Goodman,RM, Gutrella,M, Glascock,C, Weyman,K, Friedrich,L, Maddox,D, Ahlgoy,P, Luntz,T, Ward,E and Ryals,J. 1993. Increased tolerance to 2 oomycete pathogens in transgenic tobacco expressing pathogenesis related protein-1a. Proceedings of the National Academy of Sciences USA **90**:7327-7331.

Amit,AG, Boulot,G, Harper,M, Jankovic,D, Leguern,A, Mariuzza,R, Mazie,JC, Phillips,SEV and Poljak,RJ. 1986. Crystal structure of the complex between the Fab fragment of a monoclonal anti-lysozyme antibody and its antigen. Hereditas **104**:169-170.

Artsaenko,O, Peisker,M, Zurnieden,U, Fiedler,U, Weiler,EW, Muntz,K and Conrad,U. 1995. Expression of a single chain Fv antibody against abscissic-acid creates a wilty phenotype in transgenic tobacco. Plant Journal **8**:745-750.

Barbas,CF, Bjorling,E, Chiodi,F, Dunlop,N, Cababa,D, Jones,TM, Zebedee,SL, Persson,MAA, Nara,PL, Norby,E and Burton,DR. 1992a. Recombinant human Fab fragments neutralise human type-I immunodeficiency virus *in vitro*. Proceedings of the National Academy of Science USA **89**:9339-9343.

Barbas,CF, Crowe,JE, Cababa,D, Jones,TM, Zebedee,SL, Murphy,BR, Chanock,RM and Burton,DR. 1992b. Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus-F glycoprotein and neutralise infectivity. Proceedings of the National Academy of Science USA **89**:10164:10168.

Barbas,CF, Languind,LR and Smith,JW. 1993. High-affinity self-reactive human antibodies by design and selection - targeting the integrin ligand-binding site. Proceedings of the National Academy of Science USA **90**:10003-10007.

Barras,F, Vangijsegem,F and Chatterjee,AK. 1994. Extracellular enzymes and pathogenesis of soft-rot Erwinia. Annual Review of Phytopathology **32**:201-234.

Baum,TJ, Hiatt,A, Parrott,WA, and Hussey,RS. 1996. Expression in tobacco of a functional monoclonal-antibody specific to stylet secretions of the root-knot nematode. Molecular Plant-Microbe Interactions **9**:382-387.

Beachy,RN. 1997. Mechanisms and applications of pathogen derived resistance in transgenic plants. Current Opinions In Biotechnology **8**:215-220.

Beck,E, Sommer,R, Auerswald,E, Kurz,C, Zink,B, Osterburg,G, Schaller,H, Sugimoto,K, Sugisaki,H, Okamoto,T and Takanami,M. 1978. Nucleotide sequence of bacteriophage fd DNA. Nucleic Acids Research **5**:4495-4503.

Benvenuto,E, Ordas,RJ, Tavazza,R, Ancora,G, Biocca,S, Cattaneo,A and Galeffi,P. 1991. 'Phytoantibodies': a general vector for the expression of immunoglobulin domains in transgenic plants. Plant Molecular Biology **17**:865-874.

Berek,C and Milstein,C 1987. Mutation drift and repertoire shift in the maturation of the immune response. Immunological Reviews **96**:23-41.

Bevan,M. 1984. Binary Agrobacterium vectors for plant transformation. Nucleic Acids Research **12**:8711-8721.

Boulot,G, Eisele,JL, Bentley,GA, Bhat,TN, Ward,ES, Winter,G and Poljak,RJ. 1990. Crystallisation and preliminary X-ray diffraction study of the bacterially expressed Fv from the monoclonal anti-lysozyme antibody D1.3 and of its complex with the antigen, lysozyme. Journal of Molecular Biology **213**:617-619.

Broglie,K, Chet,I, Holliday,M, Cressman,R, Biddle,P, Knowlton,S, Mauvais,CJ and Broglie,R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia-solani*. Science **254**:1194-1197.

Bruyns,AM, Dejaeger,G, Deneve,M, Dewilde,C Vanmontagu,M and Depicker,A. 1996. Bacterial and plant produced scFv proteins have similar antigen binding properties. FEBS Letters **386**:5-10.

Bullock,WO, Fernandez,JM and Short,JM. 1987. XL1-Blue - a high-efficiency plasmid transforming RecA *Escherichia coli* strain with beta-galactosidase selection. Biotechniques **5**:376.

Burton,DR, Barbas,CF, Persson,MAA, Koenig,S, Chanock,RM and Lerner,RA. A large array of human monoclonal antibodies to type I Human Immunodeficiency Virus from combinatorial libraries of asymptomatic seropositive individuals. Proceedings of the National Academy of Science USA **88**:10134-10137.

Bye,J, Gorick,B, Finnern,R, Voak,D, Hughesjones,NC, Ouwehand,W, Winter,G and Marks,JD. 1993. Derivation of human scFv antibodies specific for blood group antigens using a phage display library. British Journal of Haemotology **84**(S1):8.

Carlson, JD. 1988. A new means of inducibly inactivating a cellular protein. Molecular Cell Biology **8**:2638-2646.

Carter,P, Bedouelle,H and Winter,G. 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acids Research **13**:4431.

Caton,AJ and Koprowski,H. 1990. Influenza virus haemaglutinin-specific antibodies isolated from a combinatorial expression library are closely related to the immune response of the donor. Proceedings of the National Academy of Science USA. **87**:6450-6454.

Chothia,C and Lesk,AM. 1987. Canonical structures for the hypervariable regions of immunoglobulins. Journal of Molecular Biology **196**:901-917.

Chothia,C, Lesk,AM, Gherardi,E, Tomlinson,IM, Walter,G, Marks,JD, Llewelyn,MB and Winter,G. 1992. Structural repertoire of the human VH segments. Journal of Molecular Biology **227**:799-817.

Chuck,AS and Palsson,BO. 1992. Population balance between producing and nonproducing hybridoma clones is very sensitive to serum level, state of inoculum and medium composition. Biotechnology and Bioengineering **39**:354-360.

Clackson,T, Hoogenboom,HR, Griffiths,AD and Winter,G. 1991. Making antibody fragments using phage display libraries. Nature **352**:624-628.

Clark,JR and Freedman,RB. 1994. Cloning and expression of an anti-PDI scFv in Escherichia coli. Journal Of Cellular Biochemistry **S18**:199

Collmer,A and Keen,NT. 1986. The role of pectic enzymes in plant pathogenesis. Annual Review of Phytopathology **24**:383-409.

Collmer,A, Schoedel,C, Roeder,DL, Ried,JL and Rissler,JF. 1985. Molecular cloning in *Escherichia coli* of *Erwinia chrysanthemi* genes encoding multiple forms of pectate lyase. Journal of Bacteriology **161**:913-920.

Conrad,U and Fiedler,U. 1994. Expression of engineered antibodies in plant cells. Plant Molecular Biology **26**:1023-1030.

Covell,DG, Barbet,J, Holton,OD, Black,CDV, Parker,RJ and Weinstein,JN. 1986. Pharmacokinetics of monoclonal IgG, F(ab')2 and Fab' in mice. Cancer Research **46**:3969-3978.

Dekruif,J and Logtenberg,T. 1996. Leucine zipper dimerised bivalent and bispecific scFv antibodies from a semisynthetic antibody phage display library. Journal of Biological Chemistry **271**:7630-7634.

DeNeve,M, DeLoose,M, Jacobs,A, VanHoudt,H, Kaluza,B, Weidle,U, VanMontague,M and Depicker,A. 1993. Assembly of an antibody and its derived antibody fragment in *Nicotiana* and *Arabidopsis*. Transgenic Research **2**:227-237.

Denny,TP. 1995. Involvement of bacterial polysaccharides in plant pathogenesis. Annual Review of Phytopathology **33**:173-197.

Devereux,J, Haeberlry,P and Smithies,O. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Research **12**:387.

DeWilde,C, DeNeve,M, DeRycke,R, Bruyns,A-M, DeJaeger,G, VanMontague,M, Depicker,A and Engler,G. 1996. Intact antigen-binding MAK33 antibody and Fab fragment accumulate in intercellular spaces of *Arabidopsis thaliana*. Plant Science **114**:233-241.

Dixon,RA, Harrison,MJ and Lamb,CJ. 1994. Early events in the activation of plant defense responses. Annual Review of Phytopathology **32**:479-501.

Draper,J, Scott,R, Armitage,P and Walden,R. 1988. Plant genetic transformation and gene expression : a laboratory manual. Blackwell Scientific Publications.

Duan,LX and Pomerantz,RJ. 1994. Elimination of endogenous abberant kappa chain transcripts from SP2/0 derived hybridoma cells by specific ribozyme cleavage - utility in genetic therapy of HIV-1 infections. Nucleic Acids Research **22**:5433-5438.

During,K, Hippe,S, Kreuzaler,F and Schell,J. 1990. Synthesis and self assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. Plant molecular Biology **15**:281-293.

During,K, Porsch,P, Fladung,M and Lorz,H. 1993. Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. The Plant Journal **3**:587-598.

Fecker,LE, Kaufmann,A, Commandeur,U, Commandeur ,J, Koenig,R and Burgermeister,W. 1996. Expression of single chain antibody fragments (scFv) specific for beet necrotic yellow vein virus coat protein or 25kDa protein in *Escherichia coli* and *Nicotiana benthemiana*. Plant Molecular Biology **32**:979-986.

Fiedler,U and Conrad,U. 1995. High level production and long term storage of engineered antibodies in transgenic tobacco seeds. Bio-technology **13**:1090-1093.

Firek,S, Draper,J, Owen,MRL, Gandecha,A, Cockburn,B and Whitelam,GC. 1993. Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. Plant Molecular Biology **23**:861-870.

Firek,S, Whitelam,GC and Draper,J. 1994. Endoplasmic reticulum targeting of active modified B-glucuronidase (GUS) in transgenic tobacco plants. Transgenic Research **3**:326-331.

Fitchen,JH and Beachy,RN. 1993. Genetically engineered protection against viruses in transgenic plants. Annual Review of Microbiology **47**:739-763.

Flor,HH. 1955. Host parasite interactions in flax rust - its genetics and other implications. Phytopathology **45**:680-685.

Freund,C, Ross,A, Guth,B, Pluckthun,A and Holak,TA. 1993. Characterisation of the linker peptide of the single chain Fv fragment of an antibody by NMR spectroscopy. Febs Letters **320**:97-100.

Fuqua,C, Winnans,SC and Greenberg,EP. 1996. Census and consensus in bacterial ecosystems - the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annual Review of Microbiology **50**:727-751.

Gao,S and Shain,L. 1994. Characterisation of an endopolygalacturonase produced by the chestnut blight fungus. Physiological and Molecular Plant Pathology **45**:169-179.

Gerlach,WL, LLewellyn,D and Haseloff,J. 1987. Construction of a plant-disease resistance gene from the satellite RNA of tobacco ringspot virus. Nature 328:802-805.

Glockshuber,R, Schmidt,T and Pluckthun,A. 1992. The disulphide bonds in antibody variable domains - effects on stability, folding invitro, and functional expression in E.coli. Biochemistry **31**:1270-1279.

Gray,J, Picton,S, Shabbeer,J, Schuch,W and Grierson,D. 1992. Molecular biology of fruit ripening and its manipulation with antisense genes. Plant Molecular Biology **19**:69-87.

Griffiths,AD, Malmqvist,M, Marks,JD, Bye,JM, Embleton,MJ, McCafferty,J, Baier,M, Holliger,KP, Gorick,BD, Hughesjones,NC, Hoogenboom,HR and Winter,G. 1993. Human anti-self antibodies with high specificity from phage display libraries. EMBO Journal **12**:725-734.

Hahn,MG. 1996. Microbial elicitors and their receptors in plants. Annual Review of Phytopathology **34**:387-412.

Hain,R, Reif,HJ, Krause,E, Langebartels,R, Kindl,H, Vornam,B, Wiese,W, Schmelzer,E, Schreier,PH, Stocker,RH and Stenzel,K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature **361**:153-156.

Hammondkosack,KE, Jones,DA and Jones,JDG. 1996. Ensnaring microbes - the components of plant disease resistance. New Phytologist **133**:11-24.

Harlow and Lane. 1988. Antibodies - A laboratory manual. Academic Press Ltd.

Hartman,CL, Secor,GA, Venette,JR and Albaugh,DA. 1986. Response of bean calli to filtrate from *Pseudomonas syringae* pv *phaseolicola* and correlation with the whole plant disease reaction. Physiological And Molecular Plant Pathology **28**:353-358.

Hawkins,RE and Winter,G. 1992. Cell selection strategies for making antibodies from variable gene libraries: trapping the memory pool. European Journal Of Immunology **22**:867-870.

Hiatt,A. 1990 Antibodies produced in plants. Nature **344**:469-470.

Hiatt,A, Cafferkey,R and Bowdish,K. 1989. Production of antibodies in transgenic plants. Nature **342**:76-78.

Hinton,JCD, Sidebotham,JM, Gill,DR and Salmond,GPC. 1989. Extracellular and periplasmic isozymes of pectate lyase from *Erwinia carotovora* subspecies *carotovora* belong to different gene families. Molecular Microbiology **3**:1785-1795.

Hoekema,A, Hirsch,PR, Hooykaas,PJJ and Schilperpoort,RA. 1983. A binary plant vector strategy based on separation of vir-region and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature **303**:179-180.

Holliger,P, Prospero,T and Winter,G. 1993. Diabodies - small bivalent and bispecific antibody fragments. Proceedings of the National Academy of Science USA **90**:6444-6448.

Hoogenboom,HR and Winter,G. 1992. By passing immunisation: human antibodies from synthetic repertoires of germline VH gene segments rearranged *in vitro*. Journal of Molecular Biology **227**:381-388.

Hoogenboom,HR, Griffiths,AD, Johnson,KS, Chiswell,DJ, Hudson,P and Winter,G. 1991. Multisubunit proteins on the surface of filamentous phage - methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Research **19**:4133-4137.

Hooykaas,PJJ and Beijersbergen,AGM. 1994. The virulence system of *Agrobacterium tumefaciens*. Annual Review of Phytopathology **32**:157-179.

Huston,JS, Levinson,D, Mudgetthunter,M, Tai,MS, Novotny,J, Margolies,MN, Ridge,RJ, Bruccoleri,RE, Haber,E, Crea,R and Oppermann,H. 1988. Protein engineering of antibody-binding sites - recovery of specific activity in an anti-digoxin single chain Fv analog produced in *Escherichia coli*. Proceedings of the National Academy of Science USA **85**:5879-5883.

Jach,G, Gornhardt,B, Mundy,J, Logemann,J, Pinsdorf,P, Leah,R, Schell,J, and Maas,C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant Journal **8**:97-109.

Johnston,DJ and Williamson,B. 1992. Purification and characterisation of 4 polygalacturonases from *Botrytis cinerea*. Mycological Research **96**:343-349.

Jongedijk,E, Tigelaar,H, Vanroekel,JSC, Bresvloemans,SA, Dekker,I, Vandenelzen,PJM, Cornelissen,BJC and Melchers,LS. 1995. Synergistic activity of chitinases and beta-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica **85**:173-180.

Kearney,B and Staskawicz,BJ. 1990. Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene avrBs2. Nature **346**:385-386.

Keen,NT, Dahlbeck,D, Staskawicz,B and Belser,W. 1984. Molecular cloning of pectate lyase genes from *Erwinia chrysanthemi* and their expression in *Escherichia coli*. Journal Of Bacteriology **159**:825-831.

Keen,NT and Tamaki,S. 1986. Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high level expression in *Escherichia coli.* Journal Of Bacteriology **168**:595-606.

Kitamoto,N, Kimura,T, Kito,Y, Ohmiya,K and Tsukagoshi,N. 1993. Structural features of a polygalacturonase gene cloned from *Apergillus oryzae* KBN616. FEMS Microbiology Letters **111**:37-41.

Klopmeyer,MJ and Kelman,A. 1988. Use of monoclonal antibodies specific for pectate lyase as serological probes in the identification of soft rot *Erwinia* spp. Phytopathology **78**:1430-1434.

Kohler,G and Milstein,C. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. Nature **256**:495-497.

Kotoujansky,A. 1987. Molecular genetics of pathogenesis by soft-rot *Erwinias.* Annual Review Of Phytopathology **25**:405-430.

Lei,SP, Lin,HC, Heffernan,L and Wilcox,G. 1985. Evidence that polygalacturonase is a virulence determinant in *Erwinia carotovora.* Journal Of Bacteriology **164**:831-835.

Lewis,AP, Lemon,SM, Barber,KA, Murphy,P, Parry,NR, Peakman,TC, Sims,MJ, Worden,J and Crowe,JS. 1993. Rescue, expression and analysis of a neutralising human anti-hepatitis-A virus monoclonal antibody. Journal of Immunology **151**:2829-2838.

Lin,KH and Cheng,SY. 1991. An efficient method to purify active eukaryotic proteins from the inclusion bodies in *Escherichia coli.* Biotechniques **11**:748.

Low,NM, Holliger,P and Winter,G. 1996. Mimicking somatic hypermutation - affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. Journal of Molecular Biology **260**:359-368.

Lowe,PA, Rhind,SK, Sugrue,R and Marston,FAO. 1987. Solubilisation, refolding and purification of eukaryotic proteins expressed in *Escherichia coli.* Journal Of Cellular Biochemistry **S11C**:173.

Lowman,HB, Bass,SH, Simpson,N and Wells,JA. 1991. Selecting high affinity binding proteins by monovalent phage display. Biochemistry **30**:10832-10838.

Ma,JK-C. 1995. Antibody expression in plants. ACS Symposium Series **604**:56-69.

Ma,JKC, Hunjan,M, Smith,R, Kelly,C and Lehner,T. 1990. An investigation into the mechanism of protection by local passive immunisation with monoclonal antibodies against *Streptococcus mutans.* Infection and Immunity **58**:3407-3414.

Maciejewski,JP, Weichold,FF, Young,NS, Cara,A, Zella,D, Reitz,MS and Gallo,RC. 1995. Intracellular expression of antibody fragments directed against HIV reverse transcriptase prevents HIV-infection *in vitro.* Nature Medicine **7**:667-673.

MacNicol,RJ, Williamson,B and Dolan,A. 1995. Infection of red raspberry styles and carpels by *Botrytis cinerea* and its possible role in post-harvest grey mould. Annals of Applied Biology **106**:49-53.

Marasco,WA, Haseltine,WA and Chen,SY. 1993. Design, intracellular expression and activity of a human anti-human immunodeficiency virus type-I GP120 single chain antibody. Proceedings of the National Academy of Science USA **90**:7889-7893.

Marks,JD, Tristem,M, Karpas,A and Winter,G. 1991. Oligonucleotide primers for PCR amplification of human immunoglobulin variable genes and design of family specific oligonucleotide probes. European Journal Of Immunology **21**:985-991.

Marks,JD, Hoogenboom,HR, Bonnert,TP, McCafferty,J, Griffiths,AD and Winter,G. 1991. By-passing immunisation: human antibodies from V-gene libraries displayed on phage. Journal of Molecular Biology **222**:581-597.

Marks,JD, Ouwehand,WH, Bye,JM, Finnern,R, Gorick,BD, Voak,D, Thorpe,SJ, Hughesjones,NC and Winter,G. 1993. Human antibody fragments specific for human blood group antigens from a phage display library. Bio-technology **11**:1145-1149.

Marston,FAO. 1987. The purification of eukaryotic polypeptides expressed in Escherichia coli. pp59-88 in DNA Cloning: A Practical Approach, Vol 3. IRL Press, Oxford.

Martin,ACR. 1996. Accessing the Kabat antibody sequence database by computer. Proteins- Structure Function and Genetics **25**:130-133.

Matthews,R. 1991. Plant Virology. 3rd Edition. Academic Press Ltd.

McNicol,RJ, Williamson,B and Dolan,A. 1985. Infection of red raspberry styles and carpels by *Botrytis cinerea* and its possible role in post harvest grey mould. Annals Of Applied Biology **106**:49

Meng,JX, John,TR and Kaiser,II. 1995. Specificity and binding affinity of an anti-crotoxin combinatorial antibody selected from a phage-displayed library. 1995. Biochemical Pharmacology **50**:1969-1977.

Meulemans,EV, Slobbe,R, Wasterval,P, Ramaeker,FCS and Vaneys,GJJM. 1994. Selection of phage displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. Journal Of Molecular Biology **244**:353-360.

Milstein,C and Cuello,AC. 1983. Hybrid hybridomas and their use in immunohistochemistry. Nature **305**:537-540.

Mindrinos,M, Katagiri,F, Yu,GL and Ausebel,FM. 1994. The *A thaliana* disease resistance gene Rps2 encodes a protein containing a nucleotide binding site and leucine rich repeats. Cell **78**:1089-1099.

Mitraki,A and King,J. 1989. Protein folding intermediates and inclusion body formation. Biotechnology **7**:690-697.

Mullinax,RL, Gross,EA, Amberg,JR, Hay,BN, Hogrefe,HH, Kubitz,MM, Greener,A, Altingmees,M, Ardourel,D, Short,JM, Sorge,JA and Shopes,B. 1990. Identification of human-antibody fragment clones specific for tetanus toxoid in a bacteriophage lambda immunoexpression library. Proceedings of the National Academy of Science USA **87**:8095-8099.

Nissim,A, Hoogenboom,HR, Tomlinson,IM, Flynn,G, Midgley,C, Lane,D and Winter,G. 1994. Antibody fragments from a single pot phage display library as immunochemical reagents. EMBO Journal **13**:692-698.

Orlandi,R, Gussow,DH, Jones,PT and Winter,G. 1989. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. Proceedings of the National Academy of Science USA **86**:3833-3837.

Orum,H, Andersen,PS, Oster,A, Johansen,LK, Riise,E, Bjornvad,M, Svendsen,I and Engberg,J. 1993. Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. Nucleic Acids Research **21**:4491-1198.

Owen,M, Gandecha,A, Cockburn,B and Whitelam,G. 1992. Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. Biotechnology **10**:790-794.

Perombelon,MCM. 1987. Evaluation of the black-leg potential of potato plants. Potato Research **30**:350-351.

Persson,MAA, Caothien,RH and Burton,DR. 1991. Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. Proceedings of the National Academy of Science USA **88**:2432-2436.

Pirhonen,M, Flego,D, Heikinheimo,R and Palva,ET. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. EMBO Journal **12**:2467-2476.

Plastow,GS, Border,PM, Hinton,JCD and Salmond,GPC. 1986. Molecular cloning of pectinase genes from *Erwinia carotovora* subspecies *carotovora* (strain SCRI193). Symbiosis **2**:115-122.

Pluckthun,A. 1991. Antibody engineering: advances from the use of *Escherichia coli* expression systems. Biotechnology **9**:545-551.

Portolano,S, McLachlan,SM and Rapoport,B. 1993. High affinity, thyroid specific human autoantibodies displayed on the surface of filamentous phage use V-genes similar to other autoantibodies. Journal of Immunology **151**:2839-2851.

Rasced,I and Oberer,E. 1986. FF coliphages - structural and functional relationships. Microbiological Reviews **50**:401-427.

Ravetch,JV, Siebenlist,U, Korsmeyer,S, Waldmann,T and Leder,P. 1981. Structure of the human immunoglobulin λ locus: characterisation of embryonic and rearranged J gene and D gene. Cell **27**:583-591.

Reverchon,S, Hugouvieux-Cotte-Pattat,N and Robert-Baudouy,J. 1985. Cloning of genes encoding pectolytic enzymes from a genomic library of the phytopathogenic bacterium, *Erwinia chrysanthemi*. Gene **35**:121-130.

Ridder,R, Geisse,S, Kleuser,B, Kawalleck,P and Gram,H. 1995. A cos-cell based system for rapid production and quantitation of scFv-IgC-kappa antibody fragments. Gene **166**:273-276.

Roberts,DP, Denny,TP and Schell,MA. 1988. Cloning of the egl gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity. Journal Of Bacteriology **170**:1445-1451.

Roberts,J and Devoret,R. 1983. Lysogenic Induction. p123 in Lambda III, Cold Spring Harbor.

Roine,E, Wei,WS, Yuan,J, NurmiahoLassila,EL and Kalkkinen,N. 1997. Hrp pilus : An hrp-dependant bacterial surface appendage produced by *Pseudomonas syringae* pv *tomato* DC3000. Proceedings of the National Academy of Sciences USA **94**:3459-3464.

Roitt,I.M. 1994. Essential Immunology, 8th Edition. Blackwell Scientific Publications.

Rommens,CMT, Salmeron,JM, Oldroyd,GED and Staskewicz,BJ. 1995. Intergeneric transfer and functional expression of the tomato disease resistance gene Pto. Plant Cell **7**:1537-1544.

Rosso,MN, Schouten,A, Roosien,J, Borstvrenssen,T, Hussey,RS, Gommers,FJ, Bakker,J, Schots,A and Abad,P. 1996. Expression and functional characterisation of a single chain Fv antibody directed against secretions involved in plant nematode infection process. Biochemical and Biophysical Research Communications **220**:255-263.

Rudikoff,S, Guish,AM, Cook,WD and Scharff,MD. 1982. Single amino acid substitution altering antigen binding specificity. Proceedings of the National Academy of Science USA. **79**:1979-1983.

Salmond,GPC. 1994. Secretion of exracellular virulence factors by plant pathogenic bacteria. Annual Review of Phytopathology **32**:181-200.

Salmond,GPC, Hinton,JCD, Gill,DR and Perombelon,MCM. 1986. Transposon mutagenesis of *Erwinia* using phage lambda vectors. Molecular and General Genetics **203**:524-528.

Sanz,I. 1991. Multiple mechanisms participate in the generation of diversity of human heavy chain CDR3 regions. Journal Of Immunology **147**:1720-1729.

Schots,A., De Boer,J, Schouten,A, Roosien,J, Zilverentant,JF, Pomp,H, Bouwman-Smits,L, Overmars,H, Gommers,FJ, Visser,B, Stiekma,WJ and Bakker,J. 1992. Plantibodies : a flexible approach to design resistance against pathogens. Netherlands Journal Of Plant Pathology **98(S2)**:183-191.

Schafer,W. 1994. Molecular mechanisms of fungal pathogenicity to plants. Annual Review of Phytopathology **32**:461-477.

Schouten,A, Roosien,J, Vanengelen,FA, Dejong,GAM, Borstvrenssen,AWM, Zilverentant,JF, Bosch,D, Stiekema,WJ, Gommers,FJ, Schots,A and Bakker,J. 1996. The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. Plant Molecular Biology **30**:781-793.

Sharon,J. 1990. Structural correlates of high antibody affinity: three engineered amino acid substitutions can increase the affinity of an anti-p-azophenylarsonate antibody 200-fold. Proceedings of the National Academy of Science USA. **87**:4814-4817.

Sigee,D. 1993. Bacterial plant pathology. Cell and molecular aspects. Cambridge University Press.

Skerra,A, Pfitzinger,I and Pluckthun,A. 1991. The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique. Biotechnology **9**:273-278.

Smith,GP. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science **228**:1315-1317.

Staskawicz,BJ, Dahlbeck,D and Keen,NT. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv *glyinea* determines race specific incompatibility on *Glycine-max* (L) merr. Proceedings of the National Academy of Sciences USA **81**:6024-6028.

Steele,C, Zhang,S and Shillitoe,EJ. 1994. Effect of different antibiotics on efficiency of transformation of bacteria by electroporation. Biotechniques **17**:360-365.

Strandberg,L and Enfors,SO. 1991. Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. Applied And Environmental Microbiology **57**:1669-1674.

Studier,FW and Moffatt,BA. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. Journal Of Molecular Biology **189**:113-130.

Sutherland,R, Buchegger,F, Schreyer,M, Vacca,A and Mach,J-P. 1987. Penetration and binding of radiolabelled anti-carcinoembryogenic antigen monoclonal antibodies and their antigen binding fragments in human colon multicellular tumour spheroids. Cancer Research **47**:1627-1633.

Tabor,S and Richardson,CC. 1985. A bacteriophage T7 RNA polymerase promoter system for controlled exclusive expression of specific genes. Proceedings of the National Academy of Science USA **82**:1074-1078.

Tang,PM, Foltz,LA, Mahoney,WC and Schueler,PA. 1995. A high affinity digoxin-binding protein displayed on M13 is functionally identical to the native protein. Journal of Biological Chemistry **270**: 7829-7835.

Tang,JL, Gough,CL, Barber,CE, Dow,JM and Daniels,MJ. 1987. Molecular cloning of protease genes from *Xanthomonas campestris* pv *campestris* - expression in *E.coli* and role in pathogenicity. Molecular And General Genetics **210**:443-448.

Taub,R, Gould,RJ, Garsky,VM, Ciccarone,TM, Hoxie,J, Friedman,PA and Shattil,SJ. 1989. A monoclonal antibody against the platelet fibrinogen receptor contains a sequence that mimics a receptor recognition domain in fibrinogen. Journal of Biological Chemistry **264**:259-265.

Tavladoraki,P, Benvenuto,E, Trinca,S, DeMartini,D, Cattaneo,A and Galeffi,P. 1993. Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature **366**:469-472.

Tomlinson,IM, Walter,G, Marks,JD, Llewelyn,MB and Winter,G. 1992. The repertoire of human germline VH sequences reveals about 50 groups of VH segments with different hypervariable loops. Journal of Molecular Biology **227**:776-798.

Tsror,L, Nachmias,A, Barak,Z and Keen,NT. 1991. *Escherichia coli* carrying single pectate lyase genes from the phytopathogenic *Erwinia chrysanthemi* causes disease symptoms in potato. Phytoparasitica **19**:57-63.

Vanengelen,FA, Schouten,A, Molthoff,JW, Roosien,J, Salinas,J, Dirkse,WG, Schots,A, Bakker,J, Gommers,FJ, Jongsma,MA, Bosch,D and Stiekema,WJ. 1994. Coordinate expression of antibody sub-unit genes yields high levels of functional antibodies in roots of transgenic tobacco. Plant Molecular Biology **26**:1701-1710.

Vanrooijen,GJH and Molony,MM. 1995. Plant seed oil bodies as carriers for foreign proteins. Bio-technology **13**:72-77.

Viera,J and Messing,J. 1987. Production of single stranded plasmid DNA. Methods in Enzymology **153**:3-11.

Voss,A, Niersbach,M, Hain,R, Hirsch,HJ, Liao,YC, Kreuzaler,F and Fischer,R. 1995. Reduced virus infectivity in *N.tabacum* secreting a TMV-specific full-size antibody. Molecular Breeding **1**:39-50.

Waldmann,TA. 1991. Monoclonal antibodies in diagnosis and therapy. Science **252**:1657-1662.

Wang,GL, Song,WY, Ruan,DL, Sideris,S and Ronald,PC. 1996. The cloned gene Xa21 confers resistance to multiple *Xanthomonas oryzae* pv *oryzae* isolates in transgenic plants. Molecular Plant-Microbe Interactions **9**:850-855.

Ward,ES, Gussow,D, Griffiths,AD, Jones,PT and Winter,G. 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature **341**:544-546.

Ward,L, and DeBoer,SH. 1987. A rapid procedure for purifying pectate lyase from *Erwinia carotovora* based on substrate affinity. Applied and Environmental Microbiology **53**:1189-1191.

Waterhouse,P, Griffiths,AD, Johnson,KS and Winter,G. 1993. Combinatorial infection and *in vivo* recombination - a strategy for making large phage antibody repertoires. Nucleic Acids Research **21**:2265-2266.

Wels,W, Harwerth,I-M, Zwickl,M, Hardman,N, Groner,B and Hynes,NE. 1992. Construction, bacterial expression nad characterisation of a bi-functional single chain antibody-phosphatase fusion protein targeted to the human ERBB-2 receptor. Biotechnology **10**:1128-1132.

Whitelam,GC and Cockburn,W. 1996. Antibody expression in transgenic plants. Trends In Plant Science **1**:268-272.

Williams,SC and Winter,G. 1993. Cloning and sequencing of human immunoglobulin V-lambda gene segments. European Journal of Immunology **23**:1456-1461.

Winter,G, Griffiths,AD, Hawkins,RE and Hoogenboom,HR. 1994. Making antibodies by phage display technology. Annual Review of Immunology **12**:433-455.

Winter,G and Milstein,C 1991. Man-made antibodies. Nature **349**:293-299.

Wu,GS, Shortt,BJ, Lawrence,EB, Levine,EB, Fitzsimmons,KC and Shah,DM. 1995. Disease resistance conferred by expression of a gene encoding H2O2 generating glucose oxidase in transgenic potato plants. Plant Cell **7**:1357-1368.

Xu,GW and Gross,DC. 1988. Evaluation of the role of syringomycin in plant pathogenesis by using Tn5 mutants of *Pseudomonas syringae* pv *syringae* defective in syringomycin production. Applied and Environmental Microbiology **54**:1345-1353.

Yanisch-Perron,C, Viera,J and Messing,J. 1985. Improved M13 cloning vectors and host strains : nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103.

Zebedee,SL, Barbas,CF, Hom,YL, Caothien,RH, Graff,R, Degraw,J, Pyati,J, Lapolla,R, Burton,DR, Lerner,RA and Thornton,GB. 1992. Human combinatorial antibody libraries to hepatits B surface antigen. Proceedings of the National Academy of Science USA **89**:3175-3179

Zhu,BL, Chen,THH and Li,PH. 1996. Analysis of late blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. Planta **198**:70-77.