UNIVERSITY OF SOUTHAMPTON

FACULTY OF SCIENCE
SCHOOL OF BIOLOGICAL SCIENCES

Doctor of Philosophy

Functional characterisation of the tomato *Cf-2* disease resistance gene

By Rebecca Louise Poole

July 2003

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

SCHOOL OF BIOLOGICAL SCIENCES

Doctor of Philosophy

Functional characterisation of the tomato *Cf-2* disease resistance gene

By Rebecca Louise Poole

The tomato *Cf* genes confer resistance to races of the leaf mould fungus *Cladosporium fulvum* expressing the corresponding avirulence (*Avr*) genes, in a gene-for-gene manner. The Cf proteins are predicted to be predominantly extracellular membrane bound glycoprotein receptors, which recognise fungal *Avr* gene products. The Cf-2 protein contains 38 extracellular-type leucine rich repeats, a single potential transmembrane domain and a 37 amino acid tail, predicted to be cytoplasmically located (Dixon *et al* 1996). Previous studies, on the subcellular location of the Cf proteins, have produced conflicting results. One study using an epitope-tagged version of Cf-9, demonstrated a plasma membrane localisation (Piedras *et al.*, 2000), whilst a second study, using both fusion proteins and epitope-tagging, revealed an endoplasmic reticulum localisation (Benghezal *et al.*, 2000).

To resolve the issue of subcellular localisation, 3xc-Myc epitope-tagged versions of *Cf-2* under the control of its native promoter and the strong constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter were engineered. These were stably expressed in transgenic tomato and tobacco. The majority of c-Myc:Cf-2 was demonstrated to be found in the plasma membrane and was highly glycosylated.

In addition, expression profiling of *Cf-2* and *Cf-9* was performed using promoter:GUS fusions in transgenic tomato. Both *Cf-2* and *Cf-9* were developmentally regulated showing little or no expression in very young seedlings, with expression levels increasing dramatically with the emergence of the first true leaves. Cf-9 expression was, also, dramatically induced in true leaves when exposed to crude preparations containing *C. fulvum* avirulence products.

Acknowledgements

I would first like to thank my supervisors Dr. Mark Dixon and Dr. Mark Barber for their continued help and support throughout this Ph.D. I would like to extend further thanks to Mark Dixon for providing me with laboratory training and imparting me with the wisdom of his 'Ole Granny'.

I would also like to thank members of Prof. J. Jones' lab at the John Innes Centre, Norwich. In particular, Mathew Smoker for help with tomato transformations and Miguel Torres for donating the promoter: GUS fusion lines.

Thanks also go to Prof. M. Maeshima of Nagoya University, Japan and Prof. M. Chrispeels of the University of California for the kind donations of antibodies.

Finally, I would like to thank members of the Plant labs at the University of Southampton for their friendship and support, my family and Paul Cripps whose support has been a great deal more than simply financial!

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

ACC: 1-aminocyclopropane-1-carboxylic acid

AEBSF: 4-(2-aminoethyl)-benzenesulfonyl fluoride

AOS: Active oxygen species

APS: Ammonium persulphate

ATP: Adenosine triphosphate

ATPase: Adenosine triphosphatase

Avr: Avirulence gene (*itlalics)*/ protein (normal)

BAP: 6-benzylaminopurine

BiP: Binding protein

BSA: Bovine serum albumin

CaMV: Cauliflower mosaic virus

CC: Coiled-coil

CDPK: Calcium dependent protein kinase

CLV: CLAVATA

CTAB: Cetyltrimethylammonium Bromide

DIG: Digoxygenin

DMF: Di-methylformamide

DNA: Deoxy-ribonucleic acid

ECP: Extracellular protein

EDTA: Ethylene Diamine Tetra-acetic Acid

EGTA: Ethylene glycolbis N,N-tetraacetic acid

ER: Endoplasmic reticulum

EREBP: Ethylene-responsive element binding protein

GFP: Green fluorescent protein

GST: Glutathione-S-transferase

GUS: β-D-Glucuronidase

HA: Haemaglutenin

HABS: High affinity binding site

HB: Hepatitis B

HBV: Hepatitis B virus

HR: Hypersensitive response

Hcr: Homologues of *Cf* resistance

IAA: Indole acetic acid.

IPTG: Isopropyl β-D-Thiogalactopyranosode

IF: Intercellular fluid

$L_{1/2/3}$: Lower phase 1/2/3

LABS: Low affinity binding site

LRD: Leucine-rich domain

LRR: Leucine-rich repeat

eLRR: Extracellular type LRR

MAP: Mitogen activated protein

MAPK: Mitogen activated protein kinase

MEN: Mops, EDTA, sodium acetate buffer.

MM: Money Maker

MOPS: 3-Morpholinopropanesulphonic acid

MS: Murashige and Skoog

MUG: 4-methylumbelliferyl-β-D glucuronide

MW: Milky Way

NAA: 1-naphtalene acetic acid

NBS: Nucleotide binding site

NIL: Near isogenic line

NL: Northern Lights

OD: Optical Density

ORF: Open reading frame

pBS: BluesScript

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PDZ: PSD-95, Discs large, ZO1

PG: Petit Gerard

PGIP: Polygalacturonase-inhibiting protein

PKC: Protein kinase C

PM: Plasma membrane

PR: Pathogenesis related

Prf: Pseudomonas resistance and fenthion sensitivity

PRI: Porcine ribonuclease inhibitor

PVDF: Poly-vinylidine-difluoride

PVX: Potato virus X

R: Resistance gene (italics)/ protein (normal)

RCF: Relative centrifugal force

Rcr: Required for *C. fulvum* resistance

RLK: Receptor-like kinase

RNA: Ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

S1/2/3: Arbitrary stages of cotyledon growth

T-DNA: Transfer DNA

TL: True leaves

SA: salicylic acid

SAP: Shrimp alkaline phophatase

SAR: Systemic aquired resistance

SAIK: Salicylic acid induced protein kinase

SC: Southern Cross

SDS: Sodium dodecyl sulphate

SSC: Saline-sodium citrate

T_1: Primary transformants

T_2: Second generation transformant

TAE: Tris/acetate/EDTA

TBS: Tris-buffered saline

TBST: Tris-buffered saline tween 20

TEMED: Tetramethylethylenediamine

TIR: Toll interleukine 1 receptor

TMV: Tobacco mosaic virus

$U_{1/2/3}$: Upper phase 1/2/3

VIGS: Virus induced gene silencing

Vir: Virulence gene (*italics*)/ protein (normal)

WIPK: wound induced protein kinase

X-Gal: 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

X-Gluc: 5-Bromo-4-chloro-3-indolyl β-D-glucoronide

Chapter one

1 General introduction

1.1 Introduction

Plants often come under attack from pathogens, but the majority remain resistant to disease. There are several levels of resistance e.g. general, non-host, host, varietal and race resistance. This thesis is concerned with race/cultivar resistance, in particular the interaction between tomato and the leaf mould pathogen *Cladosporium fulvum*. Fungi can be separated into groups, the first are the biotrophic fungi, which require a living host and cannot be grown in culture. The second group are the necrotrophic pathogens that live on dead host tissue and can be grown in cultures. *C. fulvum* is a facultative biotroph, it grows on living plant material, but unlike a true biotrophic fungus can be grown in culture. Resistance, in race/cultivar interaction, is inherited as single dominant or semi-dominant resistance (*R*) genes.

Pathogens unable to colonise a particular host are referred to as avirulent, whilst those able to colonise a host are termed virulent. Both virulent and avirulent pathogens secrete many proteins, some of which will enable them to infect the host. For example, they can aid penetration into the host, block plant defences or promote nutrient release from the host. These proteins are then potential targets for the host to prevent colonisation by the pathogen. Depending upon the type of pathogen protein and its mode of action the plant can either inactivate the protein/enzyme directly, as with the interaction between *Cochliobolus carbonum* and maize (described below) (Johal and Briggs, 1992) or monitor for their presence to elicit a defence response upon their recognition as seen with tomato and its pathogen *Pseudomonas syringae* pv. Tomato (Martin *et al.*, 1993). Plant genes responsible for this inactivation or detection are the, above-mentioned, *R* genes. The genes encoding these inactivated or detected proteins are termed avirulence (*Avr*) genes.

Resistance genes fall in to two categories dependent upon their mode of action, the first tends to provide protection against necrotrophic pathogens. These *R* genes often encode enzymes that detoxify a pathogen toxin or encode proteins resistant to the toxins

produced by the pathogens. An example of such an *R* gene is *Hm1* from maize, which detoxifies the HC-toxin from the necrotroph leaf spot fungus *C. carbonum* (Johal and Briggs, 1992). Detoxification of the HC-toxin protects maize from infection by this pathogen. Table 1.1 illustrates the genetics that underpins this group of race/cultivar-specific *R* genes and the corresponding pathogen genes using the *Hm1* gene and HC-toxin as an example.

Table 1.1: An example of genetic interactions between the first class of plant *R* genes and the corresponding pathogen genes.

Lower case letters indicate the absence of a gene. I indicates an incompatible host/pathogen interaction i.e. the pathogen is unable to infect the host. C indicates a compatible interaction, i.e. the pathogen is able to colonise the host. Using the maize *Cochliobolus carbonum* as an example it can be seen that for infection to ensue the pathogen must express its gene (in this case to produce the HC-toxin) in the absence of the plant *R* gene (*Hm1*).

		Host	
		HM1	***hm1***
Pathogen	**HC-toxin**	I	C
	hc-toxin	I	I

The second category of *R* genes is the gene-for-gene *R* genes and provide protection against biotrophic pathogens. These *R* genes are responsible for the recognition of secreted pathogen proteins, which upon the recognition event result in the induction of plant defences. The pathogen genes that encode the proteins detected by the host are called avirulence (*Avr*) genes. Table 1.2 illustrates the genetics behind the gene-for-gene host-pathogen interaction.

Table1.2: Genetic interactions of gene-for-gene host resistance (*R*) genes and the corresponding pathogen avirulence (*Avr*) genes.

Lower case letters indicate an absence of the gene. I indicates an incompatible host/pathogen interaction, i.e. the pathogen in unable to infect the host. C indicates a compatible interaction, i.e. the pathogen is able to colonise the host.

		Host	
		R	***r***
Pathogen	***AVR***	I	C
	avr	C	C

1.1.1 Gene-for-gene concept

The co-existence of plants with their pathogens suggests their evolution, with respect to pathogen virulence and plant resistance, is interrelated (Leonard 1994). The gene-for-gene concept encompasses this observation and provides a genetic basis for it.

The discovery of the genetic basis behind the resistance of flax against the flax rust *Melampsora lini* lead Flor to propose the gene-for-gene concept in the 1950's (Flor, 1971). The gene-for-gene concept has since been shown to operate in many plant-pathogen systems (Agrios, 1997). The gene-for-gene concept states that for each host gene that confers resistance (*R* gene) there is a complementary pathogen avirulence (*Avr*) gene. A simple receptor model can be employed to illustrate the theory behind the gene-for-gene concept (Figure 1.1). In this model the *R* genes encode receptors that recognise the *Avr* gene products. This recognition event then, in turn, initiates a cascade of signalling events that results in an incompatible interaction. Table 1.1 illustrates how this interaction operates. This is a very simplified version of the actual events leading to an incompatible interaction as often many genes are involved.

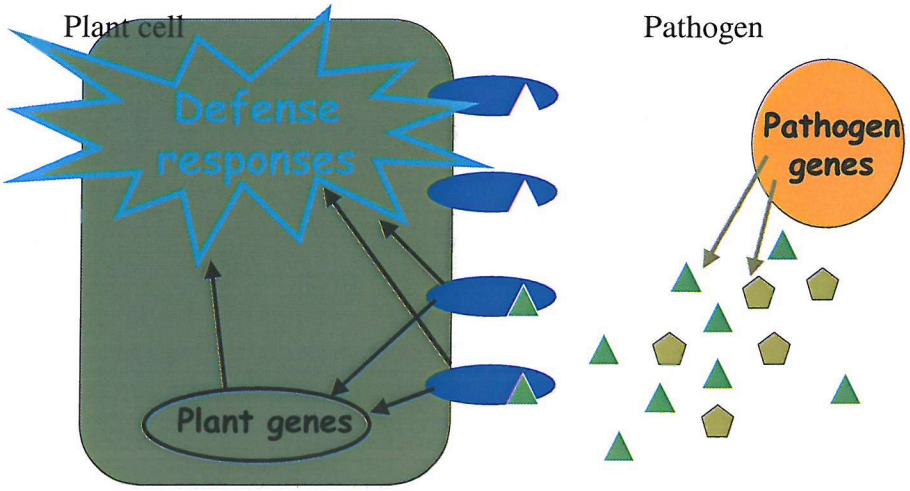


Figure 1.1: The receptor model of the gene-for-gene hypothesis.

The gene-for-gene hypothesis suggests a receptor model where *R* genes encode receptors (blue) that can either directly or indirectly recognise specific pathogen-derived molecules (green triangles). Upon the recognition event a defence response is induced. A pathogen that lacks the specifically recognised molecules is able to avoid detection and colonise the host. Similarly, a plant lacking the R protein is unable to detect the invading pathogen resulting in an infection.

1.2 Plant disease resistance genes

The three predicted properties of *R* genes are: their encoded products should recognise *Avr*-gene-dependent ligands; this recognition event should subsequently initiate a defence cascade; finally they should rapidly evolve novel recognition capabilities (Hammond-Kosack and Jones, 1997).

Plant disease resistance genes fall into two major groups; those that encode proteins with an extracytoplasmic domain and those that encode cytoplasmic proteins. The two groups can be further sub-divided (Figure1.2).

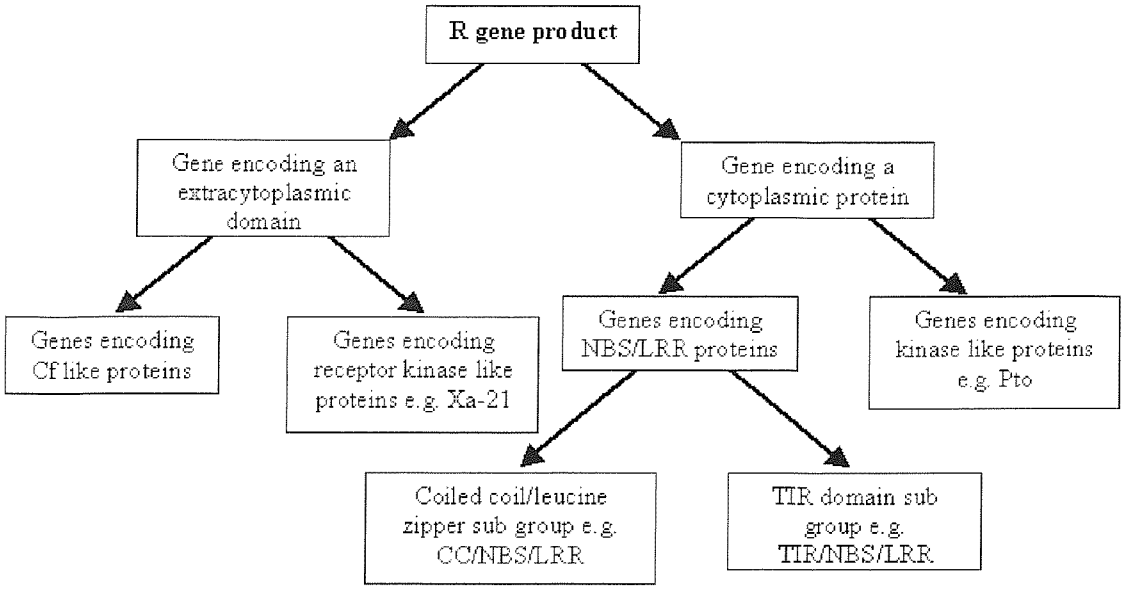


Figure 1.2: The groups and sub-groups of plant disease resistance gene products.

The *R* genes can be divided into two major groups, those encoding cytplasmic proteins and those encoding proteins with an extracytoplasmic domain. Each group can be further divided according to domains present that share homology with other proteins. TIR- abbreviation for Toll-Interleukin 1 Resistance protein.

1.2.1 *R* genes predicted to encode cytoplasmic proteins

R genes of this genre encode the nucleotide binding site (NBS)/leucine-rich repeat (LRR) (NBS/LRR) proteins. The majority of the *R* genes cloned to date fall into this category and confer resistance against a wide range of pathogens including bacteria, fungi, aphids, viruses and nematodes. These *R* genes are further subdivided on the basis of their N-terminus. The first subgroup has a leucine zipper motif at its N-terminus and the second has a motif that shares similarity with the cytoplasmic domains of the *Drosophilla* Toll and mammalian Interleukin 1 transmembrane receptors (TIR domain).

One of the first *R* genes of this group to be cloned was *RPS2* of *Arabidopsis* which confers resistance to *Pseudomonas syringae* pv. *tomato*. *RPS2* encodes a protein with a hydrophobic N-terminus, which is predicted to serve as a membrane anchor. *RPS2* also encodes a putative leucine zipper, a NBS, an internal hydrophobic domain, and 14 imperfect LRRs (Bent *et al.*,1994). A second example, again from *Arabidopsis,* is *RPM1* (for resistance against *P.s.*pv *maculicola*, which appears to share significant homology

with *RPS2*, except it lacks the N terminal hydrophobic domain (Grant *et al.*, 1995). This class of proteins are now referred to as CC-NBS (coiled-coil-NBS).

The leucine zipper comprises a series of amino acids, which form an α-helix with every 7th amino acid being a leucine. Therefore, every 2nd turn there is a leucine residue that sticks out and interacts with other leucines on a similar antiparallel α-helix. Two interacting leucine zippers can form both hetero- and homodimers.

The NBS domain is found in many ATP and GTP binding proteins, e.g. Apaf-1 of mammals which binds adenine nucleotides and plays a regulatory role in signalling of apoptosis (Zou *et al.*, 1997). Although there is no physical evidence to date of RPS2 binding to either ATP of GTP, it appears likely that it that is is a nucleotide binding protein (Tameling *et al.*, 2002). Tameling *et al.* (2002), using *Escherichia coli* expression of the NBS domain of the tomato R proteins I-2 and Mi-1, demonstrated exclusive binding of adenine nucleotides and nucleotidase activity. Due to the conserved nature of the NBS, not only between plant R proteins but also between R proteins and animal proteins with known nucleotidase activity, Tameling *et al.* (2002) postulated that the NBS/LRRs indeed have the ability to bind ATP and GTP. The NBS motif is frequently found in protein kinases however; these R proteins are not kinases themselves, although they may activate kinases (Jones and Jones, 1997).

The second subgroup of the NBS/LRRs is that of the TIR proteins, an example of which is the *N. glutinosa N* gene that confers resistance against tobacco mosaic virus (TMV) (Dinesh-Kumar *et al.*, 1995). Interestingly this group of R proteins appears to be entirely absent from monocot species (Meyers, 1999). This *R* gene is similar to *RPM1* and *RPS2*, but the N-terminus shows similarity to the cytoplasmic domains of the *Drosophilla* Toll and mammalian Interleukin-1 transmembrane receptors, suggesting that the N-terminus may play a role in signalling and not ligand recognition. Genes within this family seem to produce both full length and truncated transcripts, both of which appear to be required for full resistance (Jones and Jones, 1997). This is indeed the case with the *N* gene. One *N* gene transcript encodes a NBS followed by 16 LRRs, whilst the second encodes a truncated form of the first transcript with most of the LRRs replaced with a

novel sequence of 36 amino acids. It is postulated that this truncated protein is a negative inhibitor that controls the induced resistance response (Dinesh-Kumar *et al.*, 1995).

All of the *Arabidopsis R* genes isolated to date encode NBS/LRRs, using probes for conserved sequences in the NBS/LRR motif more than 100 homologs have been identified in *A. thaliana* (Botella *et al.*, 1997), sequencing of the *Arabidopsis* genome has revealed many more. The *RPW8* locus of *A. thaliana*, that confers resistance to the powdery mildew pathogen *Erisyphe cichoracearum*, segregates from the mapped NBS/LRR *R* gene homolog on chromosome 3 (Xiao *et al.*, 2001). This suggested that a different type of *R* gene was likely to be present at this locus. Indeed two functional *R* genes were isolated, *RPW8.1* and *RPW8.2*, which encode proteins with no significant homology to any previously characterised proteins, although they share similarity with a NBS/LRR resistance-like protein (Xiao *et al.*, 2001). They are predicted to encode a N-terminal transmembrane domain (or possibly signal peptide) and a coiled-coil (CC) domain (Xiao *et al.*, 2001). To distinguish these further from previously characterised *R* genes, *RPW8.1* and *RPW8.2* confer resistance to a wide range of pathogens. They conferred resistance to all of the Powdery mildew isolates tested, which included 15 isolates of *E. cichoracearum* and isolates from three additional species (*E. cruciferum* isolate UEA1, *E. orontii* isolate MGH and *Oidium lycopersici* isolate Oxford) (Xiao *et al.*, 2001). It seems that *RPW8.1* and *RPW8.2* do not function in a gene-for-gene manner, although it is possible that each of the pathogens tested express a corresponding *Avr* gene, which are all recognised by the genes at the RPW8 locus. Another possibility is that RPW8.1 and RPW8.2 are the virulence targets of the Avrs and that they are 'guarded' by *R* genes yet to be identified, analogous to the Pto, avrPto guard hypothesis discussed next (Van der Biezen and Jones, 1998).

The second group of *R* genes encoding cytoplasmic proteins encode cytoplasmic protein kinases. To date only one example has been found, *Pto* of tomato, which encodes a serine threonine kinase. Although recently there has been some suggestion that *Pto* is not a 'true' *R* gene. In 1993, Martin *et al.* cloned the *Pto* gene, which confers resistance to *Pseudomonas syringae* pv, *tomato* that carries the avirulence gene *AvrPto*. *R* genes are believed to encode molecular receptors, however *Pto* contains no obvious receptor domain. Interestingly, using a yeast-2-hybrid system (Fields and Sternglanz, 1994) it was

shown that Pto directly interacts with AvrPto (Scofield *et al.*, 1996). A second locus shown to be involved in resistance of tomato to *P. syringae* has also been identified. The gene involved was named *Prf* (*Pseudomonas* resistance and fenthion sensitivity) (Salmeron *et al.*, 1994). *Prf* encodes a NBS/LRR protein with a leucine zipper (Salmeron *et al.*, 1996), but as yet has not been shown to interact with Pto, or AvrPto. Overexpression of *Prf* appears to lead to the activation of plant defences and the establishment of systemic acquired resistance (SAR). It has been observed that such overexpression gives rise to enhanced resistance to a number of normally virulent bacterial and viral pathogens (Oldroyd and Staskawics, 1998).

Other proteins that interact with *Pto* have been identified, again by using the yeast-2-hybrid system. The first is Pti1 (Pto interacting 1) (Zhou *et al.*, 1995) which is a protein kinase, phosphorylated by Pto but unable itself to phosphorylate Pto and may be important in signalling for the hypersensitive response. Pti 4, 5 and 6 share some characteristics with transcription factors and are similar to the tobacco ethylene-responsive element-binding proteins (EREBPs) (Zhou *et al.*, 1997). Zhou *et al.* (1997) showed that Pti4/5/6 bind to a DNA sequence that is present in the promoter region of a number of genes encoding pathogenesis-related (PR) proteins. They demonstrated that expression of several PR proteins along with a tobacco EREBP gene is enhanced by the recognition of the Pto-AvrPto complex in tobacco, this was confirmed by Thara *et al.* (1999). It was however demonstrated that the expression of both *Pti4* and 5, unlike the *EREBP* gene, act through a pathway that is independent of salicylic acid, ethylene and jasmonic acid (Thara *et al.*, 1999). These observations reveal, in part, a pathway from the disease resistance gene to the activation of plant defence genes.

Figure 1.3 shows how the genes involved in defence against *P.syringae* were believed to interact. It was thought that the resistance to *P. syringae* was due to a cascade of phosphorylation events initiated by the recognition of AvrPto by Pto, with the role of Prf unknown.

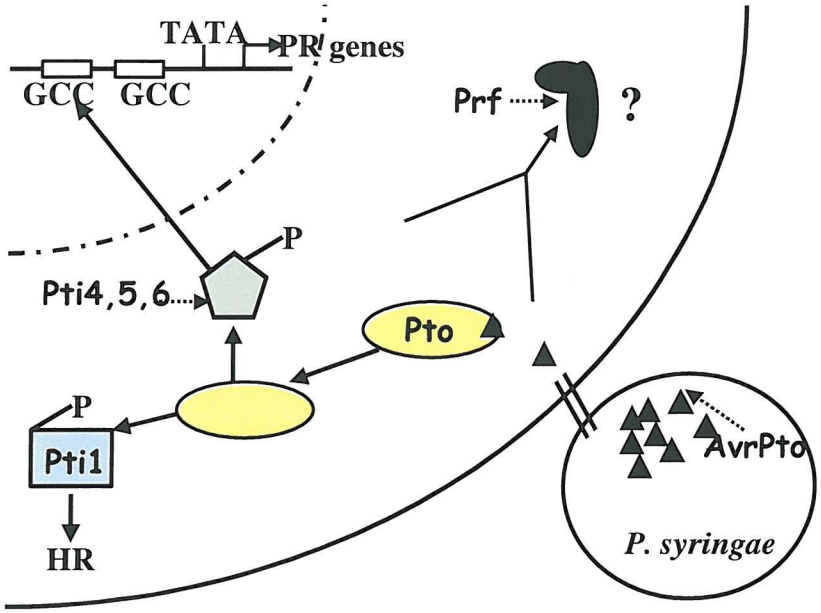


Figure 1.3: The original model of how Pto, Ptis and Prf were believed to mediate a defence response in tomatoes.

AvrPto was originally believed to initiate the defense response through an additional unkown protein that activated the Ptis which inturn activated the defence responses. The precise role of Prf was unclear. This model has more recently been revised.

Van der Biezen and Jones (1998) proposed an alternative model, termed the 'guard' hypothesis, to explain the mechanism that underpins the reistance against *P. syringae* pv. *tomato* conferred by *Prf* and *Pto* (Figure 1.4).

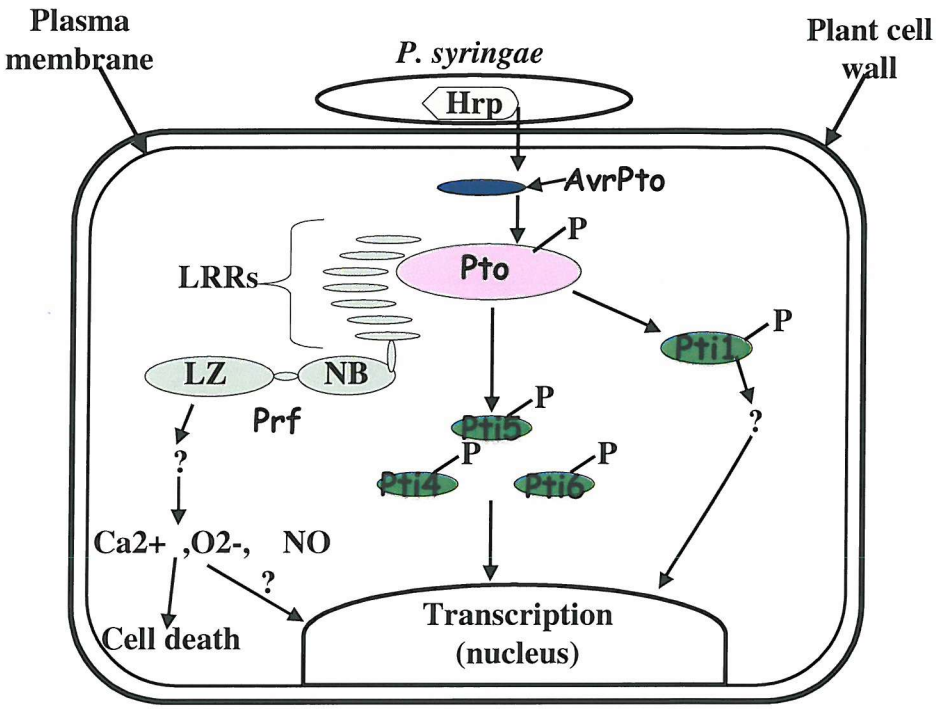


Figure 1.4: The guard hypothesis, adapted from van der Beizen and Jones (1998).

The guard hypothesis assumes the virulence target for AvrPto is Pto. Pto was at some point perhaps involved in a general defence response, which AvrPto tried to block to enable colonisation of the host. It is postulated that Prf evolved to recognise the AvrPto-Pto complex or a product of their interaction. Upon recognition of the complex a defence response ensues involving the Ptis. The Prf protein is therefore present in the cell at all times monitoring for the presence of the pathogen and in essence 'guarding' the host from attack. The exact order of events involving the Ptis still remains unclear.

Knowing that the interaction between Pto and the Ptis, and phosphorylation of Pti1 does not require AvrPto, it was proposed that the binding of AvrPto to Pto was a step taken by the pathogen to prevent it interacting with the Ptis and thus abolishes the resistance conferred by Pto. Pto could therefore be considered as the virulence target of AvrPto. It is proposed that Prf subsequently evolved to recognise the AvrPto-Pto complex and initiate the defence response. This model predicts that a recognition complex has evolved from a pathogenicity complex.

1.2.2 *R* genes predicted to encode proteins with extracytoplasmic domains

As illustrated in figure 1.2, there are two divisions to this group. The first comprises only one gene (*Xa21*) that has been cloned to date. *Xa21* confers resistance to

race 6 of the bacterium *Xanthomonas oryzae* pv. *Oryzae* that causes leaf blight in rice. Xa21 can be described as a LRR receptor-like kinase protein (LRR-RLK). This protein contains a putative signal peptide, 23 extracellular type LRRs, numerous potential N-linked glycosylation sites, a transmembrane domain and a cytoplasmically located serine/threonine protein kinase (RLK domain) (Song *et al.*,1995). The functions of other serine/threonine proteins with which Xa21 shares overall homology have not yet been determined. Xa21 can be viewed to combine the LRR feature with a kinase domain, like a combination of the Cf proteins (discussed below) and the *Pto* gene product.

Characteristic of the second group of extracellular proteins are the *Cf* genes of tomato. The *Cf* genes confer resistance to the leaf mould fungus *Cladosporium fulvum* and are predicted to encode glycoproteins that have a membrane anchor and extracytoplasmic LRRs, the consensus of which is LxxLxxLxxLxLxxNxLxGxIPxx (Jones and Jones, 1997). The membrane anchor consists of a region of acidic amino acids, which are extracytoplasmic, followed by a transmembrane domain of hydrophobic amino acids and a short basic C-terminus situated in the cytoplasm.

With the exception of *Pto* and *Hm1*, all of the plant *R* genes isolated to date encode proteins with a region of leucine-rich repeats (LRRs) (Jones and Jones, 1997). It is within the LRR encoding region of the *R* genes that the majority of sequence variation between homologues is found (Meyers, 1998). Understanding LRRs is the key to understanding many current hypothesies regarding R, and more specifically Cf, protein function. It is partly due to this observation that the LRR region of R proteins is thought to be the binding site of the Avr protein. Many LRR containing proteins are involved in specific protein-protein interactions lending support to the belief that the LRRs of R proteins are responsible for the recognition of Avr proteins (Jones and Jones, 1997). Despite efforts, there are at present, few examples of plant R and pathogen Avr proteins interacting physically. One example of a plant R protein physically interacting with a fungal Avr protein is the rice Pi-ta protein and the fungal Avr-Pita protein (Jia *et al.*, 2000). *Pi-ta* confers resistance against the rice blast fungus, *Magnaporthe grisea*, expressing *Avr-Pita* in a typical gene-for-gene manner. *Pi-ta* encodes a cytoplasmic protein that comprises of a centrally localised nucleotide binding site and a C-terminal leucine rich domain (LRD). Although it contains regions with similarity to the plant LRR motif, Pi-ta does not contain any LRRs that fit any previously described LRR concensus motifs (Jia *et al.*, 2000; Jones

et al., 1996). Using the yeast-two-hybrid system Jia *et al.* (2000), demonstrated binding between Pi-ta and Avr-Pita occurs via the LRD. This interaction occurs through the region that most closely resembles a LRR domain, suggesting that, as predicted, LRRs may be important for ligand binding.

1.2.3 Structural features of leucine-rich repeats

The leucine-rich repeat (LRR) protein superfamily can be divided into many subfamilies (Kajava, 1998) (Table 1.3 and Figure 1.5 show several of these). The LRRs of various proteins differ both in consensus sequence and length, and it is these differences that characterise each of the subfamilies. Within the different consensus sequences there are conserved residues so the subfamilies retain a similar superhelical fold. However, they differ in the three dimensional structures of individual repeats. Each type of LRR is mutually exclusive, so no two types can ever be found in one protein (Kajava, 1998). A specific network of hydrogen bonds is formed between neighbouring LRRs that could not occur between neighbours of a different subfamily. This observation implies that for one subfamily to be transformed into another requires the simultaneous mutation in several residues, which led to the conclusion of independent evolution of each of the LRR subfamilies (Kajava, 1998).

One subfamily consists of the porcine ribonuclease inhibitor (PRI)-like LRRs. The consensus sequence length is 28 or 29 amino acids and exclusively occur in intracellular proteins. The structure of the PRI protein was determined by Kobe and Diesenhofer (1994) and was believed to be a suitable model for all LRR proteins. PRI was shown to be a non-globular horseshoe structure. It was noted that proteins that contain a large number of LRRs (more than 21), would have to have a different tertiary structure to prevent collision of the termini. The LRR region of the PRI corresponds to α-β structural units, arranged to form parallel β-sheets with one surface solvent exposed (Figure 1.5). These solvent exposed residues are predicted to be the residues involved in protein binding (Kobe and Deisenhofer, 1994).

The shortest known LRRs are 20 residues long and some of these proteins consist entirely of the repeated 20-residue motif with no other domain (Kajava, 1998). These are

all extracellular proteins and originate from Gram negative bacterial and appear to be essential for virulence in the early stages of infection.

The cysteine containing, coiled coil (CC), LRRs are also found only in intracellular proteins. The CC LRRs have a characteristic 26-residue consensus (Kajava, 1998). These LRRs share sequence similarities with the PRI subfamily and are found in humans, yeast and plants. One plant example is RSP2, which is a plant R protein (Bent *et al.*, 1994).

Another subfamily of interest is the plant-specific LRRs, these are 24 residues in length, and are similar to the 'typical' 24-residue LRR (Kajava, 1998). These LRRs are extracellular domains of multidomain membrane proteins, most of which mediate plant resistance to pathogen attack (Jones and Jones, 1997). The plant R protein modelled by Kajava (1998) is *Cf2.1* of *Lycopersicon pimpinellifolium* (Dixon *et al.*, 1996). This is modelled on the typical subfamily LRRs and differs from the typical LRR in a region that corresponds to a half-turn which follows the conserved "β structure + Asn ladder" region (Kajava, 1998) (Figure 1.5). Like the PRI it is thought that the solvent exposed residues of *Cf2.1* are involved in protein binding (Kobe and Diesenhofer, 1994).

LRR subfamily	Length residues	Consensus sequence	origin	location
Typical	24	LxxLxxLxLxxNxLxxLpxxoFxx	Animal, fungi	Extracellular
PRI-like	28-29	XxxLxxLxLxxN/CxLxxxgoxxLxxoLxx	Animals	Intracellular
Coiled-Coil	26	C/LxxLxxLxLxxCxxITDxxoxxLagxx	Animals, plants, fungi	Intracellular
Bacterial	20	PxxLxxLxVxxNxLxxLPD/EL	Gram negative bacteria	Extracellular
Plant Specific	24	LxxLxxLxLxxNxLt/sGxIPxxLGx	Plants, primitive eukaryotes	Extracellular

Table 1.3: Consensus sequences of LRRs (adapted from Kajava, 1998).

Bold and uppercase letters indicate more than 70% and 40% occurrence of a specific residue. Lower case letter indicate more than 30% identity, o indicates a non-polar residue and x any residue.

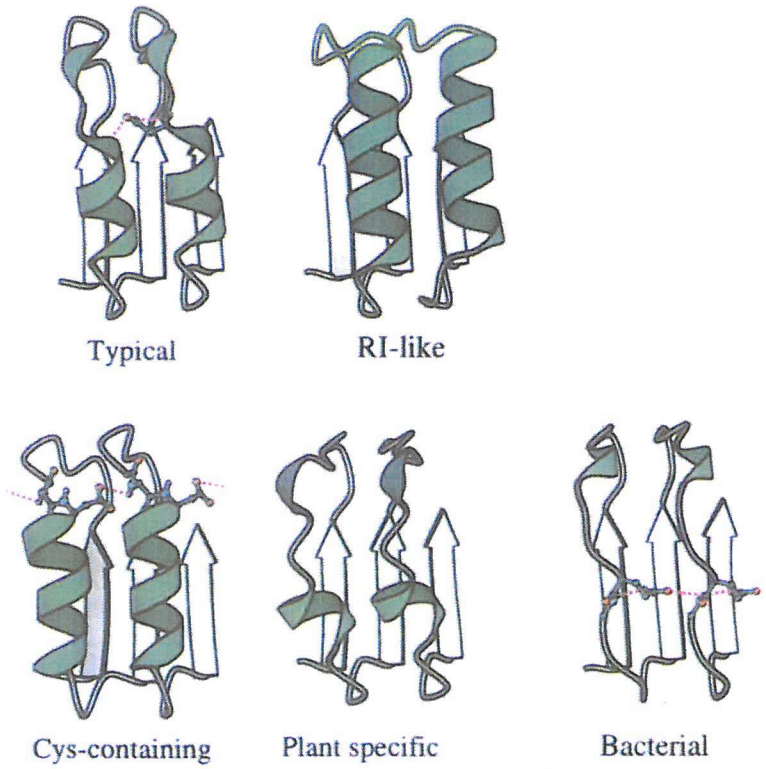


Figure 1.5: Differences, in structure, between the variable regions of the LRR subfamilies (adapted from Kajava, 1998).

Each of the LRRs are represented by 3 β strands (in white) and two variable regions. The α-helices are in green. Broken red lines denote hydrogen bonds. Carbon atoms and sticks are in dark green, nitrogen in blue and oxygen in red. (adapted from Kajava, 1998)

The previous modelling studies have all used animal proteins to predict the LRR structure of plant proteins. Recently, Di Matteo *et al.* (2003) determined the crystal structure of a polygalacturonase-inhibiting protein (PGIP), a member of the leucine-rich repeat protein family, from *Phaseolus vulgaris*. The PGIPs are plant cell wall proteins that protect plants from fungal invasion. Cf-9 was described as sharing homology with the PGIPs and that this was mailnly due to the similarity in the LRR region (Jones *et al.*, 1994), thus the structure is a more suitable model for plant proteins comprising of extracellular type LRRs (eLRRs). The overall structure was similar to that described by Kajava (1998), with a curved, elongated shape (Figure 1.6). One major difference with previous models was the presence of a second β-sheet, rather than the β-sheets and helices being connected by loops or β-turns (Di Matteo *et al.*, 2003). The second β-sheet could

represent a second ligand-binding surface, potentially involved in signal transduction. The first β-sheet (B1) consists of three strands on the N-terminal portion and seven strands on the C-terminal portion. This contains the residues that determine affinity and specificity of PGIP2 and form the concave inner side of the structure, almost parallel to B1 are nine helices (Di Matteo *et al.*, 2003). The LRR core is capped by disulphide bridges at both the C-terminus and N-terminus of the repeat region. Similarly, cysteine residues, capable of forming disulphide bridges are present at either end of the LRR region of the Cf proteins.

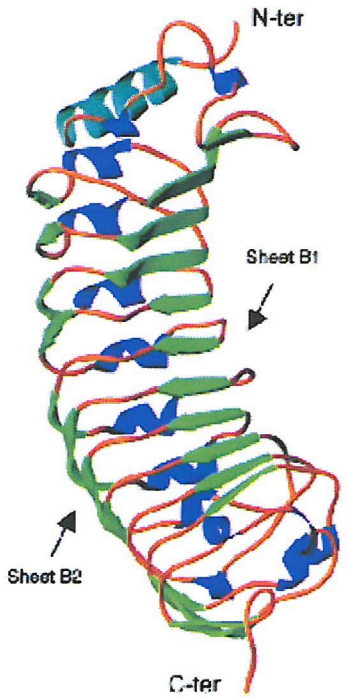


Figure 1.6 The Structure of PGIP2 from P. Vulgaris

β-Sheets 1 and 2 are coloured green, with helices coloured blue. The N-terminal α-helix is coloured light blue (adapted from Di Matteo *et al.*, 2003)

1.3 The tomato-*Cladosporium fulvum* interaction.

Cladosporium fulvum is a biotrophic fungus that causes the tomato leaf mould disease, the tomato *Cf* genes confer resistance against this pathogen. The first four *Cf* genes to be isolated were *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* (Dixon *et al.*, 1996; Dixon *et al.*, 1998; Jones *et al.*, 1994; Thomas *et al.*, 1997). Plants carrying these *Cf* genes specifically recognise races of *C. fulvum* carrying *Avr2 Avr4*, *Avr5* or *Avr9* respectively. The *Cf* and *Avr* genes interact in a typical gene-for-gene manner.

Races of *C. fulvum* are named according to the *Cf* gene they can overcome i.e. Race 2, lacks Avr2, whilst race 2,4 lacks both Avr2 and Avr4.

1.3.1 Genomic organisation of *Cf* genes

Transposon tagging was used to clone *Cf-9*, which involved a screen that identified plants that had lost the ability to recognise Avr9 (Jones *et al.*, 1994). Positional cloning, that involved using cosmid clones that contained a candidate *Cf* gene, was used to identify *Cf-2, 4* and *5* (Dixon *et al.*, 1996; Dixon *et al.*, 1998; Thomas *et al.*, 1997). Each candidate gene was stably transformed into susceptible tomatoes; the transformants were subsequently screened for resistance by pathogen challenge.

The *Cf* genes are found in complex gene clusters of tandemly repeated homologous genes called *Hcr* (homologues of Cf resistance) genes (Thomas *et al.*, 1998). *Cf-2* and *5* are essentially allelic and found on the short arm of chromosome 6, whilst *Cf-4* and *9*, also allelic, are found on the short arm of chromosome 1. *Cf* genes from the same locus encode proteins that are more than 90% identical at the amino acid level (Thomas *et al.*, 1998). One major difference between the Cf proteins is the number of LRRs, which form the putative recognition domain of the protein. Homologues of the *Cf* genes were discovered by analysing Cf near isogenic lines (NILs). The Cf NILs were established by the introgression of completely susceptible (Cf0) tomatoes (*Lycopersicon esculentum*, commercially named Money Maker (MM)) with the *Cf* genes. *Cf-2* and *Cf-9* originate from *L. pimpinellifolium*, *Cf-4* from *L. hirsutum*, and *Cf-5* from *L. esculentum* var. *cerasiforme*.

Two functional *Cf-2* genes (*Cf-2.1* and *Cf-2.2*) have been identified from a Cf2 NIL, and they differ only in 3 amino acids near the C-terminus (Dixon *et al.*, 1996). A third homologue *Hcr2-2A* has also been identified, for which no function has been assigned. The functional *Cf-5* gene (*Hcr2-5C*), is found within a cluster of other *Hcr* genes, *Hcr2-5A, B* and *D*. *Hcr2-5A, B* and *D* all have open reading frames and are probably expressed, although to date no function has been identified for them. It is plausible that analogous to antibodies they are present, anticipating a novel avr protein. A Cf0 plant although completely susceptible to *C. fulvum* challenge also carries two *Hcr2*

genes (*Hcr2-0A* and *Hcr2-0B*) on chromosome 6 and many *Hcr9s* on chromosome 1. There are in total seven homologues at the *Cf-2/Cf-5* locus, of which 6 differ in the number of LRRs ranging from 25 to 38 (Dixon *et al.*, 1998), Figure 1.7 illustrates the homologues found at the *Cf-2/Cf5* locus.

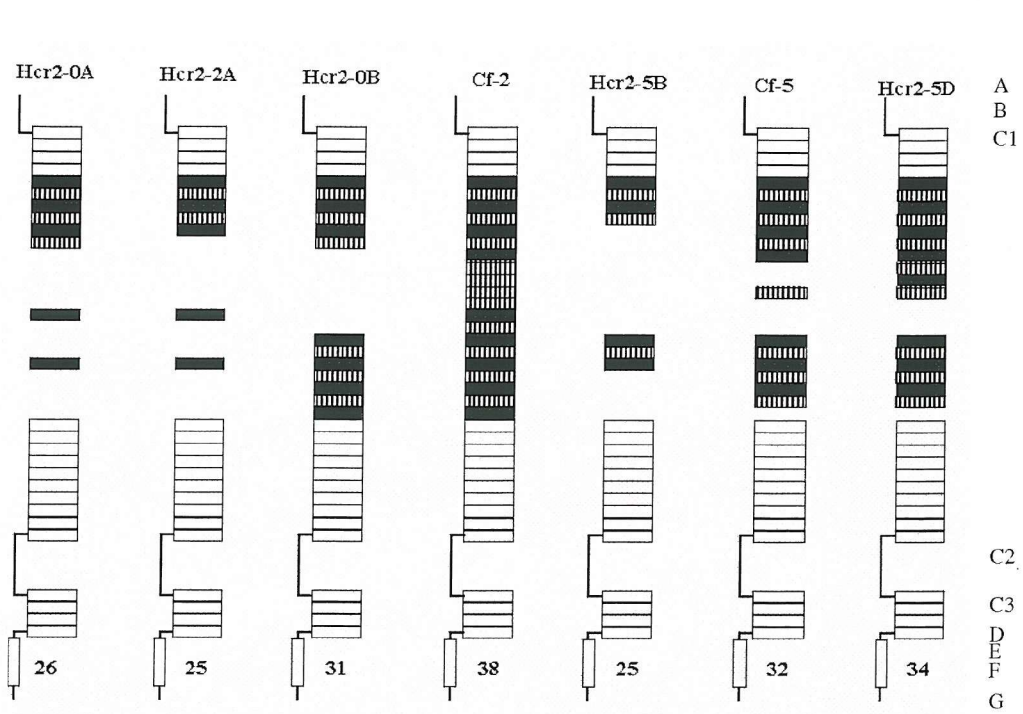


Figure 1.7: Homologues at the Cf-2/Cf-5 complex locus (Dixon *et al.*, 1998).

Schematic representation of the proteins encoded by different Hcr2s. All homologues are aligned to Cf-2. Each horizontal box represents a LRR, the shaded region indicates the hyper-variable region of the proteins. The two shading effects represent the two types (a and b) of alternating LRRs. The vertical box represents the putative transmembrane domain. The Cf proteins are divided into seven functional domains (A-G). Domain A is a putative signal peptide, domains B, C, and D have homology to the polyglacturonase inhibitor proteins (PGIPs) (Jones *et al.*, 1994). Domain C comprises all of the LRRs of the protein. Domain F is the putative transmembrane domain with domains E and G rich in acidic and basic residues respectively, forming the putative membrane anchor and responsible for orientation of the protein in the membrane.

In Cf9 plants (Cf9 NILs) there are five homologues present; *Hcr9-9A* through to *9E*, where *Hcr9-9C* is the functional gene whose product recognises Avr9 (Parniske *et al.*, 1997). In the case of Cf4 plants there are also 5 homologues, *Hcr9-4A* to *4E* where Hcr9-4D is the functional homologue that recognises Avr4 (Thomas *et al.*, 1997).

It was at first thought that *Cf-9* was the only functional gene at that locus, as mutagenesis of Cf-9 produced disease sensitive seedlings (Hammond-Kosack *et al.*, 1994a). However, it was later revealed that additional genes function later in development

(Parniske *et al.*, 1997). Inoculation of Cf9 NILs with race 5,9 of *C. fulvum* (lacking Avr9) when carried out at the eight–leaf stage resulted in an incompatible interaction. This suggests that the introgressed *L. pimpinellifolium* (the species from which *Cf-9* was identified) DNA contains an additional gene(s) that confers resistance. Transformation of MM-Cf0 plants with the *Hcr9*s revealed that either Hcr9-9A or 9B or both, and Hcr9-9E are responsible for the observed resistance (Parniske *et al.*, 1997). Parniske *et al.* (1997) also showed that when transgenic Cf0 plants expressing the *Hcr9s* were inoculated with either race 5,9 or race 4 they exhibited an identical resistant phenotype to race 5. This data reveals that neither *Avr4* nor *Avr9* are required for the induction of resistance, and that in fact there must be additional Avr proteins (e.g. Avr9A or Avr9B, and Avr9E).

Examination of a natural population of *L. pimpinellifolium* for polymorphisms within the *Hcr9s* revealed a second gene, *9DC*, with the ability to specifically recognise Avr9 (van der Hoorn *et al.*, 2001c). *9DC* is more polymorphic than *Cf-9* and is spread more widely throughout the natural population indicating it evolved before *Cf-9*.

A similar observation was made for the *Cf-4* locus using Cf-4 mutants. Despite the disruption of the ability of *Cf-4* gene product to recognise Avr4, the plants were still resistant to infection by *C. fulvum* race 5 (Takken *et al.*, 1999). By transforming Cf0 plants with the individual *Hcr9-4*s it was demonstrated that Hcr9-4E confers resistance to *C. fulvum* race 5, whilst Hcr9-4A, 4B and 4C do not. The transgenic plants expressing *Hcr9-4E* did not respond to Avr4 thus there must be an additional Avr protein that has been designated Avr4E (Takken *et al.*, 1999).

The presence of these two gene clusters gives a clue to the possible evolution and diversification of the genes. Generation of novel recognition specificities is important for long-term resistance against constantly evolving pathogens and hence a predicted property of an *R* gene (Hammond-Kosack and Jones, 1997).

1.3.2 Evolution of the *Cf* genes

It has been proposed that no single *R* gene allele is maintained at a high frequency as selection pressures exerted on a fungus may enable it to overcome the *R* gene (Dixon *et*

al., 2000). As with crops that have all been bred to carry a specific *R* gene, eventually the crop becomes vulnerable to pathogen attack by the very pathogen they were bred to resist (Leonard, 1994). It has been proposed that the observed sequence diversity of the *R* genes can be maintained by frequency dependent selection. It is obvious why the plants need a mechanism to facilitate the evolution of its *R* gene(s) in order to compete with the evolving pathogen. With each new random change in virulence or resistance exerting a selection pressure on the plant and pathogen respectively.

Frequency dependent selection may explain the maintenance of the sequence diversity, but it does not explain the mechanisms by which the changes in the sequences arise. It has been shown that many of the *R* genes, including the *Cf* genes occur in complex multigene families (Dixon *et al.*, 1996; Meyers *et al.*, 1998; Parniske *et al.*, 1997). This may give us some insight into the generation of sequence changes and ultimately the generation of novel Avr recognition specificities. An example of this is that cross over events are more likely between two sequences when the physical distance between them is small. The organisation of the tomato *Cf* genes is a good example of this. Van der Hoorn *et al.*, (2001c) suggest that the variation between Cf proteins with different Avr recognition specificities act as a reservoir of diversity as opposed to being a result of adaptive evolution. This came from the discovery of a second gene (*9DC*), which varies significantly (by 61 amino acids) from Cf-9 and yet has the same recognition specificity.

Most of the *Cf Hcr2*s, like RRP5 and RRP1 homologues of *Arabidopsis*, contain variable numbers of LRRs, suggesting that this is important in the generation of novel recognition specificities (Botella *et al.*, 1998; Dixon *et al.*, 1998; Noel *et al.*, 1999). *Hcr9*s however, contain similar numbers of LRRs, most of the sequence diversity between the *Hcr9s* is observed in the putative solvent exposed β-strand/β-turn motif of the LRR proteins (Parniske *et al.*, 1997). It is possible that the ratio of synonymous to non-synonymous substitutions at the solvent exposed residues is low, promoting variation, as oberserved within the LRR region of genes at the Dm3 locus in lettuce (Meyers *et al.*, 1998).

Another possible mechanism for the generation of sequence diversity is recombination. In the case of *RP1* this is coupled with a tendency for the genes in the

complex to mispair during meiosis (Hulbert, 1997). The *rpp8* locus of *Arabidopsis* of the susceptible Columbia (Col-0) accession contains a single chimeric gene, which also appears to have arisen through an unequal crossover event between linked genes (McDowell *et al.*, 1998).

The absence of introns from the open reading frames (ORFs) of the *Cf* genes means there is greater potential for mis-match alignment during meiosis. In addition, the highly regular nature of the *Hcr2* LRRs (i.e. exactly 24 amino acids in length), further increases the likelihood of mis-alignment. The LRRs of the *Hcr9*s are less regular in length and sequence when compared to the *Hcr2*s and therefore mis-alignment is less likely in the *Hcr9*s. This observation may explain the lack of variation in LRR copy number between the *Hcr9*s relative to the *Hcr2*s.

Comparisons of *Cf-4* and *Cf-9* haplotypes, provided evidence of sequence exchange between the *Hcr9*s, which could have contributed to the generation of novel recognition specificities (Parniske *et al.*, 1997). Parniske and Jones (1999) studied more closely three loci of tandemly repeated *Hcr9* genes; *Southern cross (SC), Milky way (MW)* and *Northern lights (NL).* It was found that the extent of sequence polymorphism within the gene family directly effects the generation of novel recognition specificities by recombination. It was found that high levels of sequence diversity are reflected in the physical separation of the *Hcr9*s. There was however one exception at *Northern lights,* where one *Hcr9* carried specific sequence characteristics for *Hcr9*s at other loci, suggesting that a recent interlocus recombination event had occurred.

There are clearly many mechanisms that operate and result in the generation of novel *R* gene sequences that could result in the generation of novel recognition specificites. This is an important feature of *R* genes, in order for plants to stay in the 'arms race' against pathogens and are discussed later.

1.3.3 *C. fulvum* virulence and avirulence proteins

In order for *C. fulvum* to cause disease on tomato plants you would expect it to produce virulence factors, and loss of one or more would result in partial or complete loss

of ability to colonise the plant. Complete or partial loss of virulence would depend upon whether the factor in question is essential for colonisation or just increases the virulence of the fungus.

Apoplastic washing fluids from tomato leaves infected by *C. fulvum* have been used to isolate various proteins secreted by the fungus. Apoplastic washes can be used as *C. fulvum* is restricted to the apoplast, and as such any plant-pathogen interactions are likely to occur extracellularly.

Extracellular proteins (ECPs) are secreted by all races of the fungus, the first to be purified and to have their corresponding genes isolated were ECP1 and EPC2 (Van den Akerveken *et al.*, 1993). These genes are highly transcribed upon colonisation of tomato leaves, suggesting a role in virulence (Lauge *et al.*, 1998b). However, deleting these genes from the fungal genome did not reduce the disease symptoms on two week old seedlings. However, when six-week-old plants were inoculated with ECP deficient strains, reduction in disease symptoms were observed. ECP2 deficient mutants poorly colonized the plant leaves, and secreted less Avr4 and Avr9. The ECP1 deficient strains colonised the leaves and appeared from the stomata of the lower epidermis, but failed to sporulate as well as the wild type strain (Lauge *et al.*, 1997). Thus both ECP1 and ECP2 can be considered virulence factors, involved, perhaps, in the suppression of host defence responses or nutrieny uptake (Lauge *et al.*, 1997).

ECP2 appears to be the most important of the two virulence factors as the reduction in virulence was greatest when the *ECP2* gene was disrupted. So a screen of tomato plants that responded to ECP2 (*ECP2* was expressed using PVX expression system) with the hypersensitive response was carried out. A single dominant gene was responsible for recognition of ECP2 and was designated *Cf-Ecp2* Lauge *et al.*, 1998a). It is thought that *Cf-ECP2* is a functional *Cf* homologue on chromosome 1, as *Cf-2* homologs segregate with *Cf-ECP2* and it is not found in the *Cf-4/Cf-9* cluster (Haanstra *et al.*, 1999). As ECP2 is important for full virulence of *C. fulvum* there would be a selection pressure for the fungus to maintain it's encoding gene, so it would appear likely that the *Cf-Ecp2* gene would be a durable line of defence and could be of great commercial benefit (Joosten and de Witt 1999).

Three additional ECPs that originate from *C. fulvum* have been identified, ECP3, 4, and 5. They too are secreted in high amounts upon colonisation of tomato leaves. However the role of these proteins as essential virulence (and/or avirulence) factors has yet to be established (Joosten and de Witt, 1999). The evidence to date points to the ECPs of *C. fulvum* as being 'antigenic' to tomatoes as indicated by them all inducing the hypersensitive response on specfic tomato genotypes.

Three of the *C. fulvum* avirulence genes (*Avr2*, *Avr4* and *Avr9*) specifically recognised by the Cf genes have been cloned to date (Joosten *et al.*, 1994; Luderer *et al.*, 2002; Vankan *et al.*, 1991). Avr4 and Avr9 were identified by separation of apoplastic fluid obtained from plant infected with *C. fulvum*. The various fractions were subsequently injected into tomato leaves of Cf4 and Cf9 near isogenic lines (NILs). The fractions that induced the hypersensitive response were identified and the proteins isolated. Avr4 induced the hypersensitive response in Cf4 tomatoes and Avr9 in Cf9 tomatoes (Joosten and de Witt, 1999). Both avirulence proteins are encoded as pre-pro-proteins that contain extracellular signalling sequences, which are subsequently cleaved to form the mature proteins. The Avr4 mature protein consists of between 86 and 88 amino acids whilst the mature Avr9 protein is 28 amino acids in length (Joosten and de Witt, 1999). Confirmation of Avr9 as an avirulence factor was obtained by disrupting the *Avr9* gene in strains of *C. fulvum* that are normally avirulent on *Cf-9* expressing plants. It was found that when the *Avr9* gene was completely deleted the fungus was able to infect the Cf9 plant; this indicates that Avr9 is not essential for vegetative growth or pathogenicity of the fungus (Marmiesse *et al.*, 1993).

A binary potato virus X (PVX)-based cDNA library was produced from race 5 of *C.fulvum* (Takken *et al.*, 2000) and subsequently used to isolate the *Avr2* cDNA. The candidate cDNAs were introduced into *Agrobacterium tumefaciens* to promote uptake of the cDNA by the plant cells, the PVX expression vector then drives expression of the cDNAs. Both Cf2 and Cf9 (as a negative control) plants were inoculated with colonies of *A. tumefaciens* carrying the candidate cDNAs. Colonies that specifically induced the hypersensitive response in the Cf2 but not the Cf9 plants were further analysed. The Avr2 protein isolated, shares no sequence homology with other proteins found in the databases, or other Avr proteins of *C. fulvum*. The cDNA is predicted to encode a protein of 78

amino acids, which includes eight cysteine residues. This is consistent with all other elicitors of *C. fulvum* characterised to date, where an even number of cysteine residues are present (Luderer *et al.*, 2002b).

Cysteine residues are important for the formation of disulphide bridges, such structures may also be important features of these elicitors. Luderer *et al.*, (2002b), using mutational analysis and the PVX expression system, studied the importance of these cysteine residues in the *C. fulvum* elicitors, ECP1, ECP2 and ECP5, carrying eight, four and six cysteine residues, respectively. It was shown that 4 of the ECP5 cysteines are absolutely essential for necrosis inducing activity and/ or stability, probably due to the formation of disulphide bridges. Seven of the eight mutants of ECP1 resulted in complete loss of necrosis-inducing activity and the eigth resulted in reduction in necrosis inducing activity. Only one of the cysteine residues in ECP2 is absolutely required for necrosis-inducing activity. As a pair of cysteine residues is required for disulphide bridges, these results indicate that in each protein, two of the cysteine residues are not involved in intramolecular disulphide bridges. It is possible that these residues form intermolecular disulphide bridges or are involved intermediate structures during protein folding. Disulphide bridges have been shown to occur during the folding of Avr9 (Van den Hooven *et al.*, 1999). Intermolecular disulphide bridges could result in protein complexes, it is also plausible that the cysteine residues are important for perception of the elicitor proteins by the plant, possibilities that are currently being investigated (Luderer *et al.*, 2002b).

The mechanisms by which the Cf proteins are able to recognise the Avr proteins and subsequently elicit a defense response is still to be determined.

1.3.4 Recognition of *C. fulvum* avirulence factors

The *Cf* genes are predicted to encode the receptors for the *C. fulvum* avirulence proteins. Many of the Cf proteins carry different numbers of LRRs, which are believed to form the recognition domain of the proteins. The LRR region is predicted to interact with proteinaceous ligands or other proteins with the LRR motif and is probably the receptor site of secreted elicitors of *C. fulvum* (Joosten and de Witt, 1999). In addition there are specific residues, the solvent exposed residues, which are hyper-variable. It is this

variable region that is predicted to be involved in ligand binding, forming the recognition domain for the Avr proteins (Parniske *et al.*, 1997).

Indeed certain LRRs and specific amino acids of Cf-4 and Cf-9 have been identified, by PCR shuffling and domain swaps, that are essential for specific recognition of Avr4 and Avr9 respectively. Using these methods, *Cf-4/Cf-9* chimeras were produced and transiently expressed in *N. benthamiana* expressing *Avr4* or *Avr9*. The chimeras were assessed for functionality by measuring levels of necrosis (Wulff *et al.*, 2001). Van der Hoorn *et al.* (2001b) employed both domain swaps and mutant constructs for the same means.

LRR number was shown to be essential for function. Only those chimeras containing 29 LRRs (the number of LRRs found in Cf-9) recognised Avr9, but could not recognise Avr4. Similarly the chimeras with two less LRRs (27 LRRs as found in Cf-4) recognised Avr4 but not Avr9 (Wulff *et al.*, 2001; van der Hoorn *et al.*, 2001b).

Sequences in the N-terminal portion of *Cf-4* appear to be important for function. Several solvent exposed amino acids in LRRs 13, 14 and 16 of Cf-4 were shown to be required for full function, implicating the importance of diversifying selection on the solvent exposed residues in the evolution of new recognition specificities (van der Hoorn *et al.*, 2001b). A 10 amino acid deletion within domain B of Cf-4 relative to Cf-9 is also required for full Cf-4 function along with additional sequences in LRRs 11 and 12 (Wulff *et al.*, 2001).

The specificity of Cf-9 resides entirely in the C1 domain (central LRRs), substituting LRRs 1 to 9 with the corresponding LRRs from Cf-4 resulted in reduced induction of HR. The region LRR 10 to LRR 18 also contains amino acids essential for Cf-9 function. Interestingly, this region also contains 15 of the 67 amino acids that distinguish it from *Cf-4* (Wulff *et al.*, 2001).

Domain swaps between Cf-2 and Cf-5 have revealed the regions of the proteins required for the recognition of the Avrs (Seear and Dixon, 2003). Portions of the *Cf-2* and *Cf-5* genes, encoding the conserved region (C-terminal portion of domain C1 and domains C2-G), variable region (majority of domain C1; LRRs 3 to 27 for Cf-2 and 3 to 21 for Cf-

5) and the signal peptide (domains A and B) were reciprocally swapped. Each of the four constructs was stably transformed into susceptible tomatoes and the transformants were tested by inoculation with *C. fulvum*. Each construct was functional and behaved according to the variable region, i.e. constructs carrying the variable region of Cf-2 behaved as Cf-2 irrespective of whether the conserved regions were derived from Cf-2 or Cf-5 and vice versa (Seear and Dixon, 2003). The sequence differences outside LRR 3-27 for Cf-2 and 3-21 for Cf-5 had little effect on the function of the R protein, demonstrating this region is important for Avr2/5 recognition (Seear and Dixon, 2003).

The importance of the Cf protein's LRRs in the recognition of Avrs is indisputable. However, whether they are directly involved in ligand binding is somewhat less certain. In fact, to date there is no evidence of a direct interaction between the Cf proteins and their corresponding Avrs. Luderer *et al.*, (2001a) investigated the possibility of a direct interaction between Cf-9 and Avr9. Firstly binding studies were performed using Cf-9 expressed in COS or insect cells and radio-labelled Avr9. A second approach was employed using tobacco expressing *Cf-9* where binding was detected by surface plasmon resonance and surface-enhanced laser desorption and ionisation. Neither approach detected any specific binding between Cf-9 and Avr9. These findings suggest that at least a third partner is involved in the recognition of Avr9. Indeed, evidence of a direct interaction between any of the R proteins and their corresponding Avr proteins is very limited. Third interacting partners have been proposed for a number of systems. One example where the third partner has been identified is the RPS5 gene of *Arabidopsis*, which encodes a NBS/LRR protein with a leucine zipper domain (Warren at al., 1998). RP5S confers resistance to races of *Pseudomonas syringae* pv. *Phaseolicola* expressing *avrPphB*. A third *Arabidopsis* gene, *PBS1* that encodes a functional kinase with similarity to Pto of tomato, is required for the perception of avrPphB (Swiderski and Innes, 2001). It is proposed that PBS1 is the virulence target of avrPphB and that PBS1 is 'guarded' by RPS5 in a system analogous to the guard hypothesis proposed for the interactions between avrPto, Pto and Prf, by Van der Biezen and Jones (1998).

One possible candidate for this interacting partner is a high affinity binding site (HABS) discovered by Kooman-Gersmann *et al.*, 1996). A HABS for Avr9 has been identified in plasma membranes of mesophyll and suspension cells of solanaceous species (Kooman-Gersmann *et al.*, 1996). These binding sites are present in tomato lines with and

without Cf-9 and appear to be specific for Avr9 as Avr4 does not compete for binding. It would seem plausible that these HABS could be the virulence target of the Avr9 peptide. Binding of Avr9 to the HABS alone however, does not induce a hypersensitive response. The Cf proteins could serve to recognise an elicitor-HABS complex. Such an elicitor-HABS complex need not be specific to plants expressing the *Cf-9* gene, but requires the Cf-9 protein in order to elicit a defence response. For example, in the case of MM-Cf0 plants the Avr9-HABS complex may be formed but no defence response will occur due to the absence of the Cf-9 protein. In support of this, a positive correlation was found between the binding affinity of synthetic and native Avr9 peptides for the HABS and the induction and spread of necrosis (Kooman-Gersmann *et al.,* 1998). Tomato seedlings expressing both a *Cf* gene and the corresponding *Avr* gene die rapidly as a result of necrosis. This response is avoided if the plants are incubated at an elevated temperature of 33^0C (de Jong *et al.*, 2002). When incubated at 33^0C, tobacco suspension cell cultures experience an 80% reduction in the amount of Avr9-HABS complexes, compared to incubation at 20^0C (de Jong *et al.*, 2002). This provides further support for the theory of the HABS being the mechanism for Avr9 recognition.

In addition to the HABS, it is possible that the Cf-9 protein is a low affinity binding site (LABS) for Avr9. In the presence of the HABS in isolated plasma membranes any binding of Avr9 to the LABS would remain undetected.

An alternative model is that more than one Avr9 molecule can bind to a LABS. The LABS and the Cf-9 protein would subsequently form a heterodimer, which would make ligand binding possible and play some role in signal transduction. The LABS would be likely to include a cytoplasmic signalling domain as Cf-9 itself lacks an obvious signalling component. It may also be expected that the LABS would contain an extracytoplasmic LRR region to allow interactions between itself and the Cf-9 protein. Thus the LABS could possibly resemble the Xa21 protein of rice as described earlier. (Joosten and de Witt, 1999).

More recently a high-affinity binding site for Avr4 has been identified (Westerink *et al.*, 2002). The HABS was isolated from tomato microsomal membranes. In contrast to the Avr9 HABS, the Avr4 HABS is of fungal origin. It is believed that the fungal origin of the HABS is related to the virulence function of Avr4 (Westerink *et al.*, 2002).

Additional candidates for the virulence targets of the Avr peptides are the *Rcr* (Required for *C. fulvum* Resistance) gene products. Hammond-Kosack *et al.* (1994a) carried out a mutagenesis screen in order to study the process of Avr9 recognition and subsequent elicitation of a defence response. The mutagenesis was carried out in MM-Cf9 plants. Two types of mutants were discovered, some with partial braking of resistance and others that were completely susceptible to infection by race 4 GUS of *C. fulvum*. Four of the six plants that were completely susceptible were found to contain a mutation in the *Cf-9* gene. Some of the partially resistant mutants had mutations in one or more genes other than *Cf-9*. Two mutants were recovered, neither of which map to *Cf-9*, nor do they appear to be linked to each other, indicating the presence of two other loci which play a role in *Cf-9* dependent resistance. These two loci were designated *Rcr1* and *Rcr2*, as they are required for full resistance.

A similar observation has recently been made for the *Cf-2* gene where a mutation, which effects Cf-2 dependent resistance, was identified at a new locus. Susceptible mutants were crossed with susceptible Cf0 plants and gave rise to resistant progeny proving the mutation was not in the *Cf-2* gene. This new gene was designated *Rcr3* and with the use of mapping populations was shown to map to chromosome 2 (Dixon *et al.*, 2000). Dixon *et al.* (2000) also revealed that there were both weak and strong mutant alleles at the *Rcr3* locus, *rcr3-2* and *rcr3-3* allowed as much fungal growth as observed in a susceptible Cf0 plant, while *rcr3-1* was less susceptible. Using a GUS marker gene assay, fungal growth could be observed in the *rcr3-1* mutant one week post infection. However, two weeks post infection the fungal growth was observed to be intermediate of that on Cf0 and Cf2 plants. Thus *rcr3-2* and *rcr3-3* are strong suppressors of the *Cf-2* function whilst *rcr3-1* is a weak suppresser (Dixon *et al.*, 2000). *Rcr3* is specifically required for full *Cf-2*-mediated resistance (Dixon *et al.*, 2000). It was revealed that *Rcr3* is not required for *Cf-9*-mediated resistance and perhaps more interestingly nor is it required for *Cf-5*-mediated resistance, despite the high homology between the Cf2 and Cf5 proteins.

Map-based cloning was used to isolate *Rcr3*. Sequencing revealed a high homology to papain-like cysteine endoproteases (Krüger *et al.*, 2002), being 44% identical to the senescence-induced cysteine protease SAG12 from *A. thaliana* (Lohmann *et al.*, 1994). It was therefore postulated that Rcr3 plays some role in general defence perhaps

programmed cell death, which is the target for Avr2. This is supported by the increase in expression levels of *Rcr3* during an infection and with increasing age (Krüger *et al.*, 2002). Cf-2 could act as a guard for Rcr3, initiating a defence response upon recognition of a processed Rcr3 or an Avr2/Rcr3 complex. This is analogous to the Pto, AvrPto, Prf 'guard hypothesis' where the *Rcr* genes are functionally analogous to *Pto*, and the *Cf* genes to *Prf*.

Further evidence in support of the 'guard hypothesis' is the observation of an Avr2 independent autonecrotic phenotype. Plants expressing *Cf-2* in the presence of the recessive *L. esculentum Rcr3* ($Rcr3_{esc}$) allele exhibited an autonecrotic phenotype, the extent of which increases with age. No such phenotype was observed in plants expressing the *L. pimpinellifolium Rcr3* ($Rcr3_{pimp}$) allele. As the L. *esculentum Rcr3* allele did not co-evolve with *Cf-2*, which originates from *L. piminellifolium*, it is possible that a 'leaky signal' that results in the observed autonecrotic phenotype is generated (Krüger *et al.*, 2002). It is possible that the differences in structure between $Rcr3_{pimp}$ and $Rcr3_{esc}$ is sufficient that $Rcr3_{esc}$ is able to interact with Cf-2 in absence of a ligand (Avr2) to generate this 'leaky signal', resulting in the activation of the defense pathways.

As for all Avrs, the exact target for Avr2 is still unclear, although, Rcr3 seems a likely candidate. Once the invading pathogen has been detected a signal must be sent, to turn on the plant's defence mechanisms.

1.3.5 Signalling of defence responses

Upon recognition of an invading pathogen, the plant must elicit a defence response. To do this, specific signalling events follow to ensure an effective and directed defence response ensues. Plants unable to either detect the pathogen or elicit a defence response, that is both specific and timely, will become colonised by the pathogen. There is to date no complete story of the signalling events that lead to the defence response in plants. There are however, many molecules that have been implicated in signalling of the defence response, these include salicylic acid (SA), ethylene, calcium ions (Ca^{2+}), active oxygen species (AOS), protein kinases, phopholipases, G proteins, and NADPH oxidase (Blumwald *et al.*, 1998; van Camp *et al.*, 1998).

Sequence comparisons of the Cf proteins revealed that the C-terminal 8.5 LRRs, along with the acidic, basic and transmembrane domains, were the most highly conserved. With the amino acid differences that distinguish Cf-4 from Cf-9 confined to the N-terminal two thirds of the proteins (Thomas *et al.*, 1997). Likewise, the Cf-2 and 5 proteins are very similar when comparing their C-termini, but differ in the N-termini. This sequence conservation, specifically in the last 8.5 LRRs, suggests a conserved role. It is plausible that this conserved region of the proteins is either directly involved in signalling or indirectly via interactions with other signalling proteins, with the acidic, basic and putative transmembrane domains forming a membrane anchor.

As with most plant defence mechanisms little is known of how the Cf proteins are involved in the signalling of a defence response. They carry no obvious signalling domains and so need to recruit other molecules to initiate a signalling pathway. It is possible that the Cf proteins form complexes like those seen in *Arabidopsis* with the CLAVATA proteins.

CLAVATA1, CLAVATA2 and CLAVATA3 (CLV1, 2 and 3) are proteins that are required for the maintenance of the balance between cell proliferation and organ formation at root and flower meristems in *Arabidopsis* (Trotochaud *et al.*, 1999). CLV1 is a receptor like protein kinase similar to the rice disease resistance gene product Xa21 (Song *et al.*,1995). *CLV2* encodes a receptor like protein, with an extracellular domain composed of LRRs (Jeong *et al.*, 1999). Trotochaud *et al.* (1999) revealed that *CLV1* is present as an inactive disulphide linked heterodimer, and that CLV3 functions to assemble the active complex. CLV2 and CLV1 are postulated to form the inactive disulphide linked heterodimer of approximately 185 kDa (Jeong *et al.*, 1999). An active complex of 450kDa is then formed upon recognition of CLV3 (the ligand) and after phosphorylation of the kinase domains of CLV1. In addition to the three CLAVATA proteins, this membrane complex comprises the protein phophatase Kapp and a Rho GTPase-related (Rop) protein. (Trotochaud *et al.*, 1999). As CLV2 and Cf-9 are similar structurally and are both activated by a small extracellular protein, it has been proposed that Cf-9 mediated defence signalling may occur by a mechanism similar to that of the CLAVATA proteins (Luderer *et al.*, 2001a).

Similar to the CLAVATA proteins Cf-4 and Cf-9 were found in membrane bound complexes, approximately 400 and 420kDa respectively (Rivas *et al.*, 2002a; Rivas *et al.*, 2002b). Each complex contained only one Cf molecule with the other components believed to be involved in signalling. Unlike the CLAVATA complexes these Cf complexes lack disulphide bridges and are formed irrespective of ligand (Avr4 or 9-elicitation) (Rivas *et al.*, 2002a, Rivas *et al.*, 2002b). However, a second protein was never purified from these complexes, casting doubt over these findings. It is also unusual that the signalling complexes are produced irrespective of the presence of the ligand. Whether the Cf proteins form membrane complexes to signal for defence in a manner similar to the CLAVATA model is still uncertain.

Only a few molecules have been positively identified as performing a role in Cf-mediated defence the first example is AOS. One of the earliest responses to elicitor recognition observed with the Cf proteins is the production of AOS. Such active oxygen species could be directly fungitoxic, induce cell wall strengthening and induce HR. In other plant/pathogen systems AOS have been demonstrated as signalling molecules contributing to defence gene activation (Clarke *et al.*, 2000; Durner *et al.*, 1998; Hancock *et al.*, 2002; van Camp *et al.*, 1998). After injection of cotyledons with apoplastic fluid containing the elicitors superoxide was noted within 2 hours, which probably marked the onset of the HR (Hammond-Kosack *et al.*, 1996). Tobacco suspension culture cells, expressing *Cf-9*, treated with pure or synthetic Avr9 produce active oxygen species within 5 minutes. This is substantially faster than that observed *in planta* (Piedras *et al.*, 1998). Inhibitor studies revealed that AOS production is almost certainly due to NADPH oxidase and is mediated by calcium uptake, protein kinase activation and phospholipases (Piedras *et al.*, 1998). Xing *et al.* (1997b) demonstrated an increase in oxidase activity and in p67-phox, p47-phox and rac2 recruitment to the plasma membrane as a result of treating *Cf-4* and *Cf-5* expressing tomato cells with Avr4 and Avr5 respectively, lending support to the theory that NADPH oxidase is responsible for the production of AOS. The assemply of the active NADPH oxidase is catalysed by a calcium dependent protein kinase that is not like protein kinase C (PKC) (Xing *et al.*, 1997b).

Several protein kinases have been identified that are important in the onset of a defence response in the Cf-9/Avr9 system. Two of the first protein kinases identified were

shown to be activated within 5 minutes of Avr9 elicitation in both tobacco plants and cell suspension cultures (Romeis *et al.*, 1999). The two proteins were shown to be similar to WIPK (wound induced protein kinase) and SAIPK (salicylic acid induced protein kinase), two mitogen activated like protein kinases (MAP like kinases) (Romeis *et al.*, 1999). Both calcium influx and phosphorylation events were shown to be important in the activation of these kinases.

In addition calcium dependent protein kinases (CDPKs) have been found in tobacco that are important in the same response. CDPKs are calcium-binding serone/threonine protein kinases which are regulated by an autoinhibitory domain joined to a regulatory calmodulin-like domain (Romeis *et al.*, 2001). It is thought that CDPKs, which thus far have not been identified in yeast and animal systems, perform the roles fulfilled by protein kinase C and calmodulin-dependent-kinases in yeast and animals (Romeis *et al.*, 2001). Romeis *et al.*, (2000), using in-gel kinase assays, identified a CDPK that was converted from a 68kDa to a 70kDa form upon elicitation with Avr9, suggesting a role in defence activation. The change in size was demonstrated to be due to a phosphorylation event (not autophosphorylation).

Two CDPKs cDNAs (*NtCDPK2* and *NtCDPK3)* were isolated from Avr9 elicited *N. tabacum* suspension cell cultures expressing *Cf-9* (Romeis *et al.*, 2001). Analysis of an epitope-tagged version of NtCDPK2 revealed a conversion between two enzyme forms induced by both abiotic (osmotic) and biotic (Avr9 elicitation) stresses. The activated form demonstrated elevated protein kinase activity (Romeis *et al.*, 2001). Silencing of the *NtCDPK2* sub-family, by virus induced gene silencing (VIGS), resulted in plants compromised in the activation of Cf-9/Avr9 and Cf-4/Avr4 HR, indicating the involvement of this CDPK sub-family in a gene-for-gene defence-related signal cascade (Romeis *et al.*, 2001). Silencing of the *NtCDPK2* sub-family, despite similar induction profiles, had no effect on the transcript levels of the MAPK, WIPK, suggesting they function by two distinct pathways (Romeis *et al.*, 2001).

The importance of the CDPKs also implicates calcium ions themselves as candidates for signalling molecules of the Cf-mediated defence response. Indeed probably the earliest of responses to elcitation is an increase in cytosolic calcium ion concentration.

Plasma membrane channels mediate calcium ion influx whilst efflux is mediated by plasma membrane calcium ATPases. Within 30 minutes of *in vivo* elicitor (Avr5) treatment, an inhibition of tomato plasma membrane calcium ATPases was observed in tomato cells expressing *Cf-5*. This inhibition was specific to Avr5 and independent from modifications of the self-inhibitory domain of the pump (Lam *et al.*, 1998). In addition to calcium ions, changes in potassium ion concentrations are important early signalling events. A rapid Avr9-induced potassium ion efflux and inhibition of influx is observed in tobacco guard cells, specifically those expressing *Cf-9* (Blatt *et al.*, 1999). The observed changes are directed towards two discrete potassium channels, controlled by phosphorylation and are irreversible (Blatt *et al.*, 1999).

The plasma membrane H^+ATPase also plays a role in Cf-mediated defence. Elicitation of tomato cells expressing *Cf-5* with preparations containing Avr5 experienced a dephosphorylation of the PM H^+ATPase, a change not observed with preparations lacking Avr5 (Xing *et al.*, 1996). The dephosphorylation event was blocked using antibodies raised against the G protein α-subunit, demonstrating a role for G proteins in the Cf-5/Avr5 mediated defence response (Xing *et al.*, 1997a). Rephosphorylation of the PM H^+ATPase occurred after treatment with elicitor preparations from an incompatible interaction after 2 hours. The rephosphorylation occurred via a calcium dependent protein kinase and was enhanced during the first hour by protein kinase C (PKC) and inhibited by PKC inhibitors (Xing *et al.*, 1996). A second kinase, inhibited by a calmodulin agonist, was responsible for the continued rephosphorylation. Activation of the second kinase was dependent upon the prior activation of the PKC-like kinase (Xing *et al.*, 1996).

Although there is no clear signalling pathway that leads from Cf-mediated pathogen detection through to pathogen containment, it seems likely that more than one pathway is involved. This is demonstrated by the involvement of both MAPKs and CDPKs. One thing seems clear that changes in calcium ion concentration plays a very important role, with calcium dependent protein kinases detecting such a change and in turn activating other enzymes that ultimately result in the defence response. Changes in other ion concentrations (e.g.potassium and hydrogen) brought about by changes in activities of PM pumps and channels is likely to be involved in the activation of Cf-mediated defence responses. However, their precise roles remain unclear.

1.3.6 Subcellular localisation of Cf proteins

The *Cf* genes are predicted to encode predominantly extracytoplasmic, plasma membrane anchored LRR glycoproteins. An ER retrieval-retention motif present in the C terminus of the Cf-4 and 9 proteins suggests an ER location. This dilysine (KKxx) sequence motif is characteristically involved in mammals and yeast with the retrieval of the protein from the golgi to the ER (Thomas *et al.*, 1998). Piedras *et al.* (2000) epitope-tagged the Cf-9 protein with the mammalian triple-c-Myc sequence to which monoclonal antibodies have been raised. Sub-cellular fractionation revealed that the Cf-9 protein enriches with the plasma membrane fraction, and that the majority of the protein is extracellular. The perception of the Avr proteins has been shown to occur extracellularly, Moneymaker (MM) Cf4 and Cf9 NILs were inoculated with PVX::*Avr4* or PVX::*Avr9*. A hypersensitive response was only recorded when the elicitor proteins contained a signal sequence for extracellular targeting (Joosten and de Witt, 1999). Using two different approaches, both a Cf-9-green fluorescent protein fusion protein, and haemaglutenin epitope tagging of Cf-9, Benghezal *et al.* (2000), demonstrated that the Cf-9 protein is retrieved and retained in the endoplasmic reticulum, a process which, is saturable, casting some doubt over the findings of Piedras *et al.* (2000).

Subsequent studies supported the findings of Piedras *et al.* (2000), the dilysine motif was shown not to be required for Cf-9 function (Van der Hoorn, *et al.*, 2001a).

Van der Hoorn *et al.* (2001a) produced Cf-9 peptides with a mutated dilysine repeat (the motif KKRY was changed to AARY). There was no significant difference observed between the mutant and wild type peptide in the recognition of Avr9. It was noted that the ER retrieval-retention signal could be weakened, in yeast, by phenlyalanine (F) or tyrosine (Y) residues at the C terminus. As the Cf-9 motif is KKRY the mutated peptide KKAA was tested, again there was no significant difference between the ability of the wild type and mutant peptide in the recognition of Avr9. Thus it was concluded that this motif (KKRY) was not essential for Cf-9 function. A truncated Cf-9 protein was also produced, with the transmembrane domain and the G domain deleted, this peptide was unable to recognise the Avr9 peptide (Van der Hoorn *et al.* 2001a). This revealed that although the dilysine motif is not required for Cf-9 function the membrane anchor is. This recent evidence indicates a plasma membrane localisation of the Cf-9 protein, confirming

the findings of Piedras *et al.* (2000). The decision to study the localisation of Cf-2, which differs from Cf-9 as it lack the dilysine motif, was made before the emergence of this recent evidence, whilst there was still some controversy over the localisation of Cf-9.

1.4 Aims

The aim of this project is to learn more about the expression of the *Cf-2* gene and biochemistry of the gene product.

There is an intrinsic logic that, like other Cf proteins, *Cf-2* is predominantly extracytoplasmic and glycosylated, the intial project aim is to investigate this.

The Cf proteins are predicted to be receptors for molecules from a fungus which lives entirely in the apoplast and which does not produce haustoria. It would therefore seem logical that the receptors should span the plasma membrane, to allow for recognition and signal transduction. Initial studies investigating the subcellular distribution of *Cf-9* were consistent with current thinking (Piedras *et al.*, 2000), however controversy resulted from a second study (Benghezal *et al.*, 2000).

The proposed studies of this project will begin with epitope-tagging of the C-terminal portion of Cf-2. A method of epitope-tagging was chosen over direct immunolabelling because there are many genes similar to *Cf-2* in both tomato and tobacco. This would make it a difficult task to raise specific antibodies, particularly if all encoded proteins are highly glycosylated. As a result a 3x c-Myc epitope-tag to which monoclonal antibodies are commercially available, was inserted into the C-terminal portion of Cf-2. The C-terminal portion was chosen as the site of epitope-tagging as this region is believed to play no part in the response to the invading pathogen, functioning only as a membrane anchor. Thus by inserting the epitope-tag in the C-terminal portion it should be possible to produce fully functional, tagged proteins.

The epitope-tagged *Cf-2* gene under the control of its own promotor and the cauliflower mosaic virus (CaMV) 35S promoter will be transformed into both tobacco and susceptible tomato plants using *Agarobacterium tumefaciens*. There is evidence to

suggest that over-expression of the *Cf-2* gene is detrimental to tomato plants. The 35S promotor was used by Piedras *et al.* (2000) and it is believed to be necessary to do the same as the level of expression of *Cf-2* under its native promoter is predicted to be too low.

Once transgenic plants have been produced it will then be possible to investigate the subcellular distribution of the protein, using cell fractionation.

Prior to investigating the subcellular distribution, the functionality of the epitope-tagged protein will be tested by inoculation with *C. fulvum* race 4*GUS*. *C. fulvum* race 4*GUS* is a transgenic race, carrying the *uidA* reporter gene (Oliver et al., 1993). This line carries AVR2 and can therefore be employed to assess the function of the epitope-tagged Cf-2. The reporter gene can be used for both qualitative and quantitative analysis of infection.

Transgenic plants, carrying a functional epitope-tagged *Cf-2* gene, would serve as a useful tool when investigating interacting proteins. Cf-2 is proposed to serve two functions, firstly to recognise the fungal avirulence protein Avr2 and secondly to signal that recognition event. So additional proteins could be involved in the perception of the fungal avirulence protein and/or be involved in signal tranduction. One potential interactor is Rcr3. There are several hypothesised roles for this gene although its function has not yet been determined. At the onset of this work *Rcr3* was close to being cloned allowing the potential for future independent or collaborative studies to be undertaken.

The second part of this project is concerned with the expression patterns of the *Cf-2* and *Cf-9* genes. Using Cf promoter GUS fusion constructs stably transformed into tomato (Torres, Smoker and Jones, unpublished). Initial experiments will investigate the onset of expression of the genes in seedlings. A similar experiment will be performed on mature plants to see if patterns exhibited in seedlings, if any, are consistent with the expression in mature plants.

In addition to investigations of the temporal pattern of gene expression, induction profiles of the genes will also be conducted. The promoter GUS fusion lines will be

crossed to Cf0, Cf2 and Cf9 and treated with intercellular fluid (IF) obtained from a compatible *C. fulvum*, tomato interaction. The aim of this is to investigate the effects of specific elicitors on gene expression.

It has become apparent that *Cf*-dependent defence is not as simple as once thought, with additional components having been discovered i.e. the *Rcr* (required for *Cladosporium* Resistance) genes, and the High Affinity Binding Site (HABS) of Cf-9 (Dixon *et al.*, 2000; Hammond-Kosack *et al.*, 1994a; Kooman-Gersmann *et al.*, 1996). Understanding the expression patterns of the *Cf* genes may give insight into their involvement in defence against *C. fulvum*.

Chapter two

2 Materials and methods

2.1 Solutions

¼ **Potato Dextrose Agar:** Quantities are per litre; 10g PDA, 6.25g agar, for selection 250µl/ml of streptomycin is added.

10x agarose gel loading dye: 0.25% (w/v) Orange G, 15% (w/v) ficoll in water

10x MEN: 0.2M MOPS, 80mM Sodium acetate, 10mM EDTA.

2x protein sample buffer: 125mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue.

20x SSC: 175.3g NaCl, 88.2g sodium citrate, H_2O to 1 litre, pH to 7.0 (NaOH or HCl).

Acetate buffer: Mix 0.2M sodium acetate and 0.2M acetic acid to the desired pH (do not autoclave).

Alkaline phenol: 1ml saturated phenol, 2ml 0.2M sodium hydroxide.

Blocking solution: 100mM maleic acid, 150mM NaCl, 1% (w/v) blocking reagent (Boehringer Mannheim, Lewes, UK).

Church and Gilbert hybridisation buffer: 0.3M sodium phosphate buffer pH 7.5, 1% (w/v) BSA, 7% (w/v) SDS (Church and Gilbert, 1984).

Denaturation solution: 0.5M NaOH, 1.5M NaCl.

Destain solution 1: 30% (v/v) methanol, 10% (v/v) acetic acid.

Destain solution 2: 10% (v/v) acetic acid.

Detection buffer: 100mM Tris-HCl, 100mM NaCl, pH 9.5.

Freezing buffer: 10mM MES (pH 6.3), 45mM $MnCl.4H_2O$, 10mM $CaCl_2.2\ H_2O$, 100mM KCl, 3mM Hexaminecobalt chloride, 10% (v/v) glycerol.

Hybridisation buffer: 7% (w/v) SDS, 2% (v/v) blocking reagent (Boehringer Mannheim, Lewes, UK), 1mM EDTA, 0.2M sodium phosphate buffer: pH 7.2.

Hybridisation washing buffer: 100mM sodium phosphate buffer, 1mM EDTA, 1% (w/v) SDS

L broth: Quantities are per litre; 10g Bacto Tryptone, 5g yeast extract, 5g sodium chloride, 1g D-glucose. 1% (w/v) agar is added for solid, autoclave after preparation.

Maleic acid buffer: 0.1M maleic acid, 0.15M NaCl, pH to 7.5 with NaOH.

Minimal A media: Quantities are per litre; 10.5g di-potassium hydrogen orthophosphate, 4.5g potassium di-hydrogen orthophosphate, 1g ammonium sulphate, 0.5g sodium citrate, 2g glucose. Prior to use add 1mM magnesium sulphate, and 1.5% (w/v) agar to make solid.

Neutralisation solution: 1.5M NaCl, 0.5M Tris-HCl pH 7.4.

PBS: 15mM sodium phosphate buffer pH 7.0 (as above), 150mM NaCl.

Protein gel running buffer: 25mM Trisma base, 3.5mM SDS and 0.2M glycine.

SOC media: Quantities are per litre; 20g Bacto Tryptone, 5g yeast extract, 0.58g sodium chloride, 2.5ml 1M potassium chloride, 4.5g D-glucose, pH to 7.0 with sodium hydroxide, and autoclave. Just prior to use 100µl/10ml of sterile 1M magnesium chloride is added.

Sodium phosphate buffer (1M): 1M Na_2HPO_4 (relatively acidic), 1M NaH_2PO_4 (relatively basic), mix appropriate amounts until desired pH is achieved.

Solution I: 50mM Tris-HCl, 10mM EDTA, 100µg RNase A/ml.

Solution II: 0.2M NaOH, 1% (w/v) SDS.

Solution III: 3M potassium, 5M Acetate.

TBS: 50mM Trisma-HCl pH 7.5, 150mM NaCl.

TBST: 0.1% (v/v) tween 20 in TBS

Tobacco regeneration media: 1x MS media with Gambourg B5 vitamins (Duchefa, The Netherlands), 3% (w/v) sucrose, pH 5.7 with KOH (0.7% (w/v) bacto-agar for solid). 500mg/l Augmentin (Duchefa, The Netherlands) and a suitable selective antibiotic (300mg/l kanamycin) were added for selection of transformants.

Tobacco suspension media: 1x MS media with Gambourg B5 vitamins, 3% (w/v) sucrose, 1mg/l 2,4-D, 2mg/l BAP, pH 5.7 (with KOH).

Tomato co-cultivation media: 1x MS media with Nitchs vitamins (Duchefa), 3% (w/v) sucrose, pH 5.7 with KOH, 1mg/l 2,4-D (0.7% (w/v) bacto-agar for solid).

Tomato regeneration media: 1x MS media with Nitchs vitamins, 2% (w/v) sucrose, pH 6.0 with KOH, 2mg/l Zeatin riboside (shooting media), 0.18mg/l IAA (rooting media), (0.7% (w/v) bacto-agar fro solid). 150mg/ml Timentin (Duchefa, The Netherlands), and a

suitable selective antibiotic (100mg/ml kanamycin) were added for selection of transformants.

Washing buffer: 100mM maleic acid, 150mM NaCl, 0.3% (v/v) Tween 20.

X-Gluc stain: 1mM EDTA, 0.5mg/ml X-gluc in DMF, 50mM phosphate buffer pH 7.0, 0.05% (v/v) Triton X-100.

2.2 Protocols

2.2.1 DNA digests

In preparation of a vector and/or insert obtained from a plasmid, at least 1µg of DNA was digested. For tests of plasmid clones 200-500ng of DNA was digested. The digest mix consisted of 1x of the appropriate buffer (according to and supplied by manufacturer) with <10% (v/v) of enzyme. To prevent Star activity, *Bam*HI digests were performed with final glycerol (and hence enzyme) concentration below 7% (v/v). For preparation of vectors and inserts the digests were left over night at 37^{0}C, all other test digests were performed at 37^{0}C for 2-5 hours (Restriction enzymes were obtained from Promega, Southampton, UK).

For partial digestion of DNA, tubes were set up containing equivalent amounts of DNA in 1x the appropriate buffer. An equal volume of enzyme in 1x buffer is added to the first tube and mixed thoroughly. Half of the solution was removed and placed in the second tube; again half of the solution is removed and placed into the third tube and so on to produce a dilution series of enzyme. The digests were carried out at 37^{0}C for no more than 1 hour.

2.2.2 Phosphatase treatment of DNA

Where vector DNA was cut with a single enzyme, to prevent self-ligation the DNA was treated with shrimp alkaline phosphatase (SAP) to remove terminal 5' phosphates. For ends with a 3' overhang 0.5u SAP/pmol of ends was added to the digest. For blunt ends 0.2u SAP/pmol of ends was added, and for ends with a 5' overhang 0.1u SAP/pmol of ends was added (Promega, Southampton, UK). SAP works in most buffers and was therefore added directly into the digest mixture. Before ligation reactions the SAP was heat inactivated at 65^{0}C for 20 minutes.

2.2.3 Agarose gel electrophoresis of DNA

Gels were prepared and electrophoresis carried out according to Sambrook *et al.*, (1989). Gels were prepared by dissolving agarose 0.7 -1.5% (w/v) in 1x TAE buffer, by bringing to the boil in a microwave. The gel was cooled to below 50^0C, and 1µg/ml (final) ethidium bromide added. The gel was poured into a gel mould, into which a well former was placed and left to set at room temperature. The DNA was prepared in 1x orange G loading dye (10x Orange G: 0.25% (w/v) Orange G, 15% (w/v) ficoll in water). Electrophoresis was carried out in 1x TAE and run at 10-15Vcm^{-1} until the desired amount of separation of the DNA fragments was achieved.

The DNA was visualised using an Alpha Imager™ 1220 (Alpha Innotech, supplied by GRI, Braintree, UK), the size of the fragments were estimated by comparison to a 1kb DNA ladder (Gibco BRL, Cheshire, UK).

2.2.4 Isolation of DNA from agarose gels (Kit method)

DNA fragments (0.5-5kb) separated by agarose gel electrophoresis were excised from the gel with a razor blade. The DNA was purified using a Machery-Nagel Nucleospin® kit (AB-Gene, Epsom, UK) in accordance with the manufacturer's instructions.

2.2.5 Isolation of DNA from agarose gels by electro-elution

Fragments that were smaller than 500bp or larger than 5kb were purified by electroelution (Sambrook *et al.*, 1989), as these fragments are not optimal for isolation using a kit (see above). The DNA fragments were excised from the agarose gel using a razor blade. Gel fragments were sealed in dialysis tubing with 50µl of TE and placed back into the electrophoresis tank. The DNA was run from the gel into the elution tube, the current was reversed for 2 seconds prior to aspirating the DNA solution. 150µl of phenol was added to the DNA solution, mixed thoroughly and centrifuged for 1 minute at 13000 x *g* in a bench top microcentrifuge. The aqueous phase (top phase) was removed and mixed with 150µl phenol chloroform (1:1) and centrifuged as before. The aqueous phase (top phase) was recovered and the DNA precipitated by ethanol salt precipitation.

2.2.6 Ethanol salt precipitation

Small volumes of DNA were made up to 20µl with water. 2.5 volumes of 100% ethanol and 1/10 volume of 3M sodium acetate pH 5.4 was added to one volume of DNA solution. Samples were mixed thoroughly (a vortex was only used on DNA fragments less than 10kb) and placed at -20^{0}C for 20-30 minutes. The DNA was pelleted by centrifugation at full speed in a bench top microcentrifuge (13000 x ***g***) for 20-30 minutes. The supernatant was discarded and salts removed by washing with 70% (v/v) ethanol. The DNA pellet was either air dried or dried in a heating block at 50^{0}C and resuspended in an appropriate volume of water or TE.

2.2.7 The polymerase chain reaction

Either AGSGold Taq kit (Hybaid, London, UK) or Biomix (Bioline, London, UK) was used for each reaction, according to manufacturers instruction. Final concentrations used; 10-20ng Genomic DNA/ 1-5ng Plasmid DNA/ 1µl of a 5ml culture grown for 1.5-2 hours at 37^{0}C (*E. coli*) or 4-6 hours at 28^{0}C (*Agrobacterium*). AGSGold method: 1x PCR buffer (Hybaid, London, UK), 1x PCR enhancer (Hybaid, London, UK), 2.0mM $MgCl_2$, 200µM dNTPs, 500nM of both primers I and II, 1µl/100µl reaction AGS Gold Taq (5u/1µ), with analar H_2O to make to volume. Bioline (London, UK) method: 1x Biomix (contains Taq, buffer, dNTPs), 500nM of each primer. The general PCR cycle was preceded by a two-minute denaturation step (94^{0}C). Each cycle began with a 15-30 second denaturation period (94 ^{0}C), followed by a 15-30 second annealing period. The annealing temperature was estimated by the following general equation:

Annealing temp = (2(Σ As and Ts in primer) + 4(Σ Cs and Gs in primer)) – 4

This was refined through trial and error. The annealing step was followed by an extension phase (1minute per Kb of DNA to be amplified, at 72^{0}C). This cycle was repeated 30-40 times. The final cycle was followed by a five-minute extension period (72^{0}C) and a 14^{0}C hold.

2.2.8 Ligations

Ligations were carried out according to manufacturers instructions. Insert and vector DNA were prepared by digestion with restriction enzymes. Vector and insert were mixed and ethanol/salt precipitated if the volumes were greater than 4µl. In general 100ng of vector was used with the insert in approximately 3-5 fold molar excess. Ligation volumes were 7µl and comprised of 1x ligation buffer (Promega, Southampton, UK or Gibco BRL, Cheshire, UK) and 0.5µl of T_4 DNA ligase (3u/µl, Promega, Southampton, UK or Gibco BRL, Cheshire, UK).

Generally ligations were carried out at 16^{0}C over night. Sticky-end ligations considered non-problematic were left at room temperature for 2 hours.

2.2.9 Production of competent bacteria ($CaCl_2$ method)

Competent bacteria were produced and subsequently transformed (2.2.9, 10 and 11) according to Sambrook *et al.*, (1989). *E. coli* strain DH5α was streaked onto a plate of L-agar and grown at 37^{0}C over night. One or two colonies were picked and used to inoculate 5ml of L-broth, and allowed to grow over night at 37^{0}C. The bacteria were diluted in L-broth (1-2% v/v) and grown to OD_{600} of between 0.5 and 0.7. Cultures were cooled on ice for 10 minutes and subsequent steps performed at 1-4^{0}C. Bacteria were recovered by centrifugation at 4000rpm (in a Sorvall SLA600 rotor) for 10 minutes at 4^{0}C. The supernatant was aspirated and the tube inverted to ensure all media drained. The bacteria were resuspended in 20ml of ice cold freezing buffer and the cells recovered by centrifugation as before. The buffer was completely removed, the bacteria resuspended in a final volume of 4ml of freezing buffer and snap frozen in 100µl aliquots on dry ice. Cells were stored at -80^{0}C.

2.2.10 Transformations ($CaCl_2$ method)

Competent cells were thawed on ice; chilled DNA (<10% of cell volume) was added and left on ice for 30 minutes. Cells were heat shocked at 42^{0}C for 90-120 seconds,

1ml of L-broth added and the cells left to recover at 37^{0}C for 45-60 minutes. The bacteria were recovered by centrifugation at full speed in a bech top microcentrifuge (13000 x *g*) for one minute and most of the supernatant discarded. Bacteria were resuspended in the remaining supernatant and spread on L-agar plates with the appropriate selection. For pGex and pBS vectors: 50µg/ml of ampicillin was added.

For the Cf-2 binary cosmid and constructs for plant transformation: 5µg/ml of tetracycline was added.

Where pBluescript II (Stratagene, UK) was used the *lac*- complemetation assay (blue/white selection) was employed to identify successfully transformed cells. This assay utilises the properties of the *E. coli lac* operon. The operon consists of an inducer (*lac* **I**) and its promoter, the *lac* promoter and operator and three genes (*lac Z*, *Y* and *A*) that encode the enzyme β-galactosidase. In the absence of lactose, a repressor protein binds the lac operator, preventing transcription of the genes in the operon. The Bluescript cloning vector contains 131 amino acids of the β-galactosidase coding sequence, interrupted by a poly linker site. IPTG, a lactose analogue, binds to the repressor protein switching on the operon, which in the presence of the substrate X-gal results in blue colonies. Insertion of DNA into the polylinker site disrupts the *lac Z* fragment resulting in white colonies.

40µl of 20mg/ml X-gal (in dimethylformamide) and 4µl of 200mg/ml IPTG were spread over the surface of the agar plates. Water was applied to the surface of each plate before adding IPTG and X-gal to ensure even coverage.

2.2.11 Preparation of electro-competent bacteria

E. coli strain DH5α was streaked onto a plate of L-agar and grown at 37^{0}C over night. One or two colonies were picked, used to inoculate 5ml of L-broth and grown over night at 37^{0}C. The 5ml o/n cultures were diluted in L-broth (1-2% v/v), and grown to an OD_{600} of 0.5 to 0.7. Cultures were chilled on ice for 15-30 minutes and the bacteria recovered by centrifugation at 4^{0}C for 10 minutes at 4000rpm (in a Sorvall SLA600 rotor).

All subsequent steps were carried out below 4^0C, care was taken that the cultures did not freeze. The supernatant was decanted and drained by inversion of the tube. To remove all salts the pelleted bacteria were resuspended in their original volume (volume of cultures at OD600 of 0.5 to 0.7) of sterile, ice-cold water. The bacteria were recovered again by centrifugation, as before, the supernatant removed and the bacteria resuspended in 50% of the original volume in cold sterile water. Once again the bacteria were recovered by centrifugation the supernatant removed and the bacteria resuspended in 2% of the original volume of cold sterile 10% (v/v) glycerol. The bacteria were recovered for a final time as before and resuspended in 0.25% of the original volume in cold sterile 10% (v/v) glycerol, snap frozen on dry ice in 40µl aliquots and stored at -80^0C.

2.2.12 Electro-transformation

Electro-competent cells were thawed on ice and DNA added (DNA was precipitated to remove salts and resuspended in 1-2µl of water prior to transformation). Cells and DNA were put into a pre-chilled electroporation cuvette and subjected to an electric shock of 12.5kV/cm. 1ml of SOC medium was added **Immediately** after the shock and cells allowed to recover at 37^0C for 45-60 minutes before being spread on plates of L-agar with the appropriate selection (as above).

2.2.13 Taking filter-lifts of colonies, for colony screening

Asymmetrical marks were made on filters (Hybond N, Amersham Pharmacia, Little Chalfont, UK) cut to fit petri dishes. Filters were laid onto colonies and identification marks were copied onto the back of the petri dishes (these marks will be used for orientating the filters after the hybridisation). After filters became damp they were removed taking care not to smear the colonies, plates were placed at a suitable temperature to allow re-growth of colonies. The filters were then placed for five minutes colony side up successively in puddles (approx 1ml) of the following solutions: 10% (w/v) SDS, Denaturation solution, neutralisation solution and 2x SSC. The filters were placed colony side up in all solutions to reduce the amount of DNA diffusion. Between solutions

the filters were placed on dry chromatography paper to remove the excess. Filters were dried completely for approximately 30 minutes at room temperature and to permanently bind the DNA baked for 2 hours at 80^{0}C sandwiched between chromatography paper.

2.2.14 Preparation of a digoxygenin (DIG) labelled probe by PCR

DIG labelled probes were produced by PCR using a suitable DNA template. 0.4µl of the final volume of a 10µl reaction was replaced with DIG-labelled dUTP (2.5mM; Amersham Pharmacia, Little Chalfont, UK). Replacing the DIG labelled dUTP with an equal amount of water made a control sample. The cycles were as for a normal PCR and the product of the control run on an agarose gel to reveal the obtained levels of amplification.

2.2.15 Hybridisation using DIG-labelled DNA probes

Hybridisations were carried using a combination the methods Church and Gilbert (1984) and the manufacturers instructions (Roche, Lewes, UK). Filters were pre-hybridised in hybridisation buffer (7% (w/v) SDS, 2% (v/v) blocking reagent (Boehringer Mannheim), 1mM EDTA, 0.2M sodium phosphate buffer: pH 7.2.) for 15 minutes; any cell debris on the filters was rubbed off with a gloved finger. The buffer was poured off and the filters placed in a hybridisation tube, with 10ml of hybridisation buffer containing 5-25ng of denatured probe/ml. The probe was denatured at 94^{0}C and quenched on ice. The filters were incubated over night with the probe at 65^{0}C, and agitated.

After incubation filters were washed with moderate stringency; three times with 20-30ml hybridisation washing buffer (100mM sodium phosphate buffer, 1mM EDTA, 1% (w/v) SDS) at 65^{0}C, followed by three washes with 2x SSC with 0.1% (w/v) SDS (65^{0}C). The rest of the procedure was carried out at room temperature. The filters were equilibrated with washing buffer (100mM maleic acid, 150mM NaCl, 0.3% (v/v) Tween 20) for 1 minute and blocked in a clean tray with blocking solution (100mM maleic acid, 150mM NaCl, 1% (w/v) blocking reagent (Roche, Lewes, UK)) for 30-60 minutes, on a shaker table. The blocking solution was poured off and 30ml of blocking solution containing anti-digoxygenin antibody (a dilution of 1:5000), was added and agitated for 30

minutes. The antibody solution was discarded and the filters washed twice with washing buffer (15 minutes per wash). The final wash was removed and the filters equilibrated in 20mls of detection buffer (100mM Tris-HCl, 100mM NaCl, pH 9.5). Filters were placed in colour detection stain, (1 NBT/BCIP tablet of complete stain and buffer (Roche, Lewes, UK) in 10ml of dH_2O) without agitation for 5-20 minutes. Washing with water arrested further colour development, filters were subsequently air-dried.

2.2.16 Alkaline lysis purification of plasmid DNA from bacteria (small scale)

As a means of screening several colonies of transformed bacteria, alkaline lysis plasmid mini-preps were performed (Sambrook *et al.*, 1989). 5ml of L-broth with 50µg/ml ampilcillin (where the BlueScript or pGex vectors were used) was inoculated with one colony of transformed bacteria and left at 37^0C overnight. Bacteria were recovered at 4^0C by centrifugation at 10000 x ***g***, for 5-10 minutes. The supernatant was poured off and the tube inverted to allow the medium to drain. The bacterial pellet was resuspended in 100µl of solution I (50mM Tris/HCl, 10mM EDTA, with RNase A, approximately 100µg/ml), and transferred to a clean eppendorf tube to which 200µl of solution II (0.2M NaOH, 1% (w/v) SDS) was added. Tubes were inverted several times and left at room temperature for 5 minutes. 150µl of cold solution III (3M potassium acetate pH 4.8) was added, the tubes inverted several times, and the samples left on ice for 15 minutes. The white precipitate was subsequently pelleted at full speed for 10 minutes in a bench top microcentrifuge. The supernatant was transferred to a clean eppendorf, to which 2 volumes of 100% ethanol was added, and left at -20^0C for 20 minutes. The DNA was recovered by centrifugation for 20 minutes (maximum speed in a bench top microcentrifuge). The supernatant was removed, the DNA pellet washed in 70% (v/v) ethanol, dried in a heating block at 50^0C and resuspended in 20µl of TE. Final DNA concentrations were determined by gel electrophoresis.

2.2.17 Alkaline lysis plasmid purification from bacteria (large scale)

40-50ml of L-broth with the appropriate antibiotic selection was inoculated with one bacterial colony and left to grow overnight at 37^0C. The bacteria were pelleted by

centrifugation at 4000rpm in a Sorval SLA600 at 4^{0}C for 10 minutes and the supernatant removed. Plasmid DNA was obtained using a Nucleobond® AX cartridge kit (AB-Gene, Epsom, UK) according to the manufacturer's instructions. The plasmid DNA was resuspended in 100µl of TE and the DNA concentration determined by gel electrophoresis.

2.2.18 DNA sequencing

DNA sequences were determined for both plasmid DNA and PCR products. The automated DNA sequencing method is based on a combination of the cycle sequencing method (Murray, 1989) and the dideoxy sequencing method (Sanger *et al.*, 1977). 10µl reactions were set up per sample as follows, 4µl of BigDye terminator cycle sequencing kit (PE Applied Biosystems), 1.6pmol of primer, an appropriate amount of DNA as described by the manufacturers instruction manual, and water. The reaction cycle was as follows; 96^{0}C for 10 seconds, 50^{0}C for 5 seconds and 60^{0}C for 4 minutes, this was repeated a total of 25 times. The reactions were subsequently ethanol/salt precipitated, and analysed in an ABI PRISM 377 DNA sequencer. The sequences were analysed visually using the computer program Chromas version 1.45.

2.2.19 Electroporation of *Agrobacterium*

20µl of competent *Agrobacterium tumefaciens* LBA 4404 cells were transformed according to manufacturers instructions (Gibco BRL, Cheshire, UK). Cells were thawed on ice and mixed with DNA to be transformed. This was placed into a pre-chilled electroporation cuvette and subjected to 12.5KV/cm. Immediately 1ml of L-broth was added and the bacteria allowed to recover at 28^{0}C for 2-3 hours. The bacteria were spread on Minimal A plates with appropriate selection and grown for 48 hours at 28^{0}C. (All cosmids carried the tetracycline resistance gene, thus 5µg/ml of tetracycline was added)

2.2.20 Plasmid purification from *Agrobacterium*

A single colony of *Agrobacterium* was grown in 5ml of Minimal A media with appropriate selection (see above) until saturated. The bacteria were recovered by centrifugation at 10000 x ***g*** and resuspended in 100µl of solution I, to which 50µl of solution I with 12mg/ml lysozyme was added mixed and left for 10 minutes at room temperature. To this 300µl of solution II was added, mixed and left for 10 minutes at room temperature. 45µl of alkaline-phenol was added and briefly vortexed before the addition of 225µl of 3M potassium acetate pH 4.8. Cell debris was pelleted in a microcentrifuge at full speed, the supernatant was collected in a fresh tube and mixed with 500µl isopropanol on ice for 10 minutes. The DNA was pelleted for 10 minutes at full speed in a microcentrifuge and washed with 70% (v/v) ethanol. The DNA pellet was dried and resuspended in 50µl TE with RNAse A (100µg/ml final).

2.2.21 Tobacco and tomato lines

All isogenic lines of the tomato *Lycopersicon esculentum* cv 'Moneymaker' were obtained from J.Jones (Sainsbury Laboratory) and were originally developed by Tigchelaar (1984). The tomato rcr3-3 was generated by mutagenesis of Moneymaker and carries a homozygous knockout mutation of the tomato gene *Rcr3* (Dixon *et al.*, 2000).

Nicotiana tabacum cv. Petit Gerard is a derivative of *N. tabacum* cv. Petit Havana obtained from J. Jones (Sainsbury Laboratory). Petit Gerard is a near isogenic line (NIL) of Petit Havana that has been introgressed with the N gene, which confers resistance to tobacco mosaic virus (TMV) (G. Bishop and J. Jones, unpublished).

2.2.22 Maintenance of tobacco suspension cultures

Two-week-old *N. benthamiana* suspension cultures were obtained from and maintained according to the Sainsbury Lab at the John Innes Centre, Norwich. 10mls of culture was diluted into fresh sterile tobacco suspension media, in 250ml conical flasks and placed at room temperature in the dark on a shaker table. This was repeated every 14 days.

2.2.23 Sterilising tomato seeds for transformation

Seeds were bathed in 70% (v/v) ethanol for 2 minutes and washed in 10% (v/v) domestos® several times, the final wash (15ml per 60 seed) was left on for 3 hours. The domestos® was removed and the seeds washed several times in sterile water until no more foaming was observed, the final wash was left on over night. The water was removed and the seeds placed onto sterile seed germination media in sealable containers. All steps were carried out in a laminar flow cabinet. The seeds were placed in the cold (4^0C) and dark for at least two weeks to synchronise germination.

2.2.24 Feeder layer plates

The day before tomato transformations 2mls of a 10-14 day old suspension culture of tobacco cells were spread over co-cultivation media plates (without selection), and placed in indirect light at 25^0C over night. Just prior to transformations a filter paper was placed over the cells to complete the feeder layer.

2.2.25 Tomato transformations A

This protocol was modified from Horsch *et al.*, (1985). Tomato seedlings were grown aseptically from seed. Cotyledons were cut on a tile and immersed in a suspension of *Agrobacterium tumefaciens*. After blotting they were placed bottom surface down on co-cultivation media plates and left for 40-48 hours, before being washed with regeneration media and transferred to regeneration media plates supplemented with 100mg/l kanamycin for selection 500mg/l augmentin to inhibit *A. tumefaciens* growth. This protocol was never successful and lead to the development of a second protocol (tomato transformations B, see below).

2.2.26 Tomato transformations B

This second protocol was obtained from Matthew Smoker, Sainsbury Lab, John Innes Centre, Norwich. Sterile seeds were removed from cold store and placed in a light room one week prior to transformation (approximately 60 seeds of *L. esculentum* Cf0 *Tm2*^{2} were used in each transformation). Seedlings that had fully expanded cotyledons but

no expansion of the first true leaves were used for the transformations. Tomato cotyledons were cut underwater in a sterile petri dish using a sterile scapel and forceps. Cotyledon tips were removed and the coteyledon cut in two. A rolling action of the scapel was used to reduce tissue damage. Once all seedlings had been cut, explants were transferred to a second petri dish of sterile water to separate them from the waste tips. Explants were blotted on filter paper and placed abaxial side up on feeder layer plates and left for 4-6 hours in indirect light at 25^{0}C. Explants were subsequently transferred to a pertri dish containing a suspension of *Agrobacterium* and each one individually submerged in the solution. The *Agrobacterium* had been grown for 36-48 hours in minimal A media with tetracycline (1-2µg/ml) at 28^{0}C. To remove the tetracycline bacteria were pelleted, the supernatant removed and resuspended in an equal volume of tomato regeneration media. Explants were blotted on sterile chromatography paper to remove excess *Agrobacterium* and placed abaxial surface up on the same feeder layer plates for 40 hours in indirect light at 25^{0}C. Explants were placed adaxial surface up onto plates of tomato regeneration media with selection and Zeatin Riboside (2mg/l). Once reasonable sized shoots had developed they were cut from the explant and placed in rooting media. Once roots had developed the plants were transferred to soil. Agar was washed from the roots under a running tap, the plants placed in soil (Levingtons F2) and into a propagator to maintain a relatively high humidity. The humidity levels were gradually reduced before the plants could be placed in the glasshouse.

2.2.27 Tobacco transformation

The plasmids pRP005 and pRP006 were mobilized in tobacco by a modified method of Horsch *et al.* (1985). Fully expanded (not senescent) tobacco leaves were harvested and placed in an autoclaved glass dish and covered with 10% (v/v) Domestos with a piece of blue roll on top to ensure every leaf surface is in contact with the solution. Leaves were left for approximately ½ an hour or until the blue roll was bleached and washed with sterile water. Approximately 1cm^2 explants were cut (avoiding major vascular tissue) on a sterile tile with sterile forceps and blades. Explants were placed in a suspension of *Agrobacterium.* (*Agrobacterium* was treated as for tomato transformations), blotted and placed on tobacco regeneration media supplemented with 0.1mg/l NAA and 1.0mg/l BAP without selection for 48 hours. To remove *Agrobacterium* from their surface, explants were washed in tobacco regeneration media, blotted and placed on

regeneration media plates with selection. Once shoots were formed they were teased from the callus and placed on regeneration media without hormones with selection in autoclaved jars. Once roots were formed the putative tobacco transformants were treated as tomatoes (see above) and planted in soil.

2.2.28 Isolation of genomic DNA from Plant material (manual method)

1 to 2 cm^2 of young leaf tissue was harvested and placed in an eppendorf with a small amount of ground glass and 500µl of nuclear extraction buffer, and ground with a small plastic pestle (values in brackets represent large scale preparations where, 1g samples were ground with a pestle and mortar under liquid nitrogen and added to 5ml of nuclear extraction buffer). To this 100µl (1ml) of 5% (w/v) sarkosyl was added and the tubes inverted several times and incubated at 65^0C for at least 20 minutes. 500µl (5ml) Phenol/chloroform (1:1) was added and the sample thoroughly mixed by inversion. The phases were then separated by centrifugation at maximum speed in a microcentrifuge (3000rpm in a centaur 1 bench top centrifuge) for 2 minutes. The upper phase was carefully removed without disturbing the interface the rest was discarded. To the upper phase (approximately 500µl (5ml)) 300µl (3ml) of isopropanol was added. To aid the precipitation of the DNA this was mixed gently by inversion so the DNA was not sheared. The genomic DNA was recovered by centrifugation at full speed in a microcentrifuge (3000rpm in a Centaur 1 bench top centrifuge) for 2 minutes, the supernatant removed and the DNA pellet washed with 70% (v/v) ethanol. The pellet was dried at room temperature and resuspended in an appropriate volume of TE. Values in brackets represent a scaled up protocol.

2.2.29 Isolation of genomic DNA from Plant material (Kit method)

Up to 100mg of fresh plant material was harvested and ground under liquid nitrogen, DNA was subsequently purified using either the Qia DNeasy kit (Qiagen, Crawley, UK) or the Nucleon® phytopure plant DNA extraction kit (Novagen, supplied by Merk Biosciences, Nottingham, UK) according to the manufacturers instructions.

2.2.30 Immobilisation of DNA

In a large volume, 6µg of tobacco or 5-6µg of tomato genomic DNA was restricted with an appropriate restriction enzyme over night at 37^{0}C. The digested DNA was concentrated by ethanol salt precipitation and resuspended in a smaller volume of 1x orange G loading dye (25µl). Samples were then loaded onto a 0.7% (w/v) agarose gel and run at 2-3V/cm overnight, until adequate separation had been achieved as visualised by ethidium bromide staining.

The gel was etched by bathing in 0.25M HCl for 15-20 minutes. The DNA was subsequently denatured in denaturation buffer, for 30 minutes, excess alkali was removed by washing with distilled water and neutralised in neutralisation buffer for at least 30 minutes. The DNA was transferred to a Hydond N membrane (Amersham Pharmacia, Little Chalfont, UK), which had been soaked in 6x SSC for a few minutes, by the technique of Southern blotting (Southern, 1975). The transfer buffer used was 6x SSC and the blot was allowed to transfer overnight. The DNA was permanently fixed to the membrane using UV cross linking or baking. The membrane was wrapped in Saran wrap, placed DNA side down on a UV transilluminator for 1.5 minutes and dried at room temperature between two pieces of 3MM paper, or blots were sandwiched in 3MM paper and baked at 80^{0}C for 2 hours.

2.2.31 Production of a ^{32}P labelled DNA probe

The gene fragment used for Southern analysis was amplified by PCR. Amplification of the correct product was tested by agarose gel electrophoresis, the probe was then excised from the gel and purified using a Machery-Nagel Nucleospin® kit (AB-Gene, Epsom, UK).

The probes was labelled according to manufacturers instructions (Amersham Pharmacia , Little Chalfont, UK). Approximately 20-25ng of Probe DNA was added to TE buffer pH 8.0 to a final volume of 50µl. The DNA was denatured at 100^{0}C for 5 minutes then immediately cooled on ice. Probe DNA was transferred to a rediprime tube (Amersham Pharmacia , Little Chalfont, UK) with 5µl (1.85MBq) ^{32}P dCTP (Amersham

Pharmacia, Little Chalfont, UK) and incubated for 10 minutes at 37^{0}C. The reaction was stopped with the addition of 5µl 0.2M EDTA.

2.2.32 Hybridisation of DNA probes

Membranes were pre-hybridised in Church and Gilbert buffer (0.3M sodium phosphate buffer pH7.5, 1% (w/v) BSA, 7% (w/v) SDS) for at least 30 minutes at 65^{0}C (Church and Gilbert, 1984). This was replaced with fresh Church and Gilbert buffer containing the denatured radioactive probe. Membranes were hybridised overnight at 65^{0}C.

Membranes were washed twice with sodium phosphate wash buffer (100mM sodium phosphate buffer pH7.5, 1% (w/v) SDS) and several washes of 0.1% (w/v) SDS in 2x SSC, until no more radioactivity was washed from the blots. Each wash was carried out at 65^{0}C for 15 minutes. Membranes were wrapped in saran wrap and subsequently autoradiographed with intensifying screens at –80^{0}C using Kodak film.

2.2.33 Testing segregation of the transgene

Tobacco plants that tested positive for the transgene by Southern, northern or RT-PCR were allowed to flower. The seed pods containing F1 self-seed were collected, one pod was sterilised by wiping the surface with 70% (v/v) ethanol. A hole was cut into the pod with a sterile scalpel and the seed sprinkled over MS media plates with 300µg/ml kanamycin. The seeds were placed in a light room at 25^{0}C, after germination the seedlings were left to grow. Each parental plant was scored by the ratio of bleached to unbleached seedlings (bleaching was caused by the absence of the kanamycin resistance gene that is linked to the transgene and hence indicated the absence of that transgene.).

Putative tomato transformants were tested by sterilisation of the seed collected from selfed plants. The seed were spread over germination media supplemented with 100µg/ml kanamycin and left for 2-3 weeks. Plants lacking the transgene were scored by eye and appeared stunted in growth and purple in colour. Confirmation of plants positive for the transgene was achieved by PCR to amplify the NPT gene for Kanamycin resitance.

2.2.34 RNA extraction

Total RNA was extracted from samples using a Qia RNeasy plant RNA kit according to manufacturers instructions (Qiagen, Crawley, UK). Samples of young leaf tissue (100mg) were ground under liquid nitrogen in an eppendorf, with a small pestle. To this, kit buffer was added, the ground material was homogenised in a kit Qia shredder column before being transferred to the extraction column. Total RNA was eluted from the column with 30-50µl of RNAse free water and stored at -80^0C. RNA was quantified on 0.7% (w/v) agarose gels by comparison with known molecular weight markers.

2.2.35 Reverse transcription PCR (RT-PCR)

RT-PCR was performed according to manufacturers instructions (Gibco BRL, Cheshire, UK). 5µg of total RNA was diluted in 10µl of water and denatured at 72^0C for 5 minutes then quenched on ice. To each tube 4µl of 5x buffer (Gibco BRL, Cheshire, UK), 2µl 0.1M DTT, 2µl 5mM dNTPs, 5U (1µl) RNase inhibitor and 1µl 500ng/µl oligo-dT was added. The mixture was heated to 37^0C for two minutes before the addition of 0.5µl of the RT enzyme superscript (Gibco BRL, Cheshire, UK). The reaction was carried out at 37^0C for one hour, the enzyme was denatured at 75^0C for 10 minutes and the cDNA products stored at -20^0C.

The cDNA was cleaned using a Machery-Nagel Nucleospin® kit (AB-Gene, Epsom, UK) according to manufacturers instructions. The cDNA was eluted in 40µl of water, 5-8µl of which was used in a 20µl PCR. The products were then separated on a 1% (w/v) agarose gel and analysed by comparison with size standards.

2.2.36 Northern analysis

Samples of 10µg of total RNA were diluted with 1.625 volumes of sample buffer (1ml formamide, 300µl formaldehyde, 250µl 10x MEN, 0.025% (w/v) orange G) and separated on a 1.5% (w/v) denaturing agarose gel (1x MEN, 16.5% (v/v) formaldehyde). The gel was run at 7V/cm for 2 hours (until adequate separation had been achieved) in 10x MEN running buffer. All samples were denatured at 65^0C for 5 minutes prior to loading.

After separation RNA was transferred to Hybond-N membrane (Amersham Pharmacia, Little Chalfont, UK) by the technique of northern blotting.

Membranes were hybridised, washed and developed according to the same protocol as Southern analysis and with the same probe.

2.2.37 Stripping blots

Blots were stripped by washing twice with water for 15-30 minutes each wash at 80^0C. Blots were then air dried between 3MM paper or placed straight into pre-hybridisation buffer ready to be re-probed.

2.2.38 Microsomal membrane preparations

Micorsomal membranes were prepared according to the modified version of Piedras *et al.* (1998). 20-30g of leaves were homogenised in 4ml/g of tissue of lysis buffer (25mM Tris-HCl pH7.5, 0.5M sucrose, 3mM EDTA, 0.5mM AEBSF, 1 tablet/50ml of complete protease inhibitor cocktail, Roche, Lewes, UK) using a food liquidiser. Samples were pulsed at maximum speed 2-4 times for 10 seconds. The homogenate was filtered through four layers of Miracloth (Calbiochem, San Diego, USA) to remove large pieces of cell debris. The flow through centrifuged at 7000 x ***g*** for 10 minutes to pellet smaller fragments of cell debris, cell nuclei and mitochondria. This cell debris pellet was resuspended in protein gel loading buffer (62.5mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol/2mM DTT, 0.001% (w/v) bromophenol blue). The supernatant containing the microsomal membranes was recovered and centrifuged at 100,000 x ***g*** for 1 hr. The pelleted microsomal fraction was resuspended in a small volume of microsome resuspension buffer (5mM potassium phosphate buffer pH7.8, 0.33M sucrose, 3mM KCl, 1-2mM DTT, 1mM AEBSF). Membranes were crudely resuspended by piprtting and homogenised in a 1ml glass homgeniser (Fischer). All steps were carried out at 1-4^0C.

2.2.39 Tobacco plasma membrane enrichment by aqeous two-phase partitioning

Plasma membranes were purified using the modified method of Yoshida *et al.* (1983). Two days prior to phase partitioning a stock of 20% (w/v) Dextran T500 was prepared (84.25g water, 22g Dextran T500) this was dissolved overnight by stirring gently. One day prior to phase partitioning a 40% (w/v) stock of PEG 3350 is made along with a bulk phase system, final composition 6.4% (w/w) dextran T500, 6.4% (w/w) PEG 3350, 330mM sucrose, 3mM KCl, 5mM potassium phosphate buffer pH 7.8. The bulk phase was mixed and placed in a separating flask and allowed to separate overnight at 1^0C. A 32g phase was also set up with the same final composition as the bulk phase; it was made up to only 28g to allow for the addition of the microsomal membranes, this tube was labelled U_1. Upper and lower phases were separated by running off the lower phase, care was taken not to disturb the interface. Microsomal membranes were prepared from 100g of tissue (as above) and resuspended in 1.6mls of, 5mM potassium phosphate buffer pH7.8, 0.33M sucrose, 3mM KCl, 1-2mM DTT (add fresh), 1mM AEBSF (add fresh). Microsomes were added to the phase system in the tube labelled U_1 and made up to 32g with water and mixed by inversion 20-30 times. Phases were separated by centrifugation at 2000 x ***g*** for 10 minutes in a swing out rotor. Upper and lower phases were repartitioned with fresh lower and upper phases respectively to produce U_2 and L_2 (i.e. the two phases were separated and fresh upper phase was added to the lower phase, and the upper phase added to fresh lower phase). U_3 and L_3 were produced by repition of the repartitioning. It was extrememly important to maintain the total phase weight at 32g (to 3dp) to ensure adequate separation. U_3 and L_3 were diluted 10 fold in 5mM potassium phosphate buffer pH7.8, 330mM sucrose, 3mM KCl, 1mM AEBSF and membranes pelleted at 100,000 x ***g*** for 1 hour. Membranes were resuspended in a small volume (as for microsomes).

2.2.40 Protein quantification by Bradfords assay

Protein extractions (microsomal or fractionated membranes) were quantified as described by Bradford (1976). Standards of 0-10µg BSA were made in 10µl of buffer (same buffer as samples to be quantified). These were placed in a 96 well plate along with 10µl of each sample (diluted appropriately) to be quantified. 100µl of coomassie solution

(0.01% (w/v) Coomassie Brilliant blue G-250, 4.7% (v/v) ethanol, 8.5% (w/v) phosphoric acid and water up to 1l) was added to each well in quick succession and read immediately on a plate reader for absorbtion at 540nm. A standard curve was drawn using the BSA controls and sample concentrations derived using the curve.

2.2.41 Glycosidase treatment of proteins

Up to 2mg of protein was denatured in 1x Denaturing buffer (New England BioLabs, Hitchin, UK, according to manufacturers instructions) at 100^0C for 10 minutes. 1/10 volume 10x G5 buffer (Endo H_f) or 1/10 volume 10x G7 buffer and 10% NP-40 (PNGase F) was added. Carbohydrates were removed by the addition of 5µl of enzyme (New England BioLabs, Hitchin, UK) to the protein buffer mix incubated at 37^0C.

2.2.42 SDS-polyacrylamide gel electrophoresis

Protein preparations were separated by SDS polyacrylamide gel electrorophoresis (Laemmli, 1970). This was performed using a Mini-Protean II apparatus (Bio-Rad, UK), producing a 0.75mm or 1mm thick gel. The gel comprised a 5cm deep resolving gel (final composition: 12.5% (w/v) or 7.5% (w/v) acrylamide (30% pre-mix acrylogel monomer, Bio-Rad), 0.001% (w/v) SDS, 0.375M Tris-HCl pH8.8, 0.001% (v/v) TEMED, 0.0005% (w/v) APS), and a 2cm deep stacking gel (4.5% (w/v) acrylamide (30% pre-mix acrylogel monomer, Bio-Rad, Hemel Hempstead, UK) 0.001% (w/v) SDS, 0.125M Tris-HCl pH6.8, 0.001% (v/v) TEMED, 0.0005% (w/v) APS). An aliquot of each sample was injected into each well alongside molecular weight markers. The samples were run in electrode buffer (25mM Tris, 3.5mM SDS and 0.2M glycine) at a constant of 100V for 2-3 hours at room temperature or at 200V for 1 hour at 4^0C.

2.2.43 Immunoblotting procedure (western blot)

After separation by SDS-PAGE proteins were electrotransferred to a membrane (hybond P, Amersham Pharmacia, Little Chalfont, UK) using a Bio-Rad mini transblot apparatus, according to the manufacturer's instructions (Bio-Rad, Hemel Hemstead, UK). The membrane was wetted in methanol for 10 seconds, followed by distilled water for 5 minutes. Membranes, filter papers and gels were soaked in cold transfer buffer (20% (v/v)

methanol, 20mM Tris and 0.15M glycine) for 30 mins at 4^0C prior to blotting. Gels were blotted for 1 to 2 hours at 90V at 4^0C. Staining the membrane with Ponceau S assessed blotting efficiency and gel loading levels. To remove methanol, the membrane was washed in Tris-Buffered saline (TBS) (50mM Tris, 0.15M NaCl, pH 7.5).

2.2.44 Immunodetection by chemiluminescence

Detection of proteins after separation and blotting described above was performed using Roche chemiluminescence western blotting kit according to manufacturers instructions (Roche, Lewes, UK). This kit uses the substrate luminol and peroxidase-labelled secondary antibodies.

To prevent non-specific protein binding membranes were incubated in a 1% (v/v) solution of kit blocking reagent diluted in TBS, for 1hr at room temperature. After washing in TBS, membranes were incubated for 1hr at room temperature in a suitable dilution of antibody in 0.5% (v/v) kit blocking reagent.

Non-bound and non-specifically bound primary antibody was removed with 2x 10 minute washes in TBST (TBS with 0.1% (v/v) polyethylenesorbitan / Tween 20). The membranes were further blocked with 2x 10 min washes in 0.5% (v/v) blocking reagent before incubation with 40mU/ml of kit horseradish peroxidase conjugated secondary antibody for 30 minutes at room temperature. Excess antibody was removed with 4x 15 min TBST washes. Probed membranes were then covered in 10 ml of detection solution for one minute. Excess solution was drained and the membrane placed between two acetate sheets. This was placed in a film cassette with a sheet of Kodak film adjacent to the protein side of the membrane. An initial exposure of 5 - 60 seconds was used, followed by exposures of up to 2 hours. Films were developed immediately. All detection and subsequent exposure was performed using a safe red light.

2.2.45 Quantification of signal (desitometry readings)

To quantify signal intensities of western blots, the blots were scanned using an Alphaimager™ 1200 and density reading taken using the PC package AlphaEase™ (Alpha Innotech, supplied by GRI, Braintree, UK). Spot areas were highlighted and all

linked to a region of background. The AlphaEase™ software produced and output spot densities, taking into account background signal intensities. Density readings were subsequently transported into an Excel spreadsheet. Here relative densities were calculated taking the control as1 (i.e. all values were divided by the control density).

2.2.46 Drying of polyacrylamide gels

Gels were fixed in de-stain solution (10% (v/v) acetic acid, 30% (v/v) methanol) for 30 minutes at room temperature. The gels were placed on a piece of 3mm paper, covered with clingfilm and placed in a vacuum gel dryer for 60 minutes.

2.2.47 Cytochrome c oxidase assay

Cytochrome C enzyme activity was determined as described by Schaller and De Witt (1995). In a 1ml cuvette, 10µg of total protein was made up to 33µl (with microsome suspension buffer) to which, 33µl of 0.3% (w/v) digitonin was added, mixed well and left for 30 seconds. 901µl of 50mM phosphate buffer pH 7.5 was added and the cuvette placed into the spectophotometer to calibrate the base line. The reaction was started by the addition of 33µl of 0.6mM cytochrome c and the reaction recored by the spectophotometer. The reaction was carried out at room temperature and the absorbance was read at 55nM with the plotter set at Km min^{-1}. The rate of reaction was determined by using the initial linear rates and the extinction coefficient for cytochrome c of 18.5 mM^{-1} cm^{-1}.

2.2.48 *In vitro* Transcription

The Promega (Southampton, UK) Ribomax SP6 transcription system was used to perform *in vitro* transcription and performed ccording to manufacturers instructions. Linearised DNA (up to 10µg) was initially processed using the Promega (Southampton, UK) Wizard PCR purification kit to remove all enzymes and any impurities. The reactions were supplemented with a 5' cap analogue: $m^7G(5')ppp(5')G$ (New England Biolabs, Hitchin, UK). Reactions were carried out in alloquots of 50µl (up to 10µg of linear template, 5µl SP6 RNA polymerase, 0.57mM GTP, 4mM ATP, 4mM CTP, 4mM UTP 3mM cap analogue and water up to 50µl). Reactions were incubated for 4 hours at

37^{0}C. The RNA was subsequently purified using Qia RNeasy purification kit (Qiagen, Crawley, UK), eluted with 50µl of RNase free water and stored at –80^{0}C.

2.2.49 *In vitro* Translation

The Promega (Southampton, UK) Nuclease treated rabbit reticulocyte lysate system was employed to perform *in vitro* translation and perfomed according to manufacturers instructions. 10µl reactions (2µl purified mRNA from *in vitro* transcription, 5µl rabbit reticulocyte lysate, 1µl amino acid mix minus methionine, 0.5µl (7.5µCi) L-[35S] methionine, 0.5µl RNasin (RNase inhibitor, Promega, Southampton, UK), 1µl water. Before addition to the reaction mixture the RNA was denatured, by heating to 65^{0}C for 5 minutes, to disrupt any secondary structure. Reactions were carried out at 30^{0}C for 90 minutes and were stopped by placing on ice.

The peptides produced were separated by SDS-PAGE, the gel dried and exposed to X-ray film (Kodak).

2.2.50 Tomato seed processing

To obtain seeds, ripe fruit was collected, cut open and the seeds squeezed out. To remove flesh, seeds were placed in 5% (v/v) HCl for 20-45 minutes. To remove all HCl seeds were washed with water in a sieve, blotted and air-dried.

2.2.51 Culturing of *Cladosporium fulvum*

Cultures of various races of *C. fulvum* were kept at room temperature on plates of ¼ PDA media. Spores were suspended in sterile water and spread over the new plate with a sterile glass spreader.

2.2.52 Inoculating tomato plants with *C. fulvum*

Water was added to the surface of two plates of fungus, spores were released by rubbing over the plate with a gloved finger and transferred to a beaker. This was repeated several times on each plate to remove a majority of the fungal spores. The spore

suspension was made up to a suitable volume using sterile water (to approximately 10^5 spores/ml). Each plant was in turn inverted and completely immersed in the spore suspension. The relative humidity was kept above 80% in a humidity tent to increase infection efficiency. This was best carried out in the glasshouse in spring and autumn (high temperatures and low light levels resulting in poor or failed infections). To prevent plants in the humidity tent growing too tall the gibberellin biosynthesis inhibitor, paclobutrazol, was added as a soil drench: 50µl of the stock solution, 100mM paclobutrazol in methanol, was diluted in 500ml of water (10^{-5}M final) and 40ml of this was added to each 8cm pot. The plants were left for 10-15 days before being scored by eye for infection. Appropriate controls were treated in the same manner. A Cf0 plant was used as a positive control for infection. and various plants carrying appropriate *Cf* genes were also inoculated as negative controls.

2.2.53 Scoring of resistance

After 10-15 days plants were scored for fungal growth by eye, looking for a white downy growth on the underside of infected leaves.

Where race 4*GUS* was used, 1 or 2 inoculated leaves were harvested and assayed qualitatively or quantitatively or both for GUS activity.

2.2.54 Qualitative analysis of GUS activity (GUS assay)

C. fulvum race 4*GUS* is a race of *C. fulvum* expresses the *uidA* reporter gene (Oliver *et al.*, 1993). The transgene *uidA* is a histochemical marker of GUS expression in cells. X-gluc is a substrate for β-D-Glucuronidase (GUS) encoded by the *uidA* transgene. Cleavage of X-gluc results in the formation of a water insoluble blue dye dichloro-dibromo-indigo precipitate, which forms at the site of enzymatic cleavage. This indigo dye can then be used to indicate the presence of the fungus qualitatively

Staining of the samples was performed using a modified protocol from Oliver et al., (1993). Samples were vacuum infiltrated with X-Gluc stain (1mM EDTA, 0.5mg/ml X-gluc in DMF, 50mM phosphate buffer pH 7.0, 0.05% (v/v) Triton X-100), which acts as

the substrate for the GUS gene to produce a blue product. Leaves were subsequently placed in 70% (v/v) ethanol for 2 days to remove chlorophyll (several changes of ethanol were used), thus facilitating visualisation of the blue stain. Images of the leaves were made using either an Alpha Imager™ 1220 (Alpha innotech, supplied by GRI, Braintree, UK) or by scanning leaves.

2.2.55 Quantitative assay of GUS activity (MUG assay)

For quantitative assessment of the level of fungal infection, the GUS substrate 4-MUG (4-methylumbelliferyl-β-D-glucuronide) is used. Cleavage of 4-MUG by GUS activity results in the generation of the fluorogenic product 4-MU (7-hydroxy-4-methylcoumarin). The 4-MU can be detected or visualised by irradiation with UV light. A fluorimeter was used to quantify GUS activity with 4-MU at a peak excitation of 365 nm (UV) and peak emission of 455 nm (blue).

Samples were prepared according to Oliver et al., (1993). Samples were ground in 0.5ml of GUS assay buffer (50mM sodium phosphate buffer pH 7.0, 5mM DTT, 1mM EDTA), snap frozen in liquid nitrogen and stored at -80^0C. 1.44ml aliquots of stop solution (0.4M Na_2CO_3) was placed into an appropriate number of Fluorometric cuvettes. Standards were prepared, a blank consisted of 1.44ml of stop solution with 180µl GUS assay buffer, a 500pmole control was prepared as the blank with the addition of 500pmoles of 4-MU. Reaction tubes (800ul GUS assay buffer, 1.25mM MUG) were placed in a heating block set at 37^0C. Samples were thawed, vortexed and debris pelleted by centrifugation at 13000 x ***g*** in a bench top centrifuge for 3 minutes. 200µl of the supernatant was added to the reaction tube, from which 180µl was removed immediately and placed in stop solution for a time zero reading. This was repeated every 30 seconds on subsequent samples. It was sometimes necessary to dilute the sample in 200µl GUS assay buffer prior to addition to the reaction tube. A second 180µl sample was removed from the reaction tubes at a suitable time determined by a pilot assay (usually between t = 5mins and t = 20 mins). Standards were used to calibrate a fluormeter (TD-360, supplied by GRI, Braintree, UK) and samples were read. Protein concentrations of the samples were calculated by a Bradfords assay and activity was calculated in the units, pmoles 4-MU/min/µg protein.

2.2.56 Preparation of intercellular fluid (IF)

For IF containing fungal elicitors susceptible plants were inoculated with *C. fulvum*. Once a high level of infection had been obtained all infected leaves were harvested. To obtain the IF, leaves were vacuum infiltrated with water, the excess water removed, rolled up and placed in to a 50ml syringe with the tip cut off. The syringe cylinder was placed in a falcon tube and then centrifuged at approximately 5000 x *g* for 5 minutes, or until the IF had collected in the falcon tube. The IF was stored at -80^{0}C until used.

2.2.57 Infiltration of plant leaves

A 1ml syringe was filled with the fluid to be infiltrated into the leaf and any air bubble pushed out. The syringe end was placed on the bottom surface of the leave between main veins. A moderate amount of pressure was applied to the other side of the leaf and the fluid pushed into the leaf. The amount of pressure used was enough to prevent the fluid running over the leaf surface but not enough to push the syringe into the leaf tissue, damaging it.

Chapter Three

3 Engineering epitope-tagged versions of Cf-2

3.1 Introduction

The *Cf* genes are predicted to encode extracytoplasmic, membrane anchored leucine rich repeat (LRR) glycoproteins (Dixon *et al.*, 1996; Dixon *et al.*, 1998; Jones *et al.*, 1994; Thomas *et al.*, 1997). They are found in two complex loci and comprise tandemly repeated homologous genes called *Hcr* (homologues of *Cf* resistance) genes (Thomas *et al.*, 1998).

The major aim of this project was to determine the subcellular localisation of Cf-2; several methods could have been employed to achieve this. The first, involves raising antibodies directly against Cf-2. The antibodies could then be used for either microscopy, e.g. using gold-coupled antibodies and electron microscopy, or for immunocytochemistry. Oshima *et al.* (2001) used antibodies raised against plant aquaporins to quantify the aquaporin content of plasma membranes and tonoplast of *Graptopetalum paraguayense*. One major problem with this approach is cross-reactivity of the antibodies; indeed the antibodies of Oshima *et al.* (2001) react with most aquaporin isoforms. Due to the presence of many Cf-2 homologues and the number of potential glycosylation sites, it would be almost impossible to produce a specific antibody.

A second possible approach involves the production of a fusion protein. Conventional fusion proteins involve the addition of a large protein such as green fluorescent protein (GFP), β-galalctosidase, alkaline phosphotase, glutathione S-transferase and protein A. The choice of fusion protein depends upon the questions to be answered. The fusion protein glutathione S-transferase can be used for the purification and immobilisation of proteins, useful for 'fishing' for interacting proteins. Hu *et al.* (2004) engineered a protein, where a portion of the Hepatitis B virus (HBV) large surface protein was fused in duplicate to GST. Antibodies against this portion of the protein possess both virus-neutralising activity and a protective effect. The fusion protein was subsequently expressed in *E. coli* and purified by affinity chromatography. The purified

fusion protein was used to detect the presence of the neutralising antibody in HB patients with satisfactory results.

A GFP fusion protein would be expressed in transgenic organisms/cells and using microscopy (and the fluorescent properties of GFP) it would be possible to visualise where in the cell the protein is targeted. This was the approach used by Kircher *et al.* (2002), who demonstrated light regulated nuclear targeting of the phytochromes A-E in *Arabidopsis*. A GFP fusion protein could not, however, be used for analysis of the glycosylation status of the protein. It is also possible that due to the size of these marker proteins that they may affect the biological properties of the target proteins.

The third approach, which was employed for this project, combines the two above methods. Firstly, a special kind of fusion protein was engineered; this involved inserting the sequence of an epitope-tag into *Cf-2*. Thus when expressed *in planta*, the Cf-2 protein carried an antigenic region. Epitope tagging was first described by Monro and Pelham (1984) in Drosphila and has some major advantages over the other approaches. Firstly, specific antibodies, both polyclonal and monoclonal, are commercially available to many-epitope-tags. This negates the need to characterise the antibodies and conditions for their use. In addition, as epitope-tags are small (generally 6-30 amino acids) and with careful selection of their insertion sites, they often do not affect the biological activity of the tagged protein (Jarvik and Telmer, 1998). Finally, epitope-tagging with immunocytochemistry provides an extremely sensitive method for detection of proteins, as multiple copies of the epitope-tag can be inserterted in tandem (Jarvik and Telmer, 1998).

There are many epitope-tags available, for example; c-Myc, a portion of the human c-Myc gene product; HA, the human influenza virus haemagglutenin, HA1; His6, a recombinant histadine-tagged fusion protein and T7 the leader peptide of phage T7 major caspid protein (Jarvik and Telmer, 1998). The choice of the epitope-tag depends upon the position and protein to be epitope-tagged and the purpose of the tagging. For example, the HA epitope is better detected when placed at the N-terminus of a protein (Rockland inc., PA, USA, anti HA antibody technical data). The His6 epitope-tag can, not only be used as a typical epitope-tag, but can also be used for the affinity purification of proteins from cell lysates. Due to the lack of anti His-6 antibodies with high specificity and low cross-reactivity, it is often used in conjunction with an additional epitope-tag (Jarvik and

Telmer, 1998). Kruger *et al.* (2002) employed a double epitope-tag approach. They tagged the tomato Rcr3 protein with both the His6 and HA tags. The HA tag was used for immuno-localization of the protein and the His6 tag for purification of it.

For this project it was decided to use the c-Myc epitope-tag tandemly repeated 3 times (3x (triple) c-Myc). Myc proteins function in cell proliferation, differentiation and neoplastic disease. Anomolous function of the c-Myc gene has been implicated in many human tumours including lung, breast and colon carcinomas. This epitope-tag was chosen as it had been employed in the same system to study the localisation of Cf-9 by Piedras *et al.* (2000). In addition, it was available free of charge.

The acidic, basic and putative transmembrane domains of the Cf proteins are proposed to serve solely as a membrane anchor. Piedras *et al.* (2000) produced two 3x c-Myc epitope-tagged versions of Cf-9 with the epitope inserted into either the N-terminal portion of the protein or in the predicted cytoplasmic tail. If the regions into which the epitope-tags were placed were important in recognition or signalling, a loss of function would be expected. Both versions were functional in tobacco, giving support to the idea that the very C-terminus serves no function other than as a membrane anchor. For this reason it was decided to insert the epitope-tag into the very C-terminus of Cf-2 between the ultimate and penultimate amino acids. Due to the relatively low expression levels of *Cf-2* (Dixon, unpublished), two constructs were built, the first using the native promoter and the second using the stronger CMV (cauliflower mosaic virus) 35S promoter.

3.2 Results

It was decided to use the 3x c-Myc epitope-tag and insert it into the putative cytoplasmic tail of Cf-2, as this strategy was successful for Cf-9 (Piedras *et al.*, 2000). In addition, insertion of an epitope-tag into the putative membrane anchor was predicted not to affect function. Due to the absence of any convenient restriction enzyme sites, one was introduced by site directed PCR mutagenesis. The first step taken to introduce the epitope-tag was to clone the portion of the gene encoding the C-terminus of the protein. A 1.2 kb *Eco*RI to *Bam*HI fragment which starts from within LRR 37 and encompasses the regions encoding the transmembrane region, the membrane anchor and the stop codon of the gene was chosen (Figure 3.1). This relatively small fragment was

chosen, as smaller fragments are easier to clone. Also subsequent steps would involve PCR, using a smaller fragment reduced the risk of spontaneous mutations caused by this process.

Site directed mutagenesis was used to create an *Aat*II site at the very C- terminus of the cytoplasmic tail, into which the 3x (triple) c-Myc epitope was inserted. The penultimate codon of *Cf-2* (TTC) encodes phenylalanine, which through PCR mutagenisis was changed to GTC, which corresponds to part of the *Aat*II restriction site and encodes valine. This change in amino acid being confined to the putative membrane anchor was predicted to have little or no effect on function of the transgene.

An internal region of the c-Myc gene was used, in triplicate, as the epitope-tag due to the availability of anti c-Myc antibodies. An *Aat*II restriction site was chosen, as it was absent from the cloned portion of *Cf-2* and the vector used for subcloning. The dark Bluescript II (pdBSII) vector was used when cloning the C-terminal portion of *Cf-2*. The dark pBS was obtained from the John Innes Centre. It was discovered accidentally when an unsuccessful attempt to fill the *Kpn*I resulted in the in frame insertion of an extra amino acid in the polylinker site. The resulting plasmids expressed in bacteria produce colonies that are a darker blue than colonies expressing the normal pBS when induced by IPTG in the presence of the substrate X-Gal. The production of a more intense colour made negative colonies easier to differentiate from transformed colonies. Due to technical difficulties several strategies were employed.

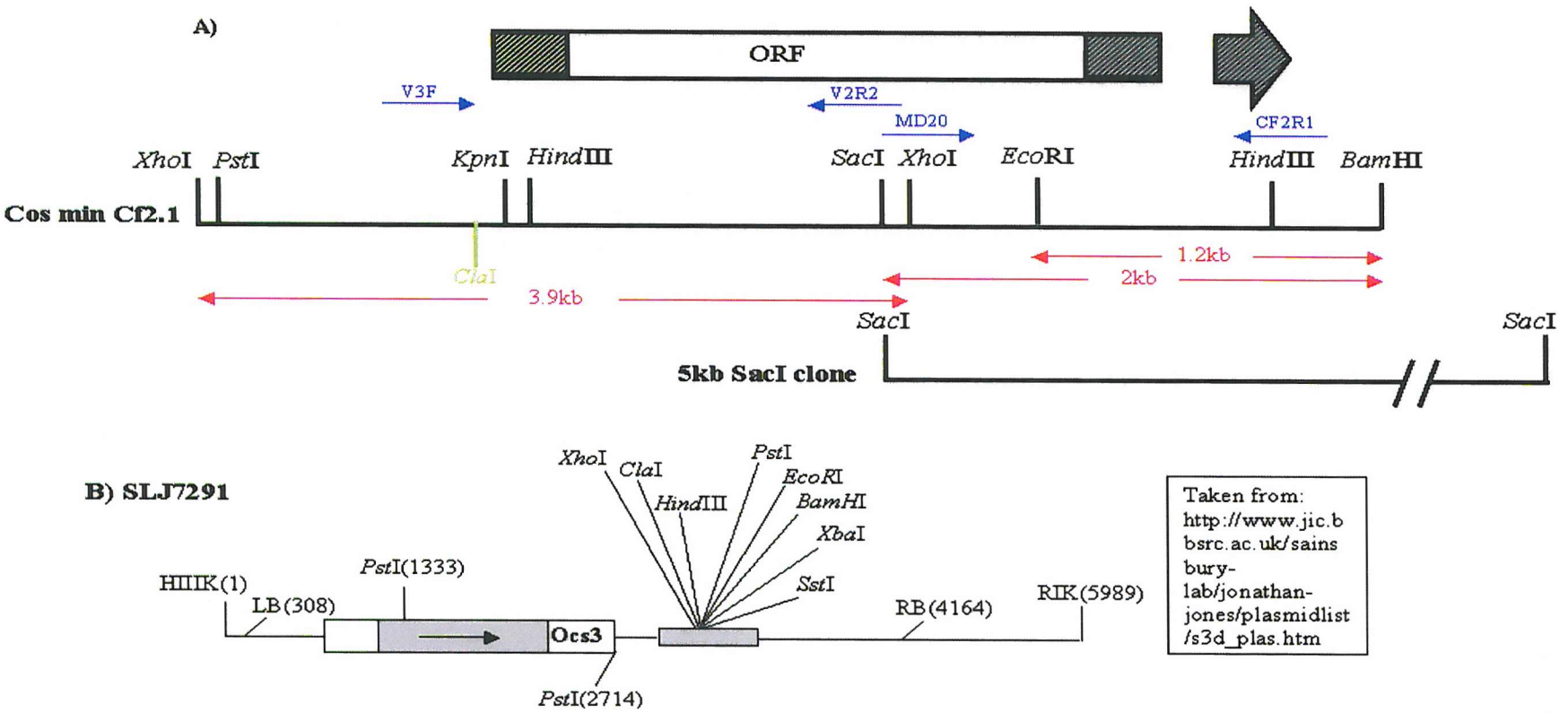


Figure 3.1: A schematic of the cosmid min Cf2.1 (Dixon et al., 1996).

The Cf2.1 gene (not to scale) (A) was engineered into the cloning vector pSLJ 7291 (B) <http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/plasmidlist/s3d_plas.htm> (Dixon *et al.*, 1996). A) The large arrow indicates the mRNA transcript with a gap indicating an intron. The shaded areas indicate untranslated regions and the inshaded area indicates the open reading frame (ORF). The position of the 5Kb *Sac*I clone is shown. Primer annealing sites and names are represented by blue arrows and text. The *Cla*I restriction site (shown in green) was introduced into the constructs described below, at the start of the ORF to aid cloning and is not found in min Cf2.1. B) HIIIK signifies a *Hind*III site filled in with Klenow polymerase and dNTPs; RIK indicates and *Eco*R1 site similarly treated; LB signifies T-DNA left border repeat sequence; RB signifies the right border repeat; nos indicates the nopaline synthase promoter; ocs 3' indicates octopine synthase 3' end; NPT indicates neomycin phosphotransferase; Gene transcriptional orientations are indicated by an arrow. All SLJ plasmids, including pSLJ 7291 are derived from the broad-host-range vector pRK290 (Friedman *et al.*, 1982).

Initially the 5kb *Sac*I clone (Figure 3.1) was restricted with *Eco*RI and *Bam*HI simultaneously in Multicore buffer (Promega, Southampton, UK). Restriction fragments were separated on a 0.7% (w/v) agarose/TAE gel and the fragment approximately 1.2kb in size was purified from the gel using a Machery-Nagel Nucleospin® kit (AB-gene, Epsom, UK). This fragment was subsequently ligated into the pdBSII vector and used to transform *E. coli* strain DH5α. Plasmid DNA was purfied from 12 putative positive colonies, those positive by size comparison with an empty vector were restricted with *Eco*RI and *Bam*HI. *Eco*RI restricted none of the clones obtained. Sequencing of these clones revealed that the initial *Eco*RI digest had occurred at a cryptic site (GAATTT) approximately 200bp downstream of the actual *Eco*RI site (GAATTC) resulting in a 1kb fragment. When restricted, this cryptic site was left with the same sequence overlap as a correctly restricted *Eco*RI fragment and thus was able to ligate with the vector (pBSII). All of the recovered clones corresponded with the insertion of the 1kb. In attempt to overcome this problem, restrictions were performed individually in the preferred buffer of each enzyme (buffer E for *Bam*HI and buffer H for *Eco*RI; Promega, Southampton,UK). After the initial digest the enzyme was heat inactivated at 60^0C for 10 mins and the restricted DNA cleaned and salts removed by ethanol salt precipitation prior to resuspension in the second restriction buffer. The restricted DNA was separated on a 0.7% (w/v) agarose/TAE gel (Figure 3.2). The band of approximately 1.2kb was excised from the gel, purified and transformed as before. The fragment excised from the gel probably contained a mixture of the 1kb and 1.2kb fragments, as several attempts resulted in the cloning of the truncated insert.

A clone of the 1kb cryptic *Eco*RI, *Bam*HI fragment in pBSIIks was named pRP001 (Figure 3.3). As a consequence, alternative strategies were devised and attempted simultaneously.

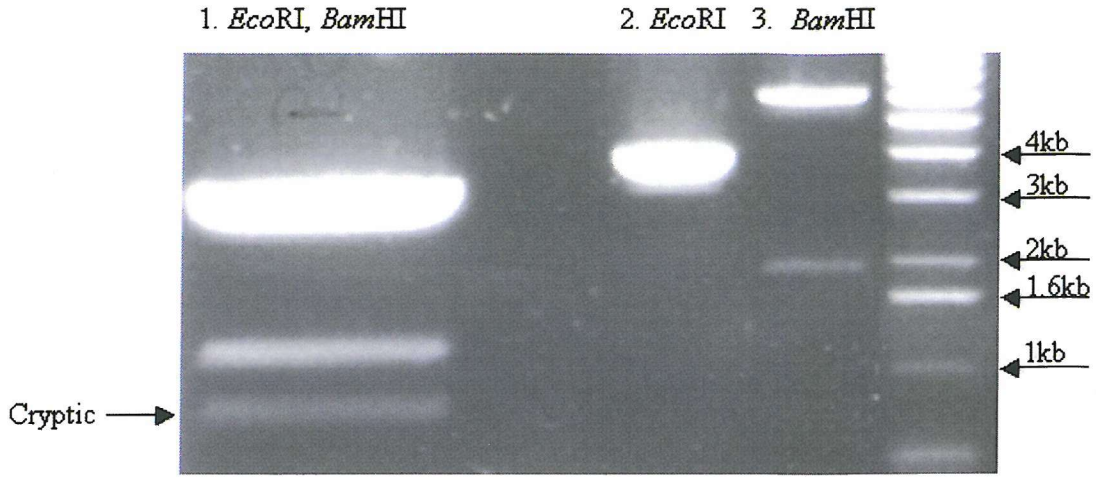


Figure 3.2: *Eco*RI restricts at a cryptic site 100bp downstream of the true restriction site.

The 5kb *Sac*I clone was digested with *Eco*RI (lane 2), *Bam*HI (lane 3) or both (lane one). Digests were performed overnight at 37^{0}C in each enzymes preferred buffer (Promega, Southampton, UK). The digests in lane 1 were performed separately with the first enzyme being denatured and digested DNA ethanol salt precipitated prior to second digest. Single digests revealed predicted restriction patterns (Figure 3.1). The double digest (1) reveals the expected 1.2kb *Eco*RI *Bam*HI fragment and a second smaller restriction fragment. M indicates the molecular size marker (1kb ladder, Gibco BRL, Cheshire, UK).

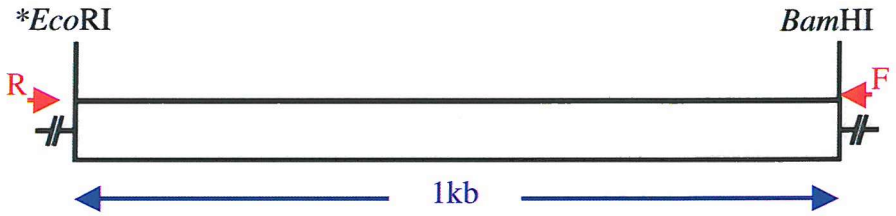


Figure 3.3: A schematic representation of pRP001.

The 1kb cryptic *EcoRI* to *Bam*HI fragment excised from the plasmid minimal Cf-2 (Dixon *et al.*, 1996) was cloned into the vector pBSsk (Stratagene, Amsterdam, The Netherlands). *Eco*RI has restricted the DNA at a cryptic site (GAA TTT), 200 base pairs downstream of the true restriction site (GAA TTC). The fragment was cloned into the vector pBSsk (Stratagene, Amsterdam, The Netherlands). Not to scale.

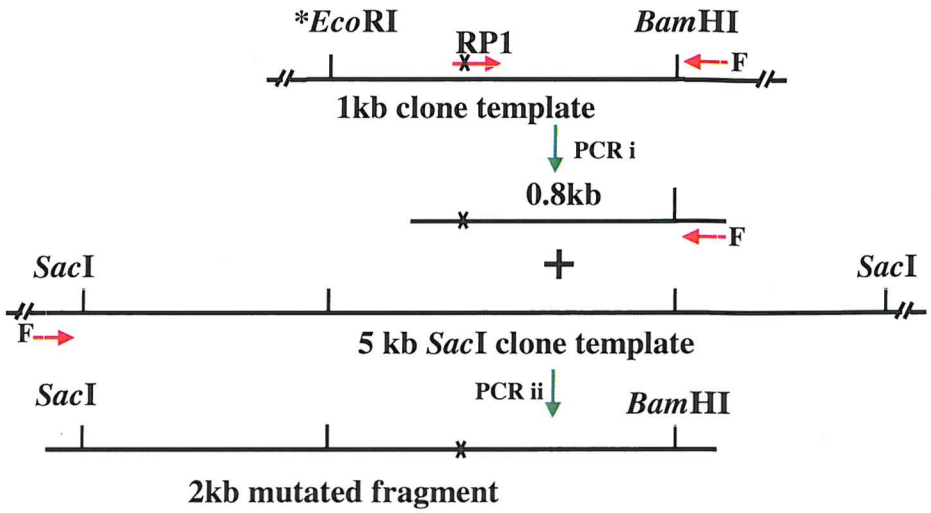
A 2kb *Sac*I, *Bam*HI fragment, encoding the C-terminal portion of *Cf-2* was cloned. This clone encompasses the original 1.2kb *Eco*RI, *Bam*HI fragment with the addition of approximately 800bp immediately upstream. The novel *Aat*II restriction site would be

introduced in the same position as described earlier, i.e between the ultimate and penultimate amino acid. This fragment was chosen as it is still relatively small, the *Sac*I site was present in the cloning site of the vector to be used and it encompasses the region to be mutatgenised. From this starting point two approaches were designed, the first was termed 'double whammy' PCR (Figure 3.4). PCR mutagenesis using the mutagenic oligos RP1 and the M13 forward primer, with pRP001 as a template was performed. This produced a mutated 0.8kb fragment. This fragment corresponded to the C-terminal end of the desired 2kb clone. The 0.8kb fragment was later employed as a primer in conjunction with the M13 forward primer, using the 5kb *Sac*I clone as a template to produce the complete 2kb *Sac*I, *Bam*HI fragment with the introduced *Aat*II restriction site.

Preliminary attempts resulted only in further amplification of the 0.8kb fragment (due to the 0.8kb fragment carrying a M13 forward primer annealing site). This was easily over come by altering the concentration of the 0.8kb fragment (to be used as a primer) relative to the template DNA. Best results were obtained when the 0.8kb primer relative to the template was in 5-10x molar excess (Figure 3.5).

Sequencing of the mutagenic PCR products revealed that not all of the population carried the desired mutagenised site. For this reason it was decided to employ an approach that would select for the introduction of an *Aat*II site. The second approach involved a three-way ligation (Figure 3.6).

A)



(*EcoRI indicates the cryptic restriction site)

B)

RP1

A AGA AAT AAT GAC GTC TAG ACA A

AGA AAT TAC AGA AGA AGA AAT AAT CAC TTC TAG ACA AGT TAC *Cf-2*

Figure 3.4: Schematic representation of 'double whammy' PCR.

A) The mutagenic primer RP1 and universal forward primers were used on pRP001 template with an annealing temperature of 40^{0}C and a 1 minute extension. The product of this PCR was purified and subsequently used as a primer on the 5kb *Sac*I clone template. The second set of PCR was set up with the addition of only one primer; the 0.8kb product of the first set. After four rounds of 40^{0}C annealing temperature for 30 seconds with a 1 minute extension the second primer, the universal forward primer, was added. 30 more cycles followed, with an annealing phase of 52^{0}C for 15 seconds and a 1 minute 30 second extension. B) Shows the mutagenic primer RP1 (red) aligned to the Cf-2 sequence (in bold) , the mutagenised bases are underlined.

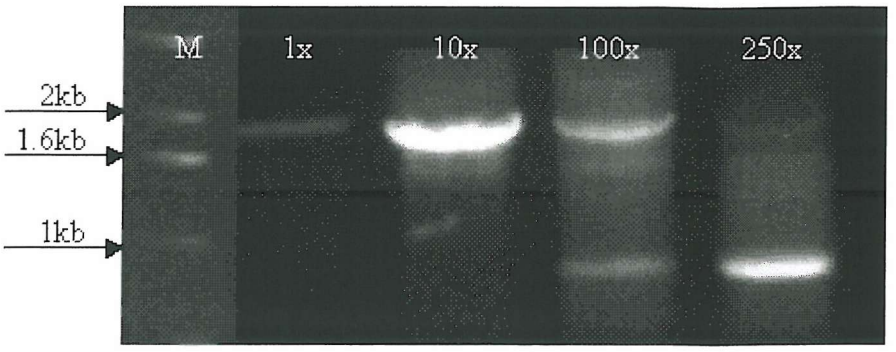
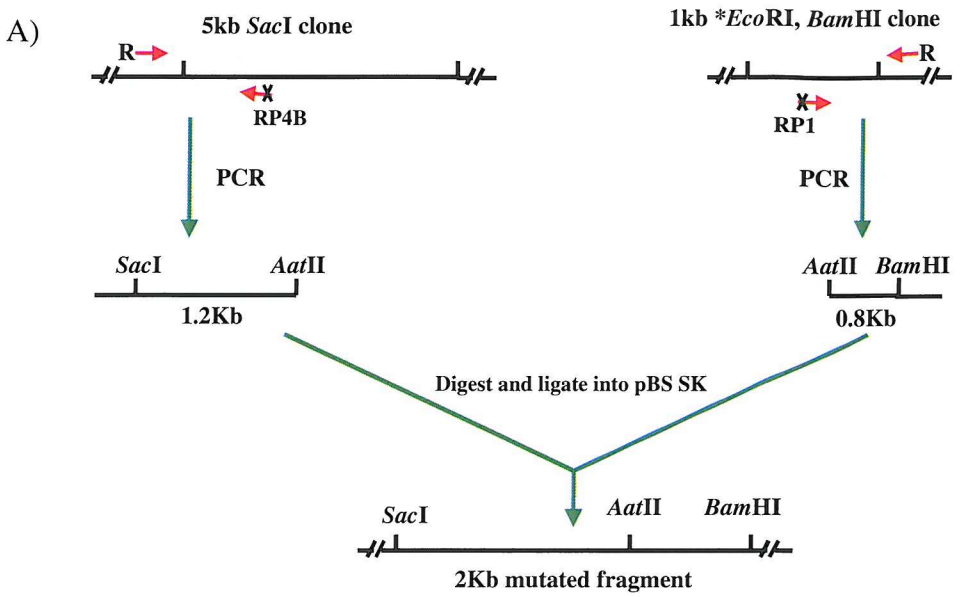


Figure 3.5: Primer concentrations affects 'double whammy' PCR specificity.

Concentrations of the 800bp primer were altered relative to template concentration, with respect to molarity, to optimise specificity. The desired PCR product is approximately 2kb. Relative primer concentrations are aligned to relevent lanes. M indicates the molecular size marker (1kb ladder Gibco BRL, Cheshire, UK).



(*EcoRI indicates the cryptic restriction site)

B)

RP1

A AGA AAT AAT GAC GTC TAG ACA A

AGA AAT TAC AGA AGA AGA AAT AAT CAC TTC TAG ACA AGT TAC

Cf-2

TCT TTA ATG TCT TCT TCT TTA TTA GTG AAG ATC TGT TCA ATG

G TCT TCT TCT TTA TTA CTG CAG ATC TGT T

RP4B

Figure 3.6: Three way ligation strategy.

Two halves of the fragment are produced by mutagenic PCR using the primers RP4B and RP1 with universal reverse primers using the 5kb *Sac*I clone and pRP001 respectively as templates. These are then ligated together with the vector pBSIISK. B) The Mutagenic primers RP1 and RP4B are shown (in red) aligned to Cf-2 sequence (bold), the mutagenised bases are shown underlined.

The three-way ligation approach involved producing two fragments by PCR mutagenesis, which together complete the desired 2kb fragment with the novel *Aat*II restriction site in place. The mutagenic primers RP4B and RP1 (Figure 3.1. Appendix 1) were employed to introduce an *Aat*II restriction site at one end of each of the composite

fragments. These were used in combination with primers flanking the *Sac*I and *Bam*HI restriction sites on the 5kb *Sac*I clone and pRP001 template DNA respectively. The PCR products were subsequently restricted and ligated to construct the 2kb *Sac*I, *Bam*HI clone, with the mutagenised site introduced. Equimolar quantities of each fragment were added to the appropriately restricted vector in 3-5x molar excess, a ligation reaction performed and the products transformed into *E. coli* DH5α, using the calcium chloride method. Transformation rates were very poor with no positive colonies ever recovered.

At this point the 1.2kb *Eco*RI, *Bam*HI fragment was successfully cloned into the vector pBSIIsk using the original double restriction protocol. Cloning success coincided with several factors; the use of ultra pure water, a reduction in restriction enzyme, and hence glycerol, concentration to below 5% (v/v) and not to be underestimated experience of the laboratory procedures. This clone was assigned pRP002 (Figure 3.7) and used for subsequent steps. PCR mutagenesis was employed to introduce an *Aat*II restriction site and a three-way ligation approach used to select for fragments carrying the desired mutation (Figure 3.8).

The mutagenic primers RP1 and RP4B were used in combination with the universal reverse and M13 forward primers respectively, using pRP002 as a template. Synthesis of the correct PCR mutagenesis products was verified by size comparison on an 0.7% (w/v) agarose/TAE gel. After gel purification using a Machery-Nagel nucleospin® kit (AB-gene, Epsom, UK) the fragments were simultaneously ligated together and into the pBS sk vector. Putative positive colonies were screened initially by size comparison with an empty vector (Figure 3.9).

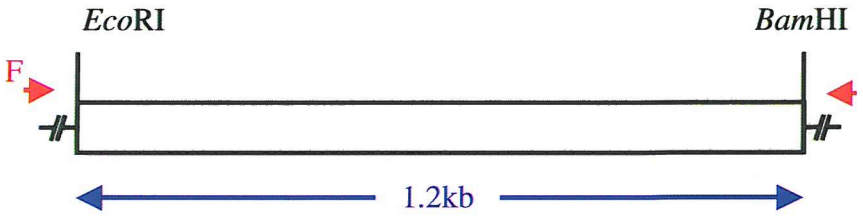


Figure 3.7: A schematic representation of pRP002.

The 1.2kb *EcoR*I to *Bam*HI fragment excised from the plasmid minimal Cf-2 (Dixon *et al.*, 1996) was cloned into the vector pBSsk (Stratagene, Amsterdam, The Netherlands). Not to scale.

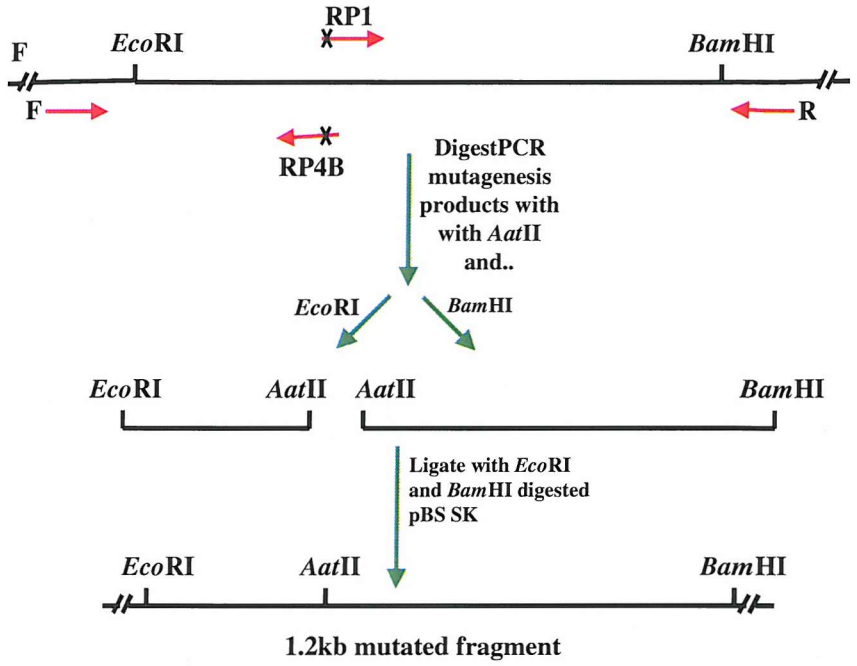


Figure 3.8: A schematic representation of the production of pRP002.

A) Two halves of the fragment, each with an *Aat*II site introduced at either end, were produced by PCR mutagenesis. These were subsequently digested and ligated together along with the appropriate vector (pBSIISK).

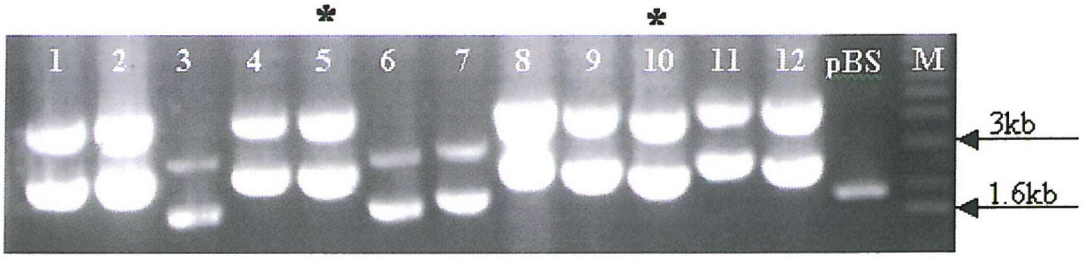


Figure 3.9: Colonies are positive for the 1.2kb *Eco*RI *Bam*HI insert.

Plasmid DNA was purified from 12 colonies and run on a 0.7% (w/v) agarose gel with the vector negative control (pBS). Only colonies 3, 6 and 7 were negative for the insert. Colonies 5 and 10 (*) were used for subsequent steps. M indicates the molecular size marker (1kb ladder, Gibco BRL, Cheshire, UK).

Individual *Eco*RI, *Bam*HI and *Aat*II restrictions revealed that 7of the above 9 clones carried the correct mutagenised insert. The two colonies designated 5 and 10

(Figure 3.7) were selected and a large-scale purification of plasmid DNA performed on each. The clones were named $p5^{3W}$ and $p10^{3W}$ respectively (Figure 3.10). These were subsequently sequenced and shown to be the desired 1.2kb clones. In each case, sequencing revealed that up to and including base 5196 (GenBank accession U42444) no sequence mutations had occurred. There was a single base change to base 5197 where a C had been replaced with a G. This base change did not confer an amino acid substitution as it had occurred within an intron nor did it generate a stop codon. This intron (bases 5051-5240, GenBank accession U42444) is the only intron in *Cf-2* and is found in the 3'untranslated region of the gene i.e. downstream of the only stop codon. Due to the positioning of this mutation it was unlikely to affect the protein or the stability of the mRNA. There were no other sequence anomalies upstream of the polyA signal and addition sites i.e. no spontaneous mutations had occurred in the transcribed region of the cloned portion of Cf-2.

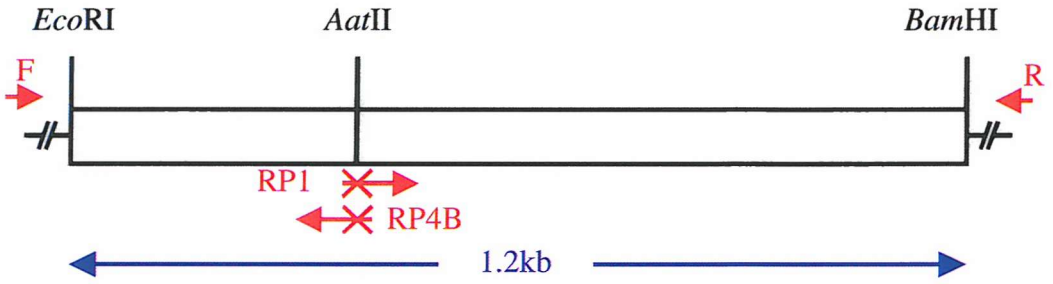


Figure 3.10: A schematic representation of $p5^{3w}$ and $p10^{3w}$.

An *Aat*II restriction site was introduced into pRP002 (Figure 3.7) by PCR mutagenesis with the primers RP1 and RP4B (Appendix I) to produce the construct $p5^{3w}$ and $p10^{3w}$. Not to scale.

Parallel to the previously described plasmid construction, the 3x c-Myc DNA sequence was prepared for insertion into the newly created *Aat*II site. The sequence encoding the 116bp 3x c-Myc epitope-tag was amplified by PCR from the plasmid LB2339 (Piedras *et al.*, 2000). It was necessary to create flanking *Aat*II sites in the correct open reading frame; this was done using the primers RP2 and RP3 (Appendix I). The PCR products were separated on an agarose gel, purified and restricted with *Aat*II. The vectors $p5^{3W}$ and $p10^{3W}$ were restricted with *Aat*II and treated with shrimp alkaline phosphatase to prevent self-ligation. Both the vector and the 3x c-Myc insert were purified from an agarose gel using a Machery-Nagel Nucleospin® kit (AB-Gene, Epsom, UK) prior to ligation. Ligation products were subsequently transformed into *E. coli* DH5α

using the calcium chloride method. Twenty putative positive colonies (10 from both 5^{3W} and 10^{3W}) were screened by PCR directly on cultures, with the primers CF2R1 and MD8 (Appendix I) (Figure 3.1). These primers amplify a 300bp product from *Cf-2* and hence an approximately 420bp product where the 3x c-Myc epitope-tag has been successfully inserted into *Cf-2*. CF2R1 and MD8 were chosen, as it would be possible to differentiate between a negative result and a failed PCR. This would not have been possible with the primer combination RP2 and RP3, which amplifies the epitope-tag alone. Purified DNA from the clones $p5^{3W}$ and $p10^{3W}$were amplified as negative controls. To screen the putative epitope-tagged contracts, 1µl of 5ml cultures was used as a template. From this screening one clone, originating from the modification of $p5^{3W}$, was identified that appeared to carry the 3x c-Myc insert (Figure 3.11).

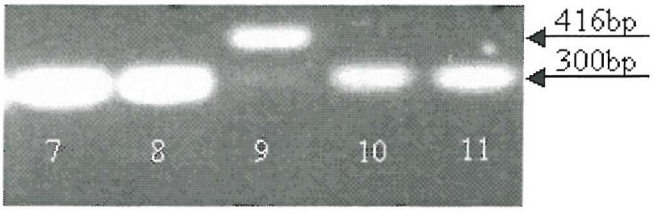


Figure 3.11: One colony carries the 3x c-Myc epitope-tag.

Twenty colonies were picked and PCR performed directly on the culture (1-10 originated from $p5^{3W}$, whilst 11-20 originated from $p10^{3W}$). The primers CF2R1 and MD8 were chosen to amplify across a 300bp region into which the epitope tag was to be inserted. One colony (9) was positive for the 3x c-Myc coding region.

The positive clone was assigned pRP004 (Figure 3.12). Sequencing across the 3x c-Myc region revealed that no sequence mutations had been introduced. The plasmid pRP004 was amplified in *E. coli* and purified using a Qiagen cartridge (Qiagen, Crawley, UK). The 1.2kb *Eco*RI, *Bam*HI epitope-tagged fragment was obtained by restriction digest, separated from its vector by gel electrophoresis and purified using a Machery-Nagel Nucleospin® kit (AB-Gene, Epsom, UK). The minimal *Cf-2* plasmid (Dixon *et al.*, 1996) was simultaneously digested with *Eco*RI and *Bam*HI and similarly separated by gel electrophoresis. Due to the size of the digested minimal Cf-2 plasmid (>5kb, see material and methods) (Dixon *et al.*, 1996) it was purified by electro-elution. A ligation reaction was performed to reconstruct the minimal Cf-2 plasmid containing the 3x c-Myc epitope-tag. *E. coli* were transformed with the ligation products by electroporation. Transformation efficiency was low, which was probably due to the size of the construct.

Two colonies were picked and the plasmid DNA purified before being screened by PCR for the epitope-tag. The primers MD8 and CF2R1 were again chosen as these amplify across the region, which encompasses the epitope-tag and would still amplify a product, although smaller, from negative clones (Figure 3.13).

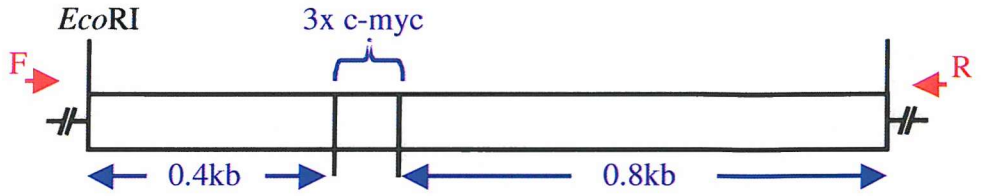


Figure 3.12: A schematic representation of pRP004.

The *Aat*II site of $p5^{3w}$ (Figure 3.10) was restricted and the 116 bp 3x c-Myc epitope-tag inserted.

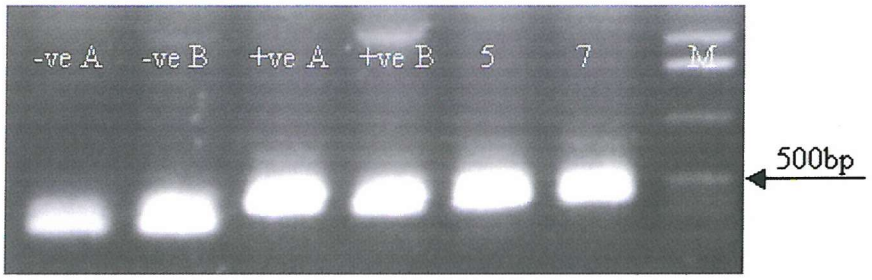


Figure 3.13: Successful construction of a 3x c-Myc epitope-tagged version of Cf-2.2.

Two putative positive colonies were screened directly by PCR for presence of the epitope tag in the reconstructed minimal Cf-2 plasmid. The primers CF2R1 and MD8 were chosen to amplify across a 300bp region into which the epitope tag was to be inserted. PCR products from positive colonies are 116bp (corresponding the epitope-tag) larger than negative controls. Minimal Cf-2 and 10^{3w} (-ve A and B respectively) plasmid DNA were used as negative template DNAs, old and new stocks of pRP004 were used as positive controls (+ve A and B respectively). Both screened colonies (5 and 7) tested positive for the epitope tagged version of the minimal Cf-2 plasmid. M indicates the molecular size marker (1kb ladder, Gibco BRL, Cheshire, UK).

Sample 7 was picked and asssigned pRP005 (Figure 5.14) and used to transform *Agrobacterium tumefaciens*. *A. tumefaciens* was transformed as plant transformations were performed using the *A. tumefaciens* method (Chapter 4). The strain LBA4404 of *A. tumefaciens* was transformed by electroporation and four putative positive colonies (A-D) were tested for the complete insert by restriction digest with the enzymes *Pst*I, *Xho*I and *Sac*I (Figure 3.15). Plasmid DNA was purified from *A. tumefaciens* putative positive

colonies and amplified in *E. coli* prior to restriction digest. The restriction digest revealed all colonies carried the complete *RP005* construct. Colony C was picked for use in plant transformations (Chapter 4)

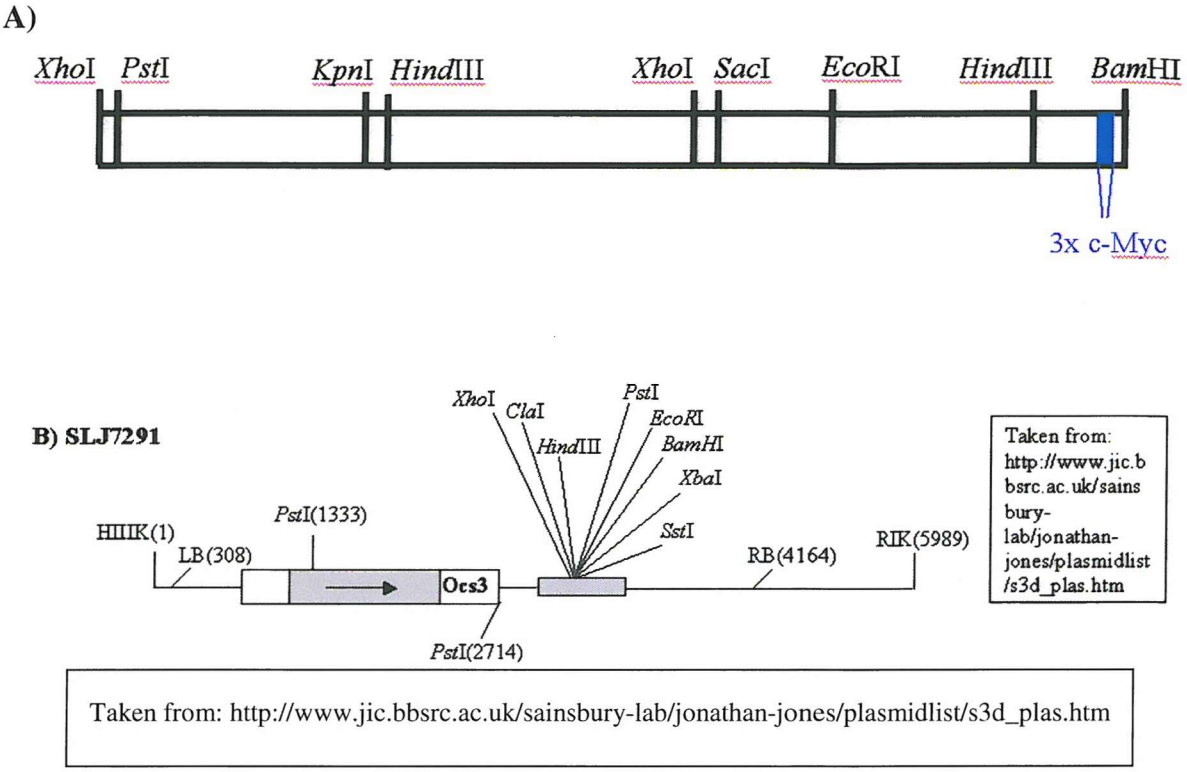


Figure 3.14: A schematic representation of pRP005.

A) The clone pRP004 (Figure 3.12) was digested with *Eco*RI and *Bam*HI and ligated into *Eco*RI, *Bam*HI restricted Minimal-Cf2.1 (Dixon *et al.*, 1996). This construct is engineered into the cloning vector SLJ 7291 (B) . http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/plasmidlist/s3d_plas.htm. B) HIIIK signifies a *Hin*dIII site filled in with Klenow polymerase and dNTPs; RIK indicates and *Eco*R1 sire similarly treated; LB signifies T-DNA left border repeat sequence; RB signifies the right border repeat; nos indicates the nopaline synthase promoter; ocs 3' indicates octopine synthase 3' end; NPT indicates neomycin phosphotransferase; Gene transcriptional orientations are indicated by an arrow. All SLJ plasmids, including SLJ 7291 are derived form the broad-host-range vector pRK290 (Friedman *et al.*, 1982).

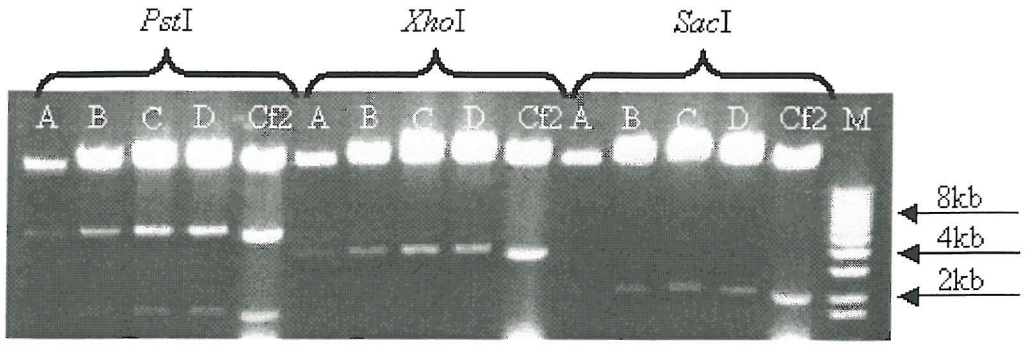


Figure 3.15: The construct pRP005 is carried in full by *A. tumefaciens* .

Four *A. tumfaciens* colonies (A-D) were picked, plasmid DNA purified and subsequently amplified in *E. coli*. Plasmid DNA was restricted with *Pst*I, *Xho*I and *Sac*I to verifiy integrity. A parallel restriction digest was performed on the plasmid minimal Cf-2 (Cf2) as a size comparison. Size shifts compared to Cf-2 are due to the epitope-tag. All four colonies were positive for the intact pRP005 plasmid. M indicates the molecular size marker (1kb ladder, Gibco BRL, Cheshire, UK).

In order to build a similar construct (*Cf-2* with the 3x c-Myc epitope-tag in the same position as pRP005 but using the CaMV 35S promoter), the majority of the *Cf-2* gene was excised from pRP005 using the restriction enzymes *Acc*65I (a neoschizomer of *Kpn*I) and *Bam*HI (Figure 3.14a). This was ligated into the vector pKO3 (Dixon, unpublished). pKO3 was engineered from the vector SLJ 7291 (Figure 3.14b). Firstly, the *Kpn*I sites outside of the left and right borders were destroyed by filling in and re-ligation. Secondly, a 1.9kb PCR product amplified from the vector SLJ8891 (http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/plasmidlist/s3d_plas.htm) was ligated into the *Eco*RI and *Bam*HI sites to create the new vector. The fragment was amplified using primers to introduce a new *Bam*HI site immediately after the *Kpn*I site. The PCR fragment contains the 35S promoter and a portion of the *Cf-2* gene (from the *Cla*I site to immediately after the *Kpn*I site). *E. coli* was subsequently transformed by electroporation. Four putative positive colonies were screened by PCR directly on liquid cultures with the primers RP1 and M13 forward. This PCR produced non-specific products of a size similar to that expected from a positive colony (approximately 1.6kb) and so was abandoned. A *Bam*HI and *Acc*65I restriction digest, after mid-scale alkaline-lysis plasmid preparation, was performed on only one putative positive colony. This enzyme combination would release a very small restriction fragment (only a few bases)

from a negative colony and a relatively large (approximately 4kb) fragment from a positive colony. The colony picked was positive for the epitope-tagged version of *Cf-2*. This construct was assigned pRP006 (Figure 3.16) and used for *A. tumefaciens* transformations. Eight (numbered 1-8) putative positive *A. tumefaciens* transformants were picked and plasmid DNA purified from them. The plasmid DNA was subsequently transformed into *E. coli* for amplification. Six of the eight transformations were successful and plasmid DNA purified from two (numbers 3 and 7). A restriction digest with the enzymes *Pst*I, *Xho*I and *Sac*I revealed that both carried the complete *RP006* construct (Figure 3.17). *A. tumefaciens* colony 7 was used for subsequent plant transformations (Chapter 4).

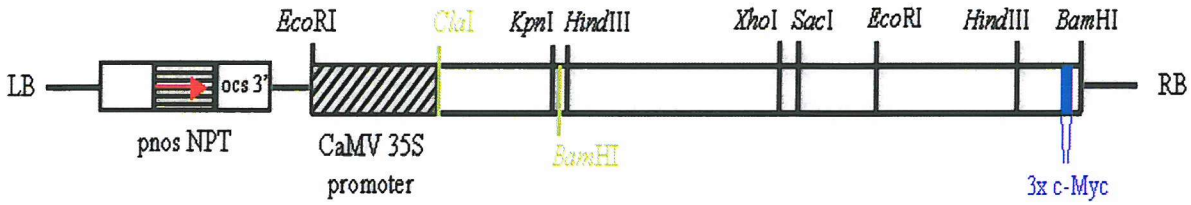


Figure 3.16: A schematic representation of pRP006.

The clone pRP005 was digested with *Acc*65I (a neoschizomer of *Kpn*I) and *Bam*HI (Figure 3.14a). This was ligated into the vector pKO3 (Dixon, unpublished). The vector pKO3 was produced from SLJ 7291 into which the 35S CaMV promoter and the initial portion of *Cf-2* (from the ATG start codon, *Cla*I site to immediately after the *Kpn*I site) were cloned. The fragment was amplified from the vector SLJ8891 (http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/plasmidlist/s3d_plas.htm) using primers to introduce a new *Bam*HI site (Green) immediately after the *Kpn*I site. This clones contains *Cf-2* up to the final *Bam*HI site (open box) and the 35S CaMV promoter (diagonal shading). Sites introduced into the *Cf-2* sequence are shown in green, *Cla*I was introduced in the contruction of SLJ8891. LB signifies T-DNA left border repeat sequence; RB signifies the right border repeat; nos indicates the nopaline synthase promoter; ocs 3' indicates octopine synthase 3' end; NPT indicates neomycin phosphotransferase; Gene transcriptional orientations are indicated by an arrow.

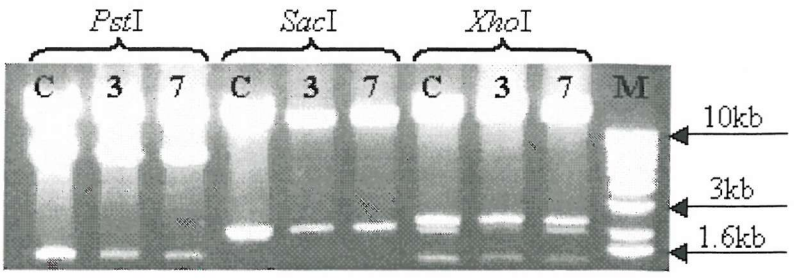


Figure 3.17: *A. tumefaciens* carries the complete *RP006* construct.

Plasmid DNA was purified from putative transformed *A. tumefaciens* colonies and amplified in *E.coli*. Two *E. coli* colonies were picked, plasmid DNA purified and restricted with *Pst*I, *Sac*I and *Xho*I. C indicates control plasmid DNA taken before transformation into *A. tumefaciens* (colony 1, Figure 3.13). Comparison with control DNA revealed both colonies carry pRP006 without any deletions. M indicates the molecular size marker (1kb ladder, Gibco BRL, Cheshire, UK).

3.3 Discussion

The aim to engineer two epitope-tagged versions of Cf-2, one using the native promoter and one using the 35S promoter, was met. These constructs were used to stably transform both tobacco and tomato and used to meet the aim of this thesis; to study the sub-cellular localisation and post-translational modification of Cf-2.

Several methods were used in an attempt to produce the epitope-tagged construct with varying degrees of success. All methods relied upon the process of introduction of mutations by PCR using mutagenic oligos. As revealed by the 'double whammy' approach, the difficulty was selecting for inserts that contained both mutations, often only one site was mutated. This unsuccessful approach required the screening of many colonies by isolation of plasmid DNA and subsequent restriction and/or sequencing, which is both costly and time consuming. As the two point mutations produced a novel restriction site (*Aat*II), the 3-way ligation approach was ultimately successful as this pre-selected for fragments with the desired mutations.

An alternative approach would have been to use an *in vitro* mutagenesis system, such as Altered sites® II (Promega, Southampton, UK). This procedure requires the cloning of the DNA to be mutagenised into a vector with the tetracycline resistance gene and a modified version of the ampicillin resistance gene. The mutagenic oligo along with the ampcillin repair oligo and the tetracycline knockout oligo, anneal with their target sequences. The mutagenised DNA is then synthesised using T4 DNA polymerase and

ligase. Contructs are subsequently transformed into *E. coli* and those carrying the desired mutated site can then be selected for using ampicillin. This procedure is best used for the introduction of one mutagenised site at a time, as if two sites were introduced at once, it would be impossible to tell the difference between clones with one or two of the desired changes. Subsequent rounds of mutagenesis can be performed by alternating the use of the tetracycline and ampcillin repair and knockout oligos. As with the 3-way ligation approach employed in this project, the Altered sites® II kit would experience the same problems with the cloning of a portion of Cf-2. This approach would have required less rounds of cloning and was less likely to have resulted in the spontaneous mutations observed with the 3-way ligation approach.

Chapter four

4 Plant transformations and characterisation

4.1 Introduction

Agrobacterium tumefaciens is one of only a few bacterial pathogens (*A. rhizogenes* and *A. vitis*) that is able to 'genetically engineer' its host. This ability of trans-kingdom DNA transfer has been exploited in plant genetic engineering.

Plant transformation by *A. tumefaciens* requires two elements located on the Ti (Tumor-inducing) plasmid. The first is the T-DNA (transferred DNA), which is the DNA between two border repeats (the left and right borders). This region normally encodes oncogenic (*onc*) genes, which *in planta* leads to tumour formation. For plant transformations the *onc* genes are removed and replaced with the gene of interest. The T-DNA does not encode any proteins important for its uptake and any DNA within the borders will be transferred to the host plant cell. The second component required on the Ti plasmid is the virulence (vir) region, which is made up of seven major loci (*virA, virB, virC, virD, virE, virG* and *virH*). The vir region encodes the machinery required for host recognition, attachment and delivery of the T-DNA into the cytoplasm of a plant cell. Localisation to the nucleus and integration into the genome requires host factors.

The first step towards T-DNA transfer requires the production of a single stranded T-DNA copy called the T-strand (Hookyaas and Beijerbergen, 1994). A complex of VirD1 and VirD2 binds to the supercoiled Ti plasmid at the T-DNA borders (24bp imperfect direct repeats) relaxes it and nicks the T-DNA to produce the T-strand. VirD2 becomes covalently bound to the 5' end of the T-strand where it remains attached during transfer into the plant cell and is involved in nuclear targeting. VirE2 has been shown to bind and package single stranded DNA (ssDNA). It is hypothesised that VirE2 in a complex with VirD2 coats the T-strand (a ssDNA) to prevent it re-annealing to its sense strand (Zupan and Zambryski, 1997).

Transport of the T-complex (T-strand, VirD2 and VirE2) occurs via a type IV secretion apparatus encoded by the *VirD4* gene and *VirB* operon (Zupan *et al.*, 1998).

VirB6 with its six transmembrane domains is postulated to be involved in the formation of the transporter pore (Zambryski, 1992), with VirB7 homodimers and VirB7/VirB9 heterodimers forming a nucleation centre for the rest of the transport complex. The functions of VirB8 and VirB10 are unknown, genetic evidence predicts interaction of VirB10 with the VirB7/VirB9 nucleation centre (Zupan *et al.*, 1998).

Once in the cell nuclear import of the T-DNA occurs. VirD2 and VirE2 (the components of the T-complex) carry nuclear localisation signals that target uptake by the nucleus (Citovsky *et al.*, 1994).

Once in the nucleus the T-DNA must integrate into a chromosome, which occurs at random. As the T-DNA doesn't encode the necessary enzymes for this, VirD2 and VirE2 proteins and/or host nuclear factors must provide these activites, indeed VirD2 has a ligase activity *in vitro* (Pansegrau *et al.*, 1993).

The right border is integrated into plant DNA with greater precision than the left border. It is not uncommon for hundreds of bases to be lost from the left border upon insertion into the plant genome. This is because the right border is integrated into the plant genome first and is better protected by the T-complex. Therefore, T-DNA nearer the left border is more exposed to nuclease activity resulting in frequent loss of sequence at the left border. One reason why the right border is better protected is because it is essential for tumorigenesis whilst the left border is dispensable (Hooykaas and Beijersbergen, 1994). It is important to take this into account when designing the construct ensuring the T-DNA is in a position and orientation less likely to be effected if a loss at the left border occurs.

Cf-2 mediates gene for gene resistance in tomato, against any race of *Cladosporium fulvum* carrying AVR2. However it seems increasingly unlikely that Cf-2 is directly the Avr2 receptor, which means other proteins, must be involved. Cf-2 absolutely requires a second plant protein Rcr3 (required for *C. fulvum* resistance) to mediate disease resistance (Dixon *et al.*, 2000). Rcr3 has been cloned and encodes an extracellular papain-like cysteine protease (Krüger *et al.*, 2002). Introgression of *Cf-2* from *L. pimpinellifolium* into *L. esculentum* requires a second *L. pimpinellifolium* gene (*Ne*) to suppress a *Cf-2* dependent auto-necrotic phenotype (Langford 1948). Krüger *et al.*

(2002) demonstrated that *Ne* and *Rcr3*^{pim} (*L. pimpenellifolium* allele of *Rcr3*) are allelic and that *Rcr3*^{esc} (*L. esculentum Rcr3* allele) corresponds to *ne*. *Rcr3*^{esc} / *ne* promotes *Cf-2*-dependent auto-necrosis, which can be prevented by the presence of *Rcr3*^{pim}. It is proposed that Cf-2 acts as a 'guard' for Rcr3 and that upon recognition of an Avr2/Rcr3 complex it triggers a defence response. Therefore, in the absence of Avr2, a plant carrying *Rcr3*^{pim} could not generate such a signal. A plant carrying *Rcr3*^{esc}, an allele that did not co-evolve with *Cf-2*, could generate a leaky signal even in the absence of Avr2 and induce auto-necrosis.

The aim of this study was to produce, if possible, functionally active epitope-tagged versions of Cf-2 to allow the study of its sub-cellular localisation. It is predicted that the cytoplasmic tail of the Cf proteins, into which the epitope tag has been inserted, (Chapter 3) serves no function other than as a membrane anchor. Therefore, the epitope tag should have little or no effect on function of the transgene. However, the Cf proteins have no obvious signalling domain, therefore, one possibility is that the cytoplasmic tail interacts with other plant molecules to elicit a signal. Piedras *et al.*, 2000 produced a version of Cf-9 with an epitope-tag in the cytoplasmic tail that was still functional. In addition Van der Hoorn *et al.* (2001a) revealed that the C-terminal dilysine repeat of Cf-9 is not required for its function, indicating that the cytoplasmic tail functions only as a membrane anchor.

Tobacco and tomato are both Solanaceous species and so tobacco (e.g. *Nicotiana tabacum*) is often used as a model plant as it is relatively quick growing, produces a large amount of vegetative material and most importantly, is easy to transform relative to tomato. This is useful for many studies. Obviously, tobacco is only useful to study Cf function (and hence epitope-tagged transgene function) if the *Cf* genes can also function in tobacco. *N. tabacum* suspension cultures carrying Cf-9 respond to Avr9 elicitation by producing active oxygen species, protein kinase activation and novel gene expression (Piedras *et al.*, 2000. Romeis *et al.*, 2001). These data suggest that Cf-Avr gene-for-gene interactions are at least to some extent functional in *N. tabacum*. Assessing the function of Cf-2 in *N. tabacum* is difficult because *Avr2* had not been cloned at the start of this project. Without purified Avr2 it would be difficult to quantitatively assess the consequences of Avr2 recognition by Cf-2. This is made more difficult as *C. fulvum* is not pathogenic on tobacco. Recent evidence suggests that Cf-2 doesn't function at all in *N.*

tabacum, but does in *N. benthamiana* (Jones, unpublished data). Even if Cf-2 does not function properly in tobacco it is still likely to be a suitable model for sub-cellular localisation studies, which is advantageous, as it is known to be easier to purify membranes from tobacco relative to tomato. Piedras *et al.*, 2000 used *N. tabacum* as a model plant to study the localisation of Cf-9. Due to the differences between Cf-2 and Cf-9 function in *N. tabacum* it appears important to perform parallel analysis in tomato to confirm that *N. tabacum* is indeed a suitable model.

The functionality of the epitope-tagged *Cf-2* constructs is easily tested in tomato by inoculation with appropriate races of *C. fulvum*. The level of resistance can be assessed using the transgenic *C. fulvum* race 4 GUS (Oliver *et al.*, 1993). Oliver *et al.* (1993) produced transgenic lines of *C. fulvum* that express the *uidA* reporter gene. The *uidA* transgene encodes the enzyme β-D-Glucoronidase (GUS), the expression of which can be assessed both qualitatively and quantitatively. The GUS gene is often used in plants to produce promoter:GUS fusions, where the promoter of a gene of interest is used to drive the expression of the GUS gene. These fusion constructs can then be used to produce expression profiles of the gene of interest, by detection of GUS activity.

Oliver *et al.* (2003) demonstrated that the GUS activity observed in leaves infected with *C. fulvum* race 4GUS is directly proportional to the amount of hyphal biomass.

To study the sub-cellular localisation of Cf-2 (Chapter 5) and the functionality of the epitope-tagged proteins both construct pRP005 and pRP006 were stably transformed into both tobacco (*N. tabacum* cv. Petit Gerard) and susceptible tomato (*Lycopersicon esculentum* Cf0). The transformations were performed using an *A. tumefaciens* mediated method. Functionality of the transgenes in tomato was assessed by inoculation with *C. fulvum* race 4GUS.

4.2 Results

To study the subcellular localisation of Cf-2 it was necessary to express the constructs pRP005 and pRP006 (Chapter 3) *in planta*. Both constructs were stably transformed into both susceptible tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum* cv. Petit Gerard) using an *A. tumefaciens* mediated method. *N.*

tabacum was transformed, primarily, because of the relative ease of transformation compared with tomato. Tomato transformation is technically challenging and time consuming by comparison to tobacco transformation. In addition, the preparation of membranes is known to be easier from tobacco relative to tomato.

4.2.1 Transforming *N. tabacum* cv. Petit Gerard with *RP005*

A strain of *A. tumefaciens* carrying the construct pRP005 (epitope-tagged Cf-2 using it native promoter, Chapter 3) was used to transform *N. tabacum*. The first transformation attempt resulted in 40 shoots, which were excised and designated T001-T040. Of these, only 16 produced roots under kanamycin selection, one later died upon transfer to soil. The recovered putative transformants were grown for both tissue and seed. DNA was extracted from each putative transformant and used for Southern blot analysis. Genomic DNA was digested with *Hin*dIII (Figure 4.1) and hybridised with a probe amplified from the minimal-Cf2 plasmid using the primers V2R2 and V3F. All but three plants (T014, T016 and T018) were positive for the transgene (Figure 4.2).

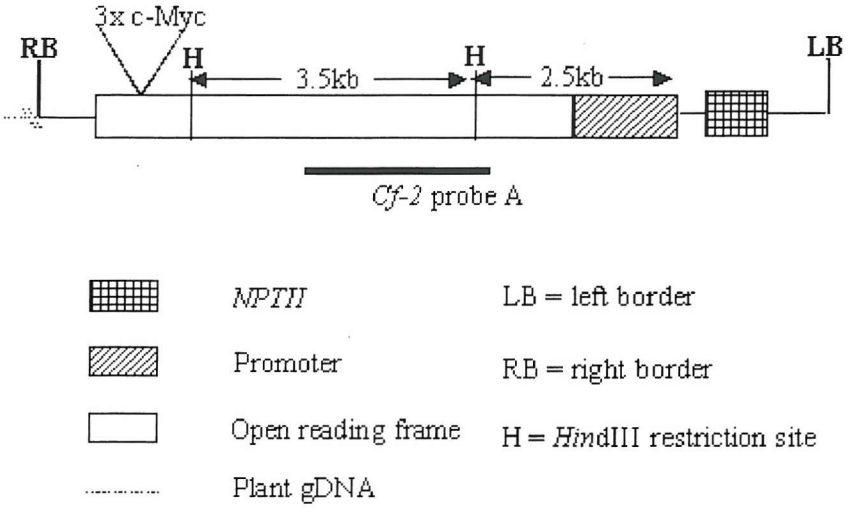


Figure 4.1: *Hin*dIII restriction map of *Cf-2*.

Schematic representation of *Cf-2*, showing the region used for a DNA probe (not to scale). The 2.4kb probe (*Cf-2* probe A) was amplified with the primers V3F and V2R2 from the minimal Cf-2 plasmid (annealing temperature: 54^{0}C, extension: 2.5 minutes, number of cycles: 35).

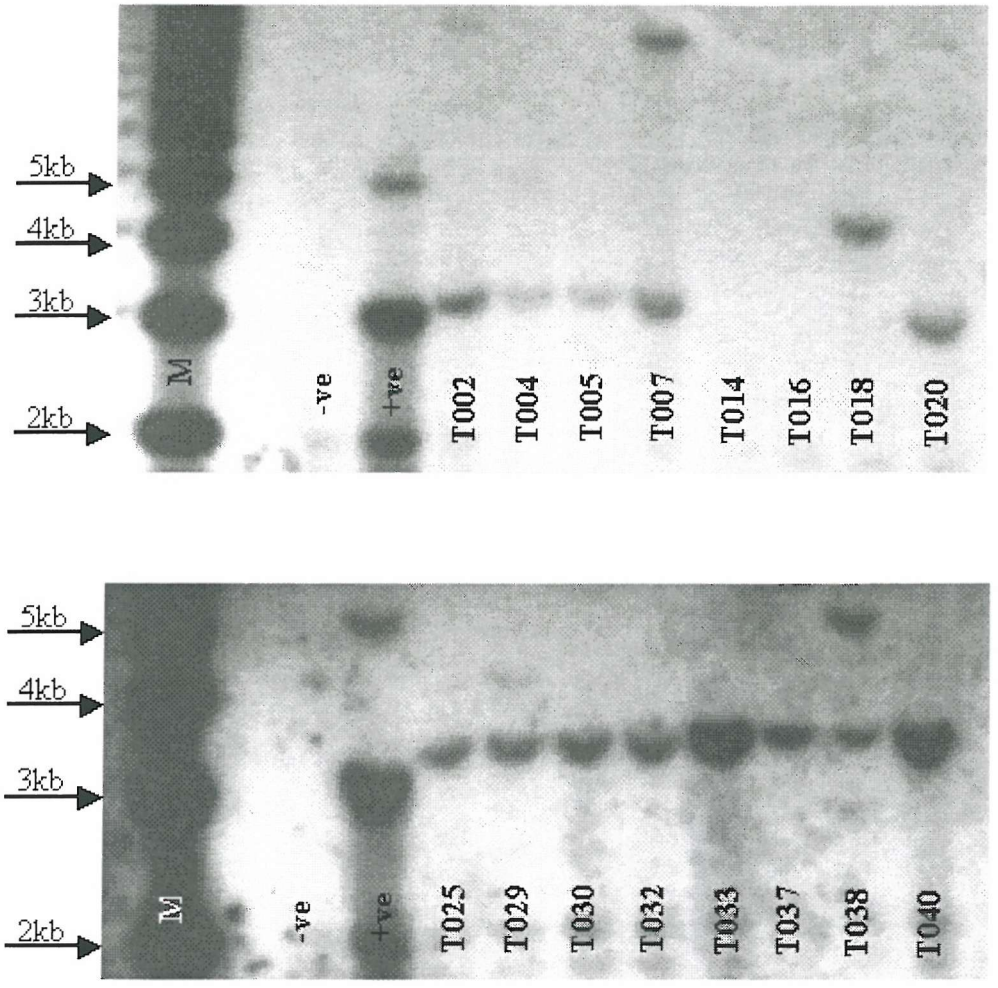


Figure 4.2: Most kanamycin resistant tobacco transformants carry the epitope tagged *Cf-2* gene.

Southern blot analysis of putative RP005 tobacco transformants. Genomic DNA (6µg) digested with *Hin*dIII and hybridised with the *Cf-2* probe A (Figure 4.1). Plants positive for the transgene should show at least two bands one 3.5kb and more than one band larger than 2.5kb. Number of additional bands indicates transgene copy number, the sizes of which depend upon the flanking region of DNA (Figure 4.1). Plants where the major 3.5kb band was present were treated as positive as the second band (>2.5kb) was not always detected. +ve indicates a positive control, D631 8891K (tomato carrying *Cf-2* transgene Dixon, unpublished). –ve indicates a negative control, *N. tabacum cv*. Petit Gerard. Plant designations are aligned to relevant lanes. M indicates molecular weight markers (1kb ladder, Gibco BRL, Cheshire, UK).

Seed was collected from selfed primary transformants (T_1 generation). Seeds were grown on kanamycin and segregation analysis was performed. Transformants carrying only one copy of the *RP005* T-DNA were selected to establish homozygous lines. Transformants carrying several copies of the T-DNA, although potentially express the protein to higher levels, were not used further. It would be very difficult to establish homozygous lines from plants carrying more than one copy of the T-DNA. Once a line

homozygous for one locus has been established it would have been challenging and time consuming to tell if the other locus (loci) is also homozygous. This would be a problem if the initial homozygous locus encodes a non-functional copy of the protein. Segregation analysis, using a Chi-squared test, revealed seven plants (T002, T005, T029, T030, T032, T037, T038) that carry just one copy of the *RP005* transgene (Table 4.1). Eight green seedlings from each of these seven plants were grown in soil for seed to establish homozygous lines.

Table 4.1: Segregation analysis of RP005 *N. tabacum cv.* Petit Gerard transformants.

To obtain segregation data self-seed of T_1 transformants were plated on MS media with 300µg/ml kanamycin. After 3 weeks the seedlings were analysed and their segregation ratios calculated. Seedlings expressing at least one copy of the transgene remained green (G) whilst those without a copy were bleached white (W). This data is combined with the results of the Southern and RT-PCR analyses. N/A = not attempted. Significance level at P = 0.05 for a Chi^2 value with one degree of freedom = 3.84 (significant values are shown in bold).

chi^2 values were calculated as follows

General formula:

x:1 $Chi^2 = \frac{(a-xb)^2}{xn}$ **Where $a \geq b$ and $a + b = n$**

Plant designations in *italics* indicate those with a single integration event

*** = Probably untransformed or protein not expressed**

‡ = Two independent integrations

§ = Probably three independent integrations.

	Segregation	Goodness of fit Chi^2		Southern analysis	RT-PCR
PLANT	G : W	3 : 1	15 : 1	+ / -	+ / -
T002	274 : 086	**0.24**	191.16	+	+
T004	072 : 037	4.65	142.68	+	N/A
T005	091 : 030	0.00	71.01	+	+
T007	179 : 040	5.30	53.95	+	N/A
T014*	000 : 187	561.00	2805.00	-	N/A
T016	003 : 185	540.26	2724.82	-	N/A
T018	074 : 016	**2.50**	20.41	-	N/A
T020	158 : 043	**1.39**	78.66	+	+
T025	160 : 036	4.60	49.12	+	N/A
T029	252 : 079	**0.23**	175.33	+	+
T030	234 : 089	**1.12**	250.20	+	+
T032	187 : 057	**0.35**	121.92	+	+
T033§	300 : 003	93.16	14.31	+	N/A
T037	256 : 090	**0.19**	230.60	+	-
T038	243 : 089	**0.58**	239.45	+	-
T040‡	270 : 019	52.33	**0.0519**	+	N/A

The seven plants carrying just one copy of the T-DNA were tested for expression by RT-PCR, a relatively sensitive protocol. Using the native Cf-2 promoter, transcript levels were predicted to be too low for detection by northern analysis (Dixon, unpublished data). The plants T002, T005, T029, T030 and T032 were all positive for expression of the T-DNA, whilst T038 was negative (Figure 4.3). The plant T037 appeared positive for expression of the transgene however, there were two additional products amplified and therefore the plant was treated as T038 and discarded. Table 4.1 contains a summary of all of the putative pRP005 transformants. The remaining plants (eight T_2 plants from each line) were grown for seed. Seeds were obtained from all selfed T_2 plants and grown under kanamycin selection, to identify homozygous lines (those which exhibited no segregation of the transgene). These homozygous lines were used for further investigation (Chapter 5).

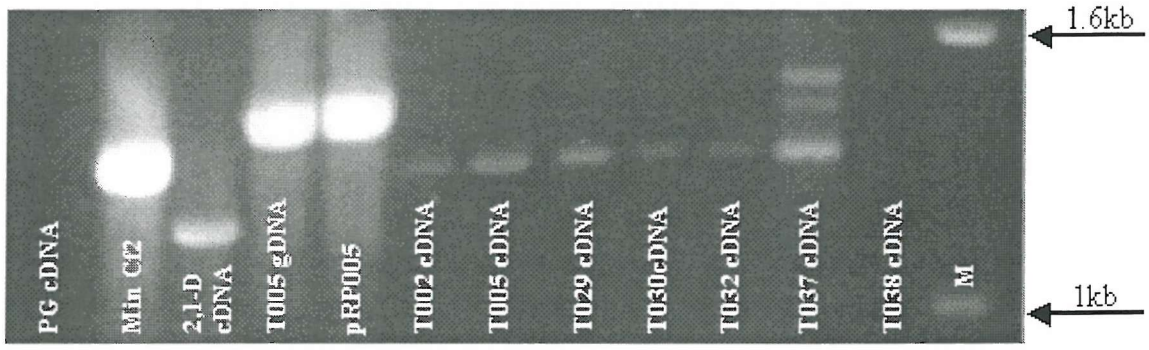


Figure 4.3: *N. tabacum* cv. Petit Gerard transformants express the *RP005* transgene.

Tobacco tranformants carrying one copy of RP005 were tested for expression of the transgene by RT-PCR. Total RNA (4-5 µg) was used as a cDNA template. The cDNA was cleaned and eluted in 30 µl. Cleaned cDNA (6 µl) was used in each 20 µl PCR and 50 ng of gDNA or 5ng of plasmid DNA were added to the control PCRs. Primers (CF2R1 and MD20) were chosen to amplify across an intron. Petit Gerard cDNA was used as a negative template control. Minimal Cf-2 plasmid and 2.1-D cDNA were used as *Cf-2* positive controls to demonstrate the size shift expected due to amplification across the intron. The plasmid pRP005 and T005 gDNA were used as positive controls to indicate the expected product size due to the presence of the intron. The products from the transformants and pRP005 were of a higher molecular weight relative to the *Cf-2* controls due to the epitope tag within the amplified region. Plant designations are aligned to relevant lanes.

UNIVERSITY OF SOUTHAMPTON LIBRARY

4.2.2 Transforming *N. tabacum* cv. Petit Gerard with *RP006.*

The second construct pRP006 (epitope-tagged Cf-2 using the relatively strong CaMV 35S promoter, Chapter 3) was stably transformed into *N. tabacum cv.* Petit Gerard. Over 100 shoots were transferred to rooting media. Under kanamycin selection 25 tobacco shoots rooted and were designated T041-T065. Putative transformants were transferred to soil and grown for seed and tissue. It was decided to test for expression (and hence presence) of the transgene by northern analysis, instead of by Southern blot and RT-PCR as before. As the construct pRP006 uses the relatively strong CaMV 35S promoter, detection by northern blot analysis was predicted to be relatively easy. It was also predicted that background *Hcr* expression would not be a problem as the native expression levels are relatively low. Of the 25 plants analysed 16 (T041, T042, T043, T046, T048, T049, T050, T053, T055, T057, T060, T061, T062, T063, T064 and T065) tested positive for transgene expression (Figure 4.4). The transformants appeared to have differing expression levels, although equal loading of the samples was not tested for the first 20 samples (Figure 4.4, A) post transfer to the membrane. However loading was assessed for the second set of samples (figure 4.4, B) by stripping the blot and re-probing with a probe designed to anneal to the relatively abundant 18S ribosomal RNA (rRNA) (Figure 4.4, C). This revealed roughly equal loading for these samples. The 18S rRNA probe is designed specifically to flax, obtained by an *Eco*RI, *Kpn*I digest of the plasmid pBG35 to release a 1.5kb fragment (Goldsborough and Cullis, 1981).

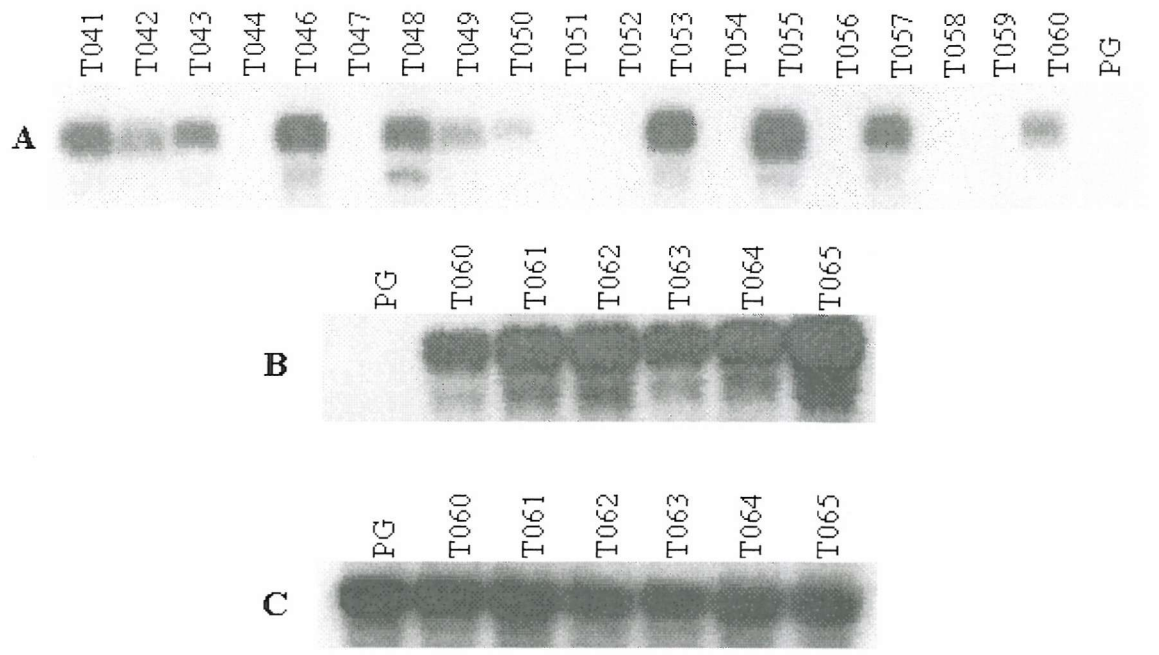


Figure 4.4: *N. tabacum* cv. Petit Gerard transformants express the *RP006* transgene.

10μg of total RNA was separated on a 1.5% (w/v) denaturing gel and subjected to northern analysis. Blots A and B were probed with 2.4kb *Cf-2* probe A (Figure 4.1). To confirm equal loading blot B was stripped and incubated with a probe for 18S ribosomal RNA (C). *N. tabacum cv.* Petit Gerard (PG) total RNA was used as a negative control to test for the expression of *Hcrs* in tobacco. Plant designations are aligned to the relevant lanes. A shows a 50 minute exposure, B and C show results from a 4 hour exposure.

Segregation analysis of the transgene in the T_2 generation was performed on all transformants that were positive by northern analysis. Seven transformants (T041, T042, T060, T061, T063, T064 and T065) carried just one copy of the transgene (Table 4.2). Due to contamination of the seedlings grown on kanamycin, the segregation data obtained for the plant T041 came several weeks later. Therefore, it was decided to carry on the analysis with the remaining 6 single insertion lines. A positive correlation was observed between copy number and intensity of signal on the northern blot. Eight green seedlings of each of the above lines were grown in soil for seed. From these plants homozygous lines were established and used for further investigation (Chapter 5).

Table 4.2: Segregation analysis of RP006 *N. tabacum* cv. Petit Gerard transformants.

Self-seed of T_1 transformants were plated on MS media with 300µg/ml kanamycin selection. After 3 weeks the seedlings were analysed and their segregation ratios calculated. Seedlings expressing at least one copy of the transgene remained green (G) whilst those without a copy were bleached white (W). Significance level at P = 0.05 for a chi^2 value with one degree of freedom = 3.84 (significant values are shown in bold).

chi^2 values were calculated as follows

General formula:

x:1 $chi^2 = \frac{(a-xb)^2}{xn}$ **Where $a \geq b$ and $a + b = n$**

Plant designations in *itallics* indicate those with a single integration event

*** = Probably untransformed or protein not expressed.**

‡ = Two independent integrations

§ = Three independent integrations.

	Segregation	Goodness of fit chi^2		
Plant	**G : W**	**3 : 1**	**15 : 1**	**63:1**
T041	222:072	**0.04**	166.93	1004.78
T042	444:140	**0.33**	313.05	1906.86
T043*	000:100	300.00	1500.00	6300.00
T046§	354:003	111.13	17.83	**1.21**
T048§	261:003	80.18	11.78	**0.31**
T049*	000:100	300.00	1500.00	6300.00
T050	356:069	17.41	72.32	594.89
T053‡	336:014	82.32	3.02	13.52
T055§	280:004	84.30	11.36	**0.04**
T060	117:035	**0.32**	73.01	455.28
T061	305:101	**0.00**	240.41	1434.80
T062‡	293:021	56.16	**0.10**	53063
T063	231:084	**0.47**	224.09	1290.69
T064	173:053	**0.29**	114.13	704.00
T065	225:062	**1.77**	115.45	749.39

4.2.3 Transforming *L. esculentum* with *RP005.*

Tomato transformation was technically challenging, with many failed attempts. The first unsuccessful protocol (tomato transformations A, Chapter 2) always resulted in the death of explants, neither shoots nor callus were ever observed. After repeated failed attempts a new protocol (tomato transformations B, Chapter 2) was devised with the kind help of Matthew Smoker in the Sainsbury Laboratory at the John Innes Centre, Norwich. This protocol made use of feeder layer plates for the *A. tumefaciens* / tomato explant co-cultivation phase. Feeder layer plates were made up of co-cultivation media with a layer of tobacco suspension cells covered with filter paper onto which the explants were placed. This protocol proved a success. Further problems were encountered when subsequent attempts resulted in no shoot formation. This was overcome by the use of a fresh stock of the shooting hormone zeatin riboside. In addition to this, the antibiotic augmentin (Duchefa, The Netherlands) was replaced with 150mg/l Timentin (Duchefa, The Netherlands), as this antibiotic has been shown to be non-toxic and significantly promotes shoot formation (Ling *et al.*, 1998). Once shoots were formed they were cut from the explants and placed in rooting media. The first two attempts resulted in very low rooting efficiency, with approximately 10% of shoots producing roots. Green shoots are capable of producing their own hormones for root growth; however, to increase rooting efficiency 0.18mg/l of the auxin indole-3- acetic acid (IAA) was used to supplement the rooting media (Seear, unpublished data).

L. esculentum Cf0 tomatoes were transformed with the construct pRP005. During the first few attempts, approximately 40 shoots were obtained of which only six rooted, one of which lacked an apical meristem. The five putative positive transformants were designated T001, T002, T003, T004 and T005. These were subsequently transferred to soil and placed in a propagator to maintain high humidity levels. The propagator was gradually opened over a period of a few days to allow the plants to adapt to a drier environment. The second round of transformation produced seven putative transformants designated T212-T218, plant T217 was later lost.

The plants were screened for presence of the transgene by inoculation with *C. fulvum* race 4*GUS* and by PCR and RT-PCR analysis concurrently. Inoculation with *C. fulvum* also served to assess the functionality of the transgene.

Presence of the T-DNA was analysed by PCR, using the primer combination of RP2 and CF2R1. This primer combination amplifies a region of the transgene from within the epitope-tag. This revealed that only two plants (T002 and T213) were positive for the T-DNA, *RP005* (Figure 4.5 and 4.6). This was repeated several times with the same result.

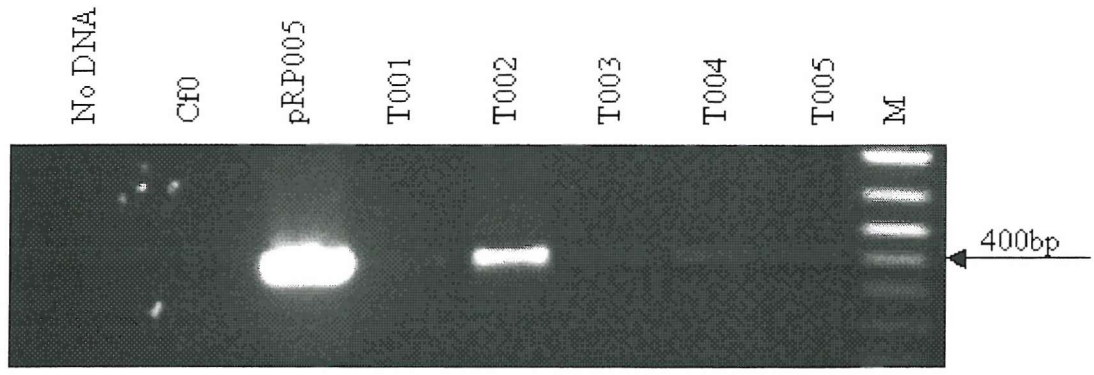


Figure 4.5: *L. ecsulentum* putative transformants carry the T-DNA *RP005*.

PCR was carried out on 100ng of template genomic DNA extracted from the putative tomato transformants and controls. 5ng of pRP005 was used as a positive control, with Cf0 and no template making up the negative controls. The primers (RP2 and CF2R1) were chosen to amplify a region starting within the 3x c-Myc region for increased specificity. Plant designations are aligned to relevant lanes. M indicates molecular weight markers (1kb Plus ladder, Invitrogen, Paisley, UK).

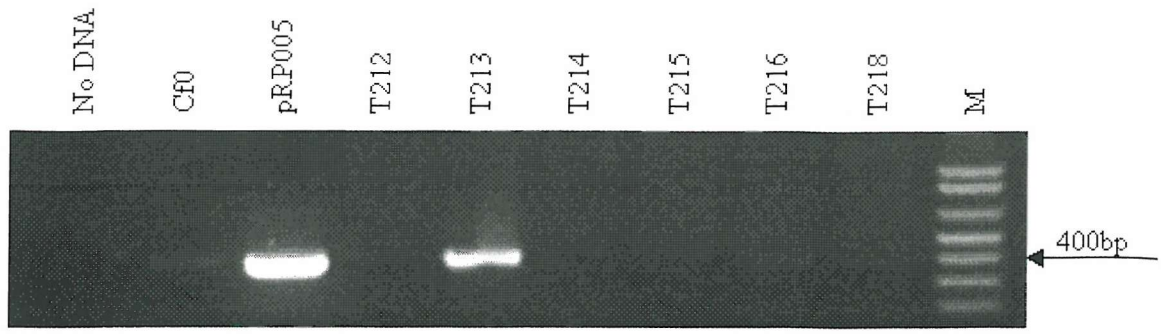


Figure 4.6: *L. esculentum* putative transformants carry the T-DNA *RP005*.

PCR was carried out on 100ng of template genomic DNA extracted from the putative tomato transformants and controls. 5ng of pRP005 was used as a positive control, with Cf0 DNA and no DNA template making up the negative controls. The primers (RP2 and CF2R1) were chosen to amplify a region starting within the 3x c-Myc region for increased specificity. Plant designations are aligned to relevant lanes. M indicates molecular weight markers (1kb plus ladder, Invitrogen, Paisley, UK).

Expression of the T-DNA was tested by RT-PCR. Optimisation of the RT-PCR was difficult, probably due to the predicted low levels of transcript and the presence of additional *Hcr2s*. Initially the same primer combination, as used on tobacco transformants, was used. Problems were encountered with this primer combination (CF2R1 and MD20). This primer combination amplifies across the *Cf-2* intron but is situated in the most conserved region of the gene resulting in the amplification of Cf0 homologues (as revealed by sequencing data, not shown). To circumvent amplification of the *Hcrs* the primers RP2 and CF2R1 were chosen. This primer combination amplifies a region of the transgene from within the epitope-tag and spanning the intron. Although only one plant (T002), from the initial group of transformants, was shown to be positive by PCR all five plants were analysed for expression to verify the above result. Two plants (T002 and T005) appeared to express the transgene as shown by RT-PCR (Figure 4.7). However, non-specific bands were also amplified from both Cf0 and *Cf-2* negative controls. As the non-specific band was present in the tobacco 2,1-D, which expresses *Cf-2* and carries no Hcrs, it could be assumed that this is due to amplification of *Cf-2* itself. Repetition of RT-PCR revealed only T002 to be consistently positive for expression of the transgene. This is what was to be exprescted as only T002 was shown to be positive by PCR. Sequencing of the T002 RT-PCR product confirmed it to be a specific product, the epitope-tagged transgene (data not shown). RT-PCR was performed on only T213 from the second group of transformants and revealed that this plant also expresses the transgene (Figure 4.8 A). The Primers RP2 and CF2R1 were chosen to access expression of the

transgene for reasons described above. In addtion the cDNAs and the gDNA controls were amplified using the primers MD8 and CF2R1 as this amplifies the most conserved region of Cf-2 and had previously been shown to anneal preferentially to Cf0 at lower annealing temperatures (50-52^{0}C). These reactions served as a positive control for the quality of the cDNAs and gDNAs demonstrating that amplification from these templates was possible (Figure 4.8B).

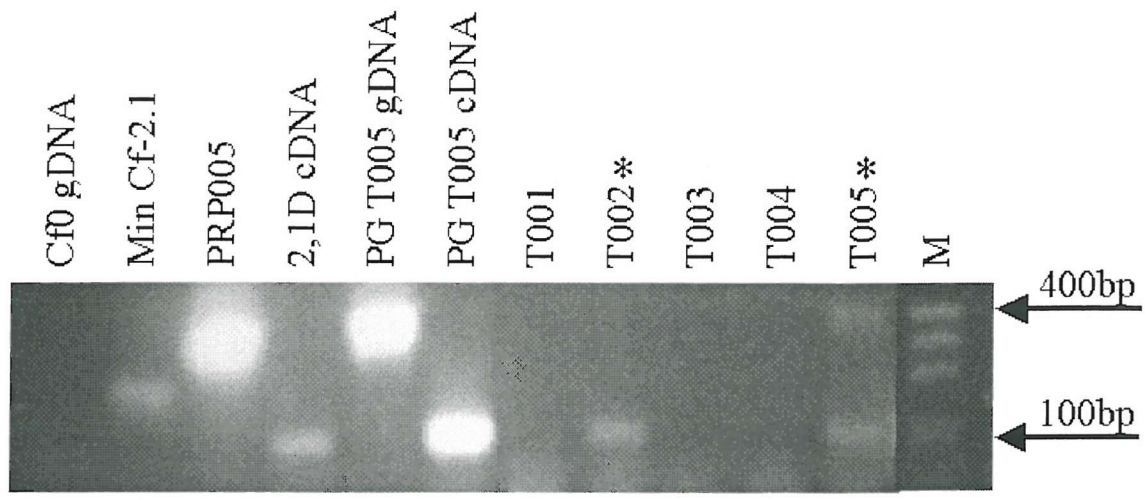


Figure 4.7: *L. esculentum* Cf0 expresses the transgene RP005.

All five putative transformants were assessed for expression of the T-DNA by RT-PCR. Total RNA (4-5 µg) was used as a cDNA template. The cDNA was cleaned and eluted in 30 µl. Cleaned cDNA (6 µl) was used in each 20 µl PCR and 50 ng of gDNA or 5ng of plasmid DNA were added to the control PCRs. Primers (CF2R1 and RP2) were chosen to amplify across an intron. Cf0 gDNA, min Cf-2.1 plasmid and 2,1D cDNA (Petit Gerard tobacco transformed with *Cf-2*) were used as a negative template controls. PG T005 gDNA and cDNA (tobacco transformed with *RP005*) were used as *Cf-2* positive controls to demonstrate the size shift expected due to amplification across the intron. The products from the transformants and pRP005 were of a higher molecular weight relative to the *Cf-2* controls due to the epitope tag within the amplified region. Plant designations are aligned to relevant lanes and * indicates plants positive for expression of the transgene. M indicates molecular weight markers (1kb ladder, Gibco BRL, Cheshire, UK)

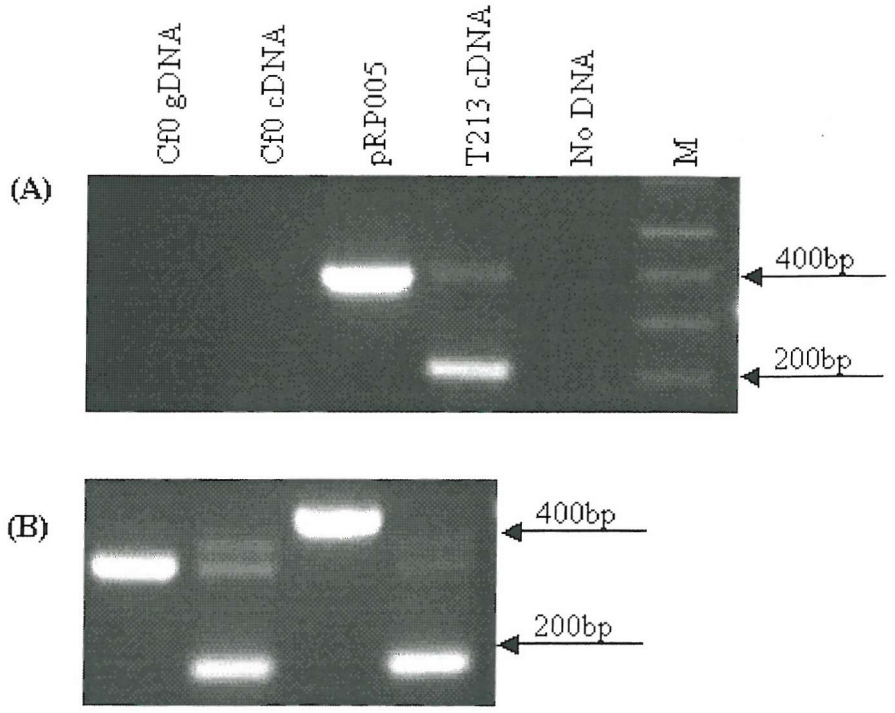


Figure 4.8: *L. esculentum* Cf0 transformants express the *RP005* transgene.

Tomato transformants positive by PCR (T213) were tested for expression of the RP005 transgene by RT-PCR. Total RNA (2µg) was used as a cDNA template. 2µl of the 20µl reverse transcription (RT) reaction was used in each 20µl PCR and 100 ng of gDNA or 5 ng of plasmid DNA were added to the control PCR tubes. The primers (RP2 and CF2R1) were chosen to amplify a region starting within the 3x c-Myc region and spanning an intron (A). Cf0 gDNA and cDNA were used as negative controls and pRP005 as a positive control. Plants expressing the transgene should show a band with a molecular weight approximately 120bp smaller than the plasmid control. Primers MD8 and CF2R1 were chosen (B) as a positive control for the RT reaction as these were predicted to amplify a region of Cf0 (as well as Cf-2) as they amplify the most conserved region of the gene. Plant designations are aligned to relevant lanes. M = Size marker (1kb plus ladder, Invitrogen, Paisley, UK).

Concurrent with the PCR and RT-PCR analysis the first five putative, *RP005*/Cf0, transformants (T001-T005) were inoculated with *C. fulvum* race 4*GUS*. Initial qualitative results indicate that T001-T005 were all susceptible to some degree, though not the same degree as a completely susceptible (Cf0) tomato (Figure 4.9). Unfortunately a completely resistant (Cf2) tomato was not inoculated as a control, previous inoculations revealed that no GUS staining is observed in such plants indicating the GUS staining observed on the putative transformants was due to breaking of resistance. In addition as only T002 carries the transgene all the others should be completely susceptible. Repeat inoculations were performed to allow quantitative analysis to be performed. This revealed that none of the

plants were as susceptible as a Cf0 tomato, but that T002 may be partially resistant (Figure 4.10).

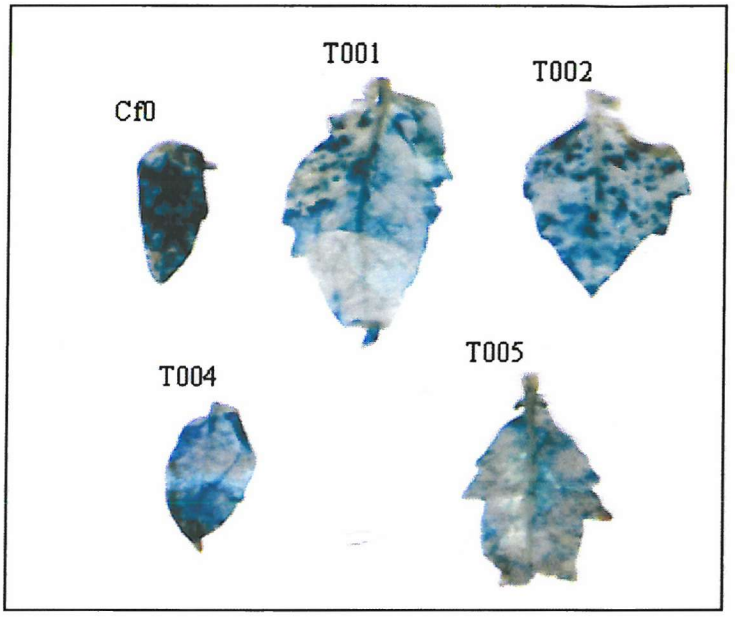


Figure 4.9: *L. esculentum* RP005 transformants are susceptible to *C. fulvum* race 4 GUS.

Equivalent leaves were harvested from each plant 17 days post infection. GUS activity was assayed qualitatively by incubation with X-gluc. Leaf tissue was soaked in 70% (v/v) ethanol to remove chlorophyll. Cf0 is included as a completely susceptible control.

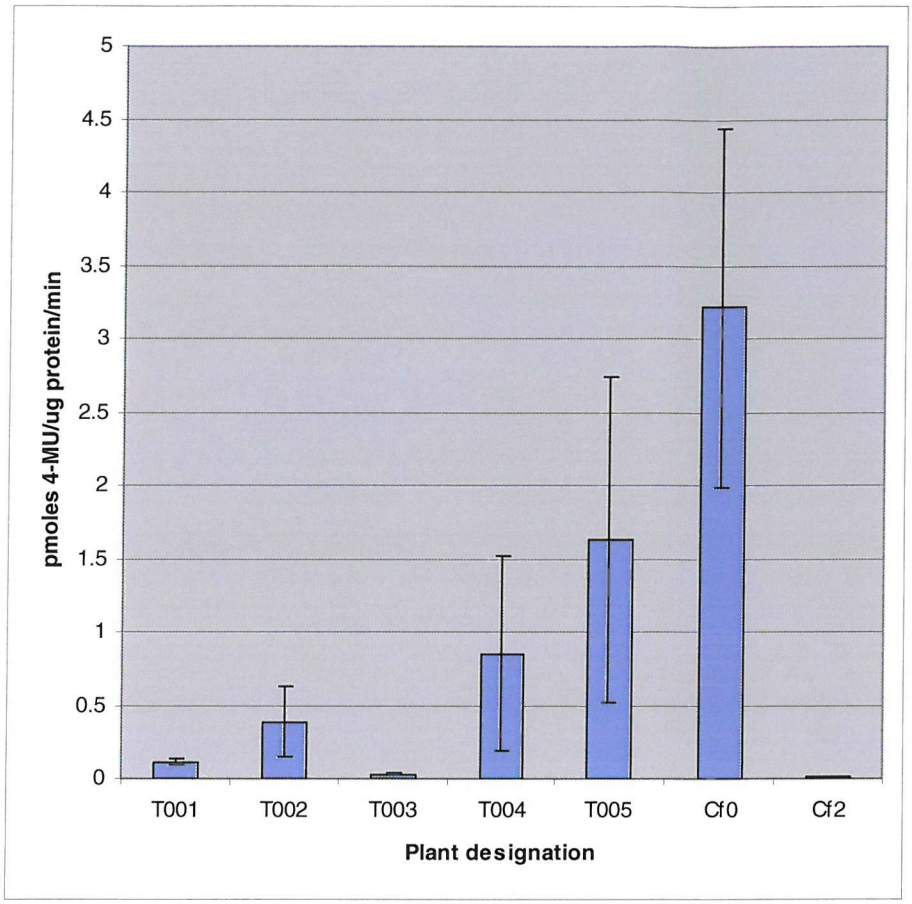


Figure 4.10: *L. esculentum* RP005 transformants are susceptible to *C. fulvum* race 4 GUS.

Equivalent leaves were harvested from each plant 17 days post infection and assayed for infection levels by GUS quantification. Three leaves were harvested and assayed for each plant, plants T001 and T003 lack the transgene. Error bars indicate the standard error of the mean, in each case n = 3.

The second group of putative, *RP005*/Cf0, primary transformants (T212-T218) were also inoculated with race 4*GUS*. Tomato seedlings obtained from selfed primary transformants were inoculated and incubated for 15 days. Second generation (T_2) plants were used simply due to timing. Inoculations were not performed during the shorter days of the winter period as light quality and day length affects inoculation efficiency. When conditions were good for inoculations the primary transformants had already set seed. One consideration to be made was that some of the plants would not carry the transgene; this was easily determined by PCR. During the incubation period it was discovered that only one plant (T213) expressed the transgene, so only this plant was analysed. Although no adequate controls are included, it is clear the plants are at least partially susceptible to *C. fulvum* race 4*GUS* infection (Figure 4.11). Due to the lack of a susceptible control the level of infection is unclear. Comparisons to previous inoculation would suggest that

these are only partially susceptible, however, it is impossible to tell whether this is due to some level of resistance or a poor inoculation. To determine whether the susceptible plants carried the transgene PCR analysis was performed revealing 2 (B and C) carried the transgene (Figure 4.12).

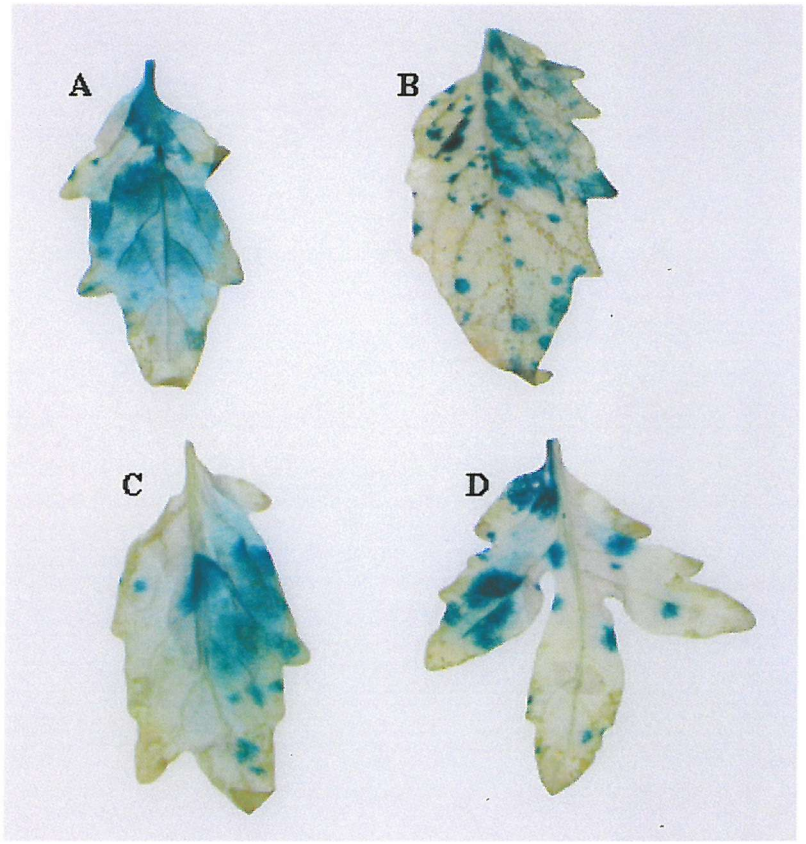


Figure 4.11: Second generation T213 tomatoes are susceptible to *C. fulvum* race 4*GUS*.

Equivalent leaves were harvested from four T_2 T213 tomatoes at 17 days post infection. GUS activity was assayed qualitatively by incubation with X-gluc. Leaf tissue was soaked in 70% (v/v) ethanol to remove chlorophyll.

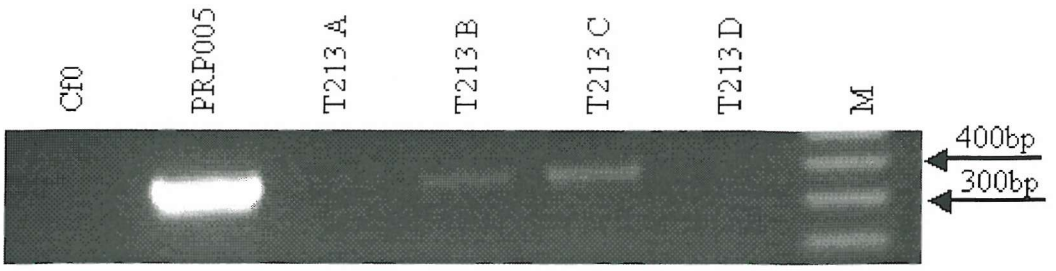


Figure 4.12: T213 tomatoes susceptible to *C. fulvum* race 4*GUS* carry the transgene *RP005*.

DNA was extracted from four T_2 T213 tomatoes shown to be susceptible to *C. fulvum* race 4*GUS*. Presence of the transgene was assessed by the PCR using the primers RP2 and CF2R1. Plants B and C are both positive for the transgene. Plant designations are aligned to the appropriate lanes. Cf0 and pRP005 DNA was used as negative and positive controls respectively. M indicates molecular size marker (1kb plus ladder, Invitrogen, Paisley, UK).

4.2.4 Transforming *L. esculentum* with *RP006*.

Previous attempts to over-express *Cf-2* in *L. esculentum* Cf0 have been relatively unsuccessful, with only one resistant transformant out of 13 recovered. It was hypothesised that only plants with low levels of *Cf-2* expression might be viable (Dixon unpublished). This observation is likely to be due to the auto-necrosis that results from the leaky signal generated by $Rcr3^{esc}$. To avoid such auto-necrosis the construct pRP006 was transformed into *L. esculentum* rcr3-3, an Rcr3 loss of function mutant (Dixon *et al.*, 2000), as well as Cf0.

Ten shoots were produced from pRP006/Cf0 transformations, two of which rooted under kanamycin selection (T010 and T011). These plants were provisionally tested for expression of the transgene by RT-PCR (Figure 4.13). RT-PCR and subsequent sequencing of the putative transformants revealed that only T010 was positive for expression of the transgene.

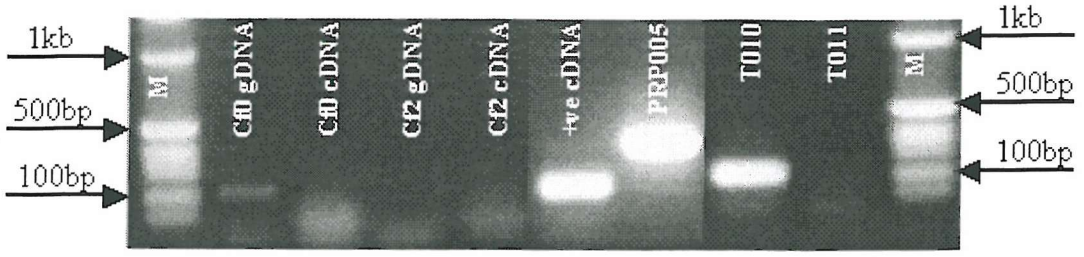


Figure 4.13: One *L. esculentum* (Cf0) transformant expresses the *RP006* transgene.

Putative tomato transformants were tested for expression of the RP006 transgene by RT-PCR. Total RNA (4-5µg) was used as a cDNA template. The cDNA was cleaned and eluted in 30 µl. Cleaned cDNA (6 µl) was used in each 20 µl PCR and 50 ng of gDNA or 5 ng of plasmid DNA were added to the control PCRs. The primers (RP2 and CF2R1) were chosen to amplify a region starting within the 3x c-Myc region and spanning an intron. Cf0 and Cf2 plants were used as negative controls. The Petit Gerard transformant T005 (+ve cDNA) and the plasmid pRP005 were used as positive controls. Plants expressing the transgene should show a band with a molecular weight approximately 120bp smaller than the plasmid control. Plant designations are aligned to relevant lanes. M indicates molecular weight markers (1kb ladder, Gibco BRL, Cheshire, UK).

Of 15 shoots from the RP006/rcr3-3 transformations, four rooted under kanamycin selection and were designated T006-T009. As these plants carry a loss of function mutation in Rcr3, a protein absolutely required for Cf-2 mediated resistance, presence of a copy of Cf-2 cannot be detected by inoculation with *C. fulvum*. Also as the plants carry a copy of Cf-2 the previously described Southern blot protocol could not be used. The plants were provisionally tested by RT-PCR. To ensure that *Cf-2* was not amplified primers were chosen to amplify from within the epitope tag. Of the four putative transformants, three were positive for expression of the transgene (Figure 4.14). Sequencing of the RT-PCR products revealed products specific for the transgene.

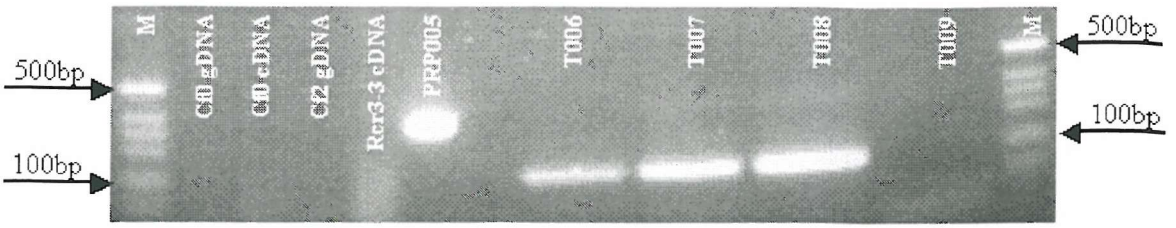


Figure 4.14: *L. esculentum* transformants (rcr3-3) express the *RP006* transgene.

Putative tomato transformants were tested for expression of the RP006 transgene by RT-PCR. Total RNA (4-5µg) was used as a cDNA template. The cDNA was cleaned and eluted in 30 µl. Cleaned cDNA (6 µl) was used in each 20 µl PCR and 50 ng of gDNA or 5 ng of plasmid DNA were added to the control PCRs. The primers (RP2 and CF2R1) were chosen to amplify a region starting within the 3x c-Myc region and spanning an intron. Cf0, Cf2 and rcr3-3 plants were used as negative controls and the plasmid pRP005 as a positive. Plants expressing the transgene should show a band with a molecular weight approximately 120bp smaller than the plasmid control. Plant designations are aligned to relevant lanes. M indicates molecular weight marker (1kb ladder, Gibco BRL, Cheshire, UK)

To assess expression levels of the RP006 transgene in both a Cf0 and rcr3-3 background northern analysis was performed (Figure 4.15 A). To test for equal loading of the samples the blot was stripped and re-incubated with the 18S probe described above (Figure 4.15 B). Two sets of RNA preparations were made from different cuttings of the same plant and assessed together for replication. Due to unequal loading, density readings of the signals obtained were made so relative expression levels could be calculated. It appears that expression levels in an rcr3-3 background are at least twice of that seen in a Cf0 background. This observation should be treated with caution as only one RP006/Cf0 transformant was recovered and analysed.

An interesting observation is the two transcripts present only in the plants expressing the transgene; this second transcript can also be seen in the tobacco northern blot analyses (Figure 4.4). As no size markers were run with these samples it is impossible to know which of the two is the desired transcript.

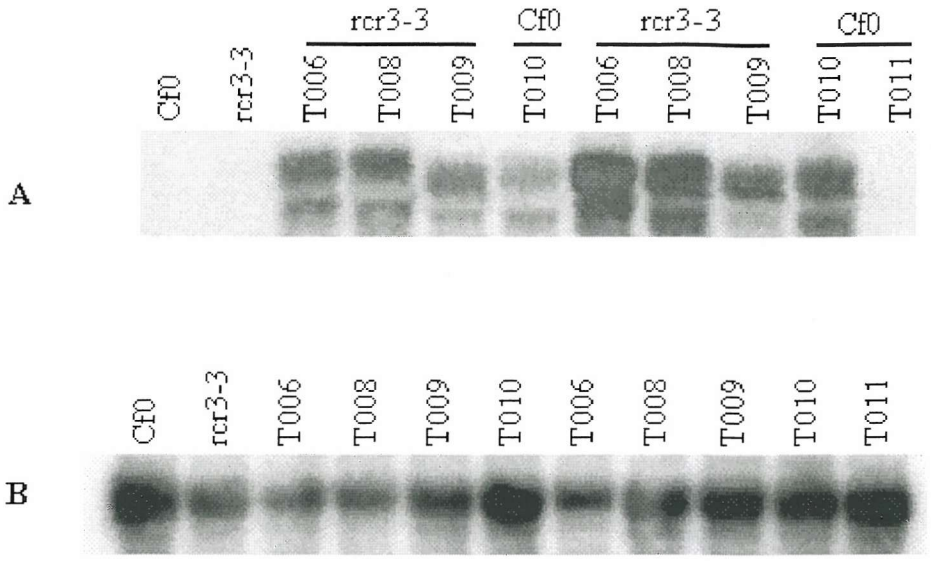


Figure 4.15: *L. esculentum* Cf0 and rcr3-3 express the transgene *RP006*.

10μg of total RNA was separated on a 1.5% (w/v) denaturing gel and subjected to northern analysis. The blot was initially probed with 2.4kb *Cf-2* probe A (Figure 4.1) (A). To confirm equal loading of samples the membrane was stripped and probed for 18S ribosomal RNA (B). Cf0 and rcr3-3 total RNA were loaded as negative controls for specificity of the probe and expression levels of native *Cf-2* respectively. Plant designations are aligned to relevant lanes and background genomes indicated. Both images represent a 4 hour exposure.

Cuttings taken from the plant T010 were inoculated with race-4GUS to test for the function of the transgene. Quantitative analysis revealed that the plant was indeed resistant to pathogen challenge indicating the transgene is functional (Figure 4.16).

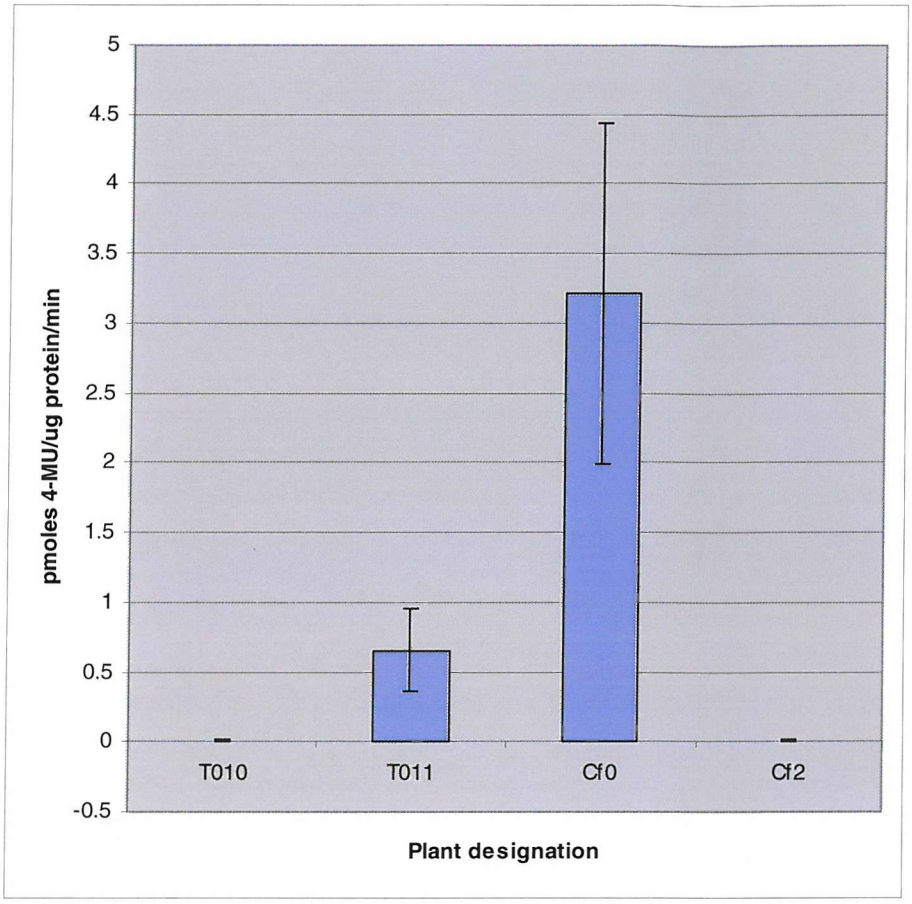


Figure 4.16: The *L. esculentum RP006* primary transformant T010 is resistant to *C. fulvum* race-4GUS.

Equivalent leaves were harvested from each plant 17 days post infection and assayed for infection levels by GUS quantification. Three leaves were harvested and assayed for each plant. Error bars indicate the standard deviation from the mean, in each case n = 3.

To test for segregation of the transgene, seed obtained from the selfed primary transformants were sterilised and grown on seed germination media supplemented with kanamycin (100mg/l). T009 did not set seed readily, producing only 4 seed. All lines (ecept T008) were shown to carry just one copy of the T-DNA (Table 4.4). To test for the function of the transgene in a Cf0 background and to verify the segregation analysis approximately 20 seed were sown in soil and inoculated with *C. fulvum* race-4GUS. Although the rcr3-3 lines are not expected to be resistant to pathogen challenge they were also inoculated and used as additional negative controls to assess the success of the inoculations. All plants were completely susceptible to the pathogen as revealed by GUS staining, suggesting the transgene in the T010 line is non-functional in the next generation (Figure 4.17). Four of the susceptible T_2 T010 plants were selected at random and DNA purified. PCR analysis revealed that the transgene was present in all four plants (Figure

4.18). The primary transformant, T010 appeared to be resistant to *C. fulvum* challenge. This could be due to either the failure of the inoculation on the primary transformant and hence the generation of a false positive or through gene silencing in the second generation. To test this Northern analysis was performed on the four T_2 T010 plants and showed clearly strong expression of the epitope-tagged *Cf-2* gene (Figure 4.19).

Table 4.4: Segregation analysis of RP006 *L. esculentum* transformants.

Self-seed of T_1 transformants were plated on MS media with 100µg/ml kanamycin selection. After 3 weeks the seedlings were analysed and their segregation ratios calculated. Seedlings expressing at least copy of the transgene remained green (G) whilst those without a copy were stressed with purple colouration and stunted root growth (P). Significance level at P = 0.05 for a chi^2 value with one degree of freedom = 3.84 (significant values are shown in bold).

chi^2 values were calculated as follows

General formula:

x:1 $chi^2 = \frac{(a-xb)^2}{xn}$ **Where a ≥ b and a + b = n**

*** = Probably untransformed or protein not expressed.**

‡ = Two independent integrations

	segregation	Goodness of fit chi^2	
Plant	**G : P**	**3: 1**	**15: 1**
T006	43:15	**0.02**	38.07
T008‡	84:08	13.04	**0.94**
T009	01:01	**0.67**	6.53
T010	30:07	**0.73**	10.14

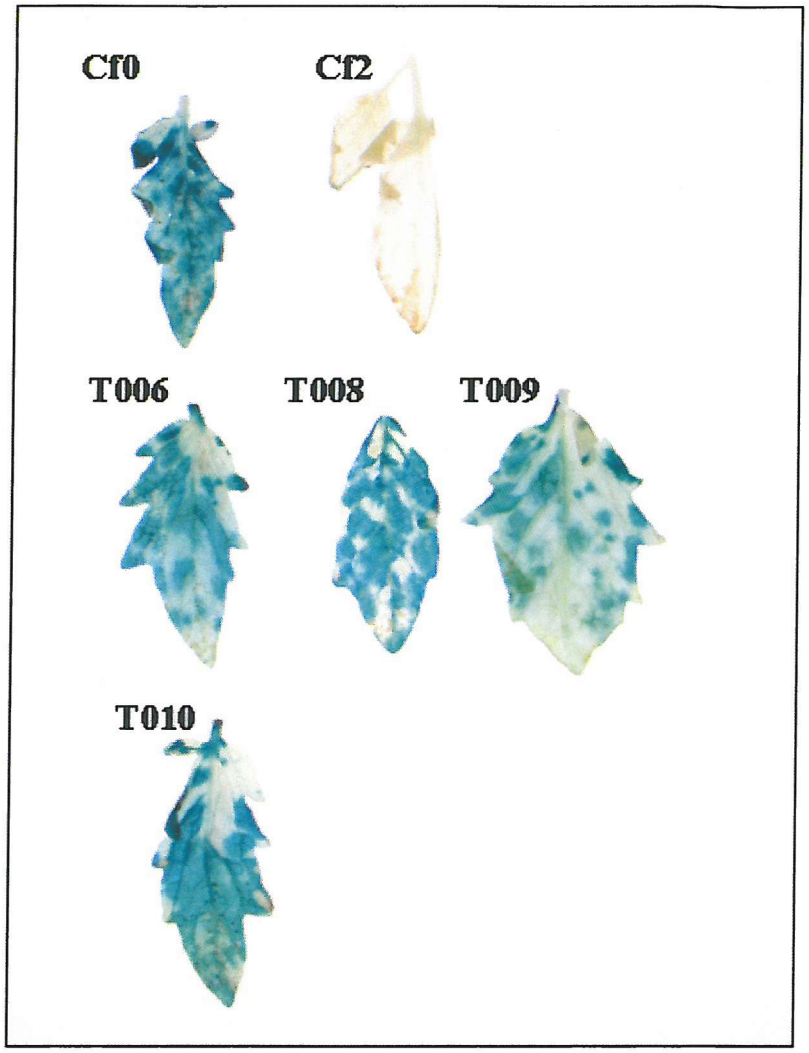


Figure 4.17: c-Myc:Cf-2 using the 35S promoter is non-functional in the T_2 seedlings.

Seed was collected from the selfed primary transformants and grown on media supplemented with kanamycin. Eight plants resistant to kanamycin were transferred to soil allowed to recover and inoculated with *C. fulvum* race 4*GUS*. Leaves were selected, infiltrated with X-gluc and incubated over night at 37^0C. Leaves were de-stained in 70% (v/v) ethanol before being photographed. T006, T008 and T009 are all in an rcr3-3 background and therefore included as completely susceptible controls. In addition Cf0 was included as a susceptible control and Cf2 as a completely resistant control.

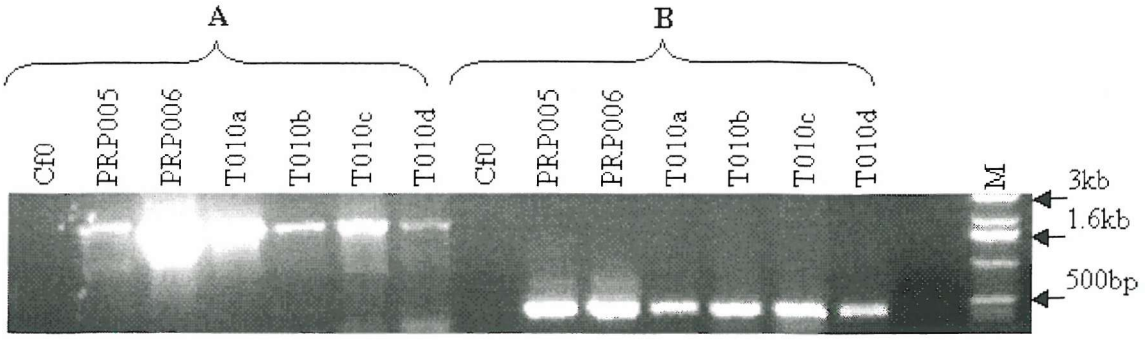


Figure 4.18: T_2 generation T010 transformants susceptible to *C. fulvum* carry the transgene *RP006*.

Genomic DNA was purified from four of the plants resistant to kanamycin but susceptible to *C. fulvum* race-4GUS. PCR analysis was chosen to test for presence of the transgene. Two primer combinations were used; universal reverse and 8891R2 (A), RP2 and CF2R1 (B). Primer combination A amplifies the promoter of the construct and combination B the epitope-tag insert. Cf0 gDNA was used as a negative control and pRP005 and pRP006 as positive controls. There is little size difference between the native and 35S (pRP005 and pRP006) promoters using primer combination A. PCR conditions used were an annealing temperature of 52^{0}C, an extension period of 1 minute 30 seconds, cycled 35 times. M indicates the molecular weight size markers (Gibco BRL 1kb marker, Cheshire, UK).

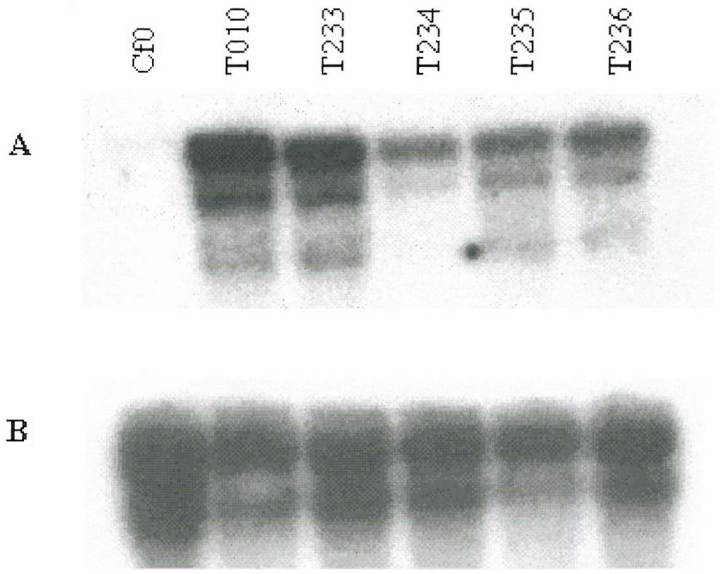


Figure 4.19: T010 second generation (T_2) transformants, susceptible to *C. fulvum* race 4*GUS* express the transgene *RP006*.

RNA was purified from four of the tomatoes, resistant to kanamycin but susceptible to *C. fulvum* race 4*GUS*, from the T010 line of transformants. 10µg of total RNA was separated on a 1.5% (w/v) denaturing gel and subjected to northern analysis. The blot was initially probed with 2.4kb *Cf-2* probe A (Figure 4.1) (A). To confirm equal loading of samples the membrane was stripped and probed for 18s ribosomal RNA (B). Cf0 was loaded as negative control for specificity of the probe. Plant designations are aligned to relevant lanes. Image A represents an overnight exposure at –80^{0}C with an intensifying screen, image B represents a 45 minute exposure at room temperature without an intensifying screen.

Table 4.5 shows a summary of all transgenic plants obtained that were positive for the expression of one copy of the transgene, also detailed are the homozygous lines established through the selfing of these plants.

Table 4.5: Summary of transgenic lines

Listed are all transgenic plants generated that carry and express a single copy of the transgenes *RP005* or *RP006*. Also included is the background genetics of each plant and plant designations where homozygous lines were established. N/E = not established

The plants listed	**Transgene**	**Background**	**Homzygous T_2 lines**
T004	*RP005*	N. Tabaccum	N/E
T002	*RP005*	*N. Tabaccum*	T105
T005	*RP005*	*N. Tabaccum*	T066, T069
T029	*RP005*	*N. Tabaccum*	T080, T087
T030	*RP005*	*N. Tabaccum*	T088, T094
T032	*RP005*	*N. Tabaccum*	T096, T099
T041	*RP006*	*N. Tabaccum*	T146, T149, T150
T042	*RP006*	*N. Tabaccum*	T111
T060	*RP006*	*N. Tabaccum*	T155, T158, T159
T061	*RP006*	*N. Tabaccum*	N/E
T063	*RP006*	*N. Tabaccum*	T168
T064	*RP006*	*N. Tabaccum*	N/E
T065	*RP006*	*N. Tabaccum*	T177
T002	*RP005*	L. esculentum/ *Cf0*	*N/E*
T005	*RP005*	*L. esculentum/* Cf0	N/E
T213	*RP005*	*L. esculentum/* Cf0	N/E
T010	*RP006*	*L. esculentum/* Cf0	N/E
T006	*RP006*	*L. esculentum/* rcr3-3	N/E
T009	*RP006*	*L. esculentum/* rcr3-3	N/E

4.3 Discussion

Differences were noted concerning the relative ease of transformation between tobacco and tomato and between the tomato backgrounds Cf0 and rcr3-3. Tomato transformations were always substantially less successful than tobacco, with the generation of fewer shoots and lower rooting efficiencies. This was due, in part, to the technical difficulty of the transformation protocol. Tomato explants were much smaller and more fragile than tobacco explants and therefore more tissue damage occurred. After approximately two weeks both tobacco and tomato explants became harder and therefore easier to handle and less prone to damage. The tomato protocol involved the additional co-cultivation period, which lead to more handling of the explants at an early stage and resulted in substantially more tissue damage. This tissue damage often led to death of the explants. Important for the success of the tomato transformations were the tobacco suspension cultures used for a feeder layer. The maintenance of a healthy suspension culture was difficult and could have affected the transformation efficiency.

Due to the low transformation rate many attempts of tomato transformations were performed with each construct. Still relatively few transformants were ever recovered. Only one *RP006*/Cf0 transformant was recovered, possibly due to the leaky signal generated by $Rcr3^{esc}$ resulting in necrosis, as observed by Krüger *et al.* (2002). *L. esculentum* Cf0 cells that were successfully transformed with *RP006* would have become necrotic and hence shoots were not regenerated. Transient transfection of *N. tabacum* with two strains of *Agrobacterium* carrying either *Avr2* or *Cf-2* did not result in necrosis, which was observed in an *Rcr3* dependent manner in *N. benthamiana* and tomato (Jones *et al.*, unpublished). This is believed to be due to *N. tabacum* lacking a functional Rcr3, explaining the relative ease with which pRP006 was transformed into *N. tabacum*. This is supported by the recovery of more *RP006* expressing transformants in an rcr3-3 background than in a Cf0 background with fewer tranformation attempts. This should be treated with caution due to the low number of transformants recovered.

When characterising the putative transformants, for the presence and functionality of the T-DNA, many controls were ommited. This could have resulted in false negatives. For example, where RT-PCR was used to assess expression of the transgene, positive controls testing for expression of constitutive genes (e.g. actin genes) are lacking.

Therefore, it is possible that some of the plants were labelled as negative for expression where, in fact, the problem could have been with the RNA sample being impure or degraded preventing amplification. The same is true where PCR was used to assess the presence of the transgene. Many controls are also lacking for the pathogen challenge tests (inoculation with *C. fulvum* race 4*GUS*). In every case a completely susceptible (Cf0) and resistant (Cf2) plants should also have been inoculated for comparisons. Where they have been included, it was always the case that no staining could be seen in the resistant controls and a lot in the susceptible controls. Due to the lack of controls, caution should be taken when interpreting the results. In particular, comparisons of expression levels using the RT-PCR data presented cannot be treated as wholly quantitative. RNA concentrations were estimated, so the same amount of RNA was included in each reaction and every sample was treated identically. However, due to lack of amplification controls it is not known if some samples amplified better than others, irrespective of true expression levels.

Expression levels of the two constructs in tomato appeared to vary greatly; RT-PCR on plants carrying the *RP006* transgene was easier and more consistent than those carrying *RP005*. Although this RT-PCR was semi-quantitative, the same amount of starting material (total RNA) was used with the same protocol in each case. This would suggest that expression levels in *RP006* transformants is higher in than in *RP005* transformants. Previous attempts to obtain tomatoes over-expressing *Cf-2* have been unsuccessful, with only one transformant, predicted to be a relatively low expresser, recovered (Dixon, unpublished data). Northern analysis of the *RP006* tomatoes both in a Cf0 and an rcr3-3 background indicated that it is possible to express *Cf-2* to higher levels in an rcr3-3 background. This may provide some explaination for the limited success observed in the generation of Cf0 tomatoes over-expressing *Cf-2*. However, this should be treated with caution, as comparison with only one Cf0 transformant may not be representative of reality. This is also based on the assumption that the transgenes are functional, something that was brought into question by the *RP005* transformants and in the second generation T010 (*RP006*) plants.

Northern blot analysis of both the tomato and tobacco transformants carrying *RP006* revealed a second transcript (Figures 4.4 and 4.15). As no size markers were present it is impossible to tell which of the two transcripts is that of the desired epitope-

tagged Cf-2. However, as the second transcript is seen solely in the positive transformants, it appears that the second transcript is most likely to be a truncated version of the full transcript. This second transcript is seen only once in each transformant, irrespective of copy number, indicating it is unlikely to be as a result of a second truncated construct. There is no evidence of a second transcript in the lines carrying the *RP005* construct. These transformants were however assessed for expression by RT-PCR. As this only amplifies a small portion of the transgene, it is quite possible that a second truncated transcript is present, but that the deleted portion is in another part of the transgene.

The primary transformant T010 appeared to be resistant to infection by *C. fulvum* race 4*GUS* indicating that the transgene was functional. This was supported by the observation of autonecrosis as an adult plant. However, inoculation of second generation plants (T213 line of *RP005* transformants and the T010 line of *RP006* transformants) revealed the plants to be susceptible to *C. fulvum* challenge. The infection level was not quantified due to the inconsistent nature of previous inoculations. The qualitative staining of the plants indicates that they were fully susceptible, although it is not possible to tell the difference between a completely susceptible plant and one with severe loss of resistance function. These data reveal that insertion of the epitope-tag at the C-terminus of Cf-2 affects its function. In support of these data, Piedras *et al.* (2000) found that plants with the 3x c-Myc inserted in the G domain of Cf-9 showed a greater reduction in resistance compared to transformants with the same tag in the B domain (near the N- terminus). It is possible that insertion of the epitope-tag into the cytoplasmic tail of Cf-2 prevents it from interacting with additional, possibly signalling, proteins. This could be due to changes in the physical properties of the cytoplasmic tail, brought about by the insertion of the highly charged acidic c-Myc epitope-tag into the basic part of the Cf-2 protein. This would also explain why insertion of the c-Myc epitope-tag into the G domain of Cf-9 saw a greater reduction in function relative to the B domain tagged version (Piedras *et al.*, 2000).

Thomas et al. (1995) demonstrated that the *Cf-9* membrane anchor (domains E ,F and G) is absolutely required for induction of HR, when they transiently expressed a truncated version of the protein in tobacco. To investigate the importance of the dilysine motif, Van der Hoorn *et al.* (2001a) produced mutated Cf-9 proteins (the dilysine motif KKRY

was changed to AARY). This motif is at the C-terminus of Cf-9. There was no significant difference observed between the mutant and wild type peptide in the recognition of Avr9. It was noted that the ER retrieval-retention signal could be weakened, in yeast, by phenlyalanine (F) or tyrosine (Y) residues at the C-terminus. As the Cf-9 motif is KKRY the mutated peptide KKAA was tested, again there was no significant difference between the ability of the wild type and mutant peptide to recognise Avr9. Thus it appears that this motif (KKRY) is not essential for Cf-9 function. These Cf-9 constructs used the relatively strong CaMV 35S promoter, with a high inoculum of Avr9. It is possible that with native Cf-9 expression levels and elicitor levels equivalent to that upon infection, differences may have been observed with function of the mutated proteins. Insertion of epitope-tags into different positions of the Cf-9 cytoplasmic tail also does not abolish Cf-9 mediated resistance (Piedras *et al.*, 2000. Jones, unpublished data). To establish the importance of the membrane anchor, a truncated Cf-9 protein was produced with the transmembrane domain and the G domain deleted. This peptide was non-functional in Avr9 recognition, indicating that the presence of the cytoplasmic tail is essential for function (Van der Hoorn *et al.* 2001a). Seear and Dixon (2003) identified a Cf-5 mutant, where a truncated Cf-5 protein lacking the membrane anchor resulted. The protein was non-functional, further demonstrating the importance of the membrane anchor, probably as it is responsible for sub-cellular localisation and orientation in the membrane. The data available for Cf-9 would suggest that although the membrane anchor is essential for function, the sequence of the cytoplasmic tail of the anchor is not so important.

The 3x c-Myc epitope-tag was inserted between the ultimate and penultimate amino acids of Cf-2 in both constructs. To test if the epitope-tag affects function, tomato transformants (with a Cf0 background) were tested for resistance to *C. fulvum* challenge. Initial qualitative analysis, staining for GUS activity in T002 challenged with *C. fulvum* race 4*GUS*, suggested that the plant was partially resistant. These inoculation experiments were conducted on the first generation transformants, plants that were heterozygous for the transgene and hence carrying just one copy of *Cf-2*. Hammond-Kosack and Jones (1993) showed that the *Cf* genes are incomplete in their dominance. Tomato plants homozygous for a *Cf* gene were more effective in containing infections and responded to a two-fold lower concentration of race-specific elicitor than heterozygotes (Hammond-

Kosack and Jones, 1993). *Cf-2* in the heterozygous state is weaker in function than when in the homozygous state. Fungal hyphae were observed to progress up to twice the distance from their point of entry in the *Cf-2* heterozygote plants (up to two mesophyll cell lengths from the substomatal cavity) compared to the homozygote plants (less than one mesophyll cell lengths from the substomatal cavity). This was due to the delay in the onset of the responses, the ultimate resistant phenotypes were however similar for both homo- and heterozygotes (Hammond-Kosack and Jones, 1993). However, no significant difference in MUG activity was observed between *Cf-2* heterozygotes and homozygotes. It is important to remember that *Cf-2* homozygotes carry four functional copies of *Cf-2* and heterozygotes two functional copies, whilst first generation transformants carry only one. This may go some way to explain why the first generation T002 plants appear to be only slightly susceptible, although this seems unlikely as susceptibility was analysed by MUG activity. Although the quantitative analysis indicated that T002 was not as completely susceptible as a Cf0 tomato, it was generally no more or less susceptible than the other putative transformants (T001,3,4and5) despite the latter being shown to lack the transgene. This was likely to be due to poor inoculations of the plant cuttings. Cuttings were rooted for two weeks in a mister, during this time the leaves became very hairy. The hairs caused air bubbles to be trapped around the leaves when they were immersed in a spore suspension. This appears to reduce the infectivity of *C. fulvum* (Seear, unpublished data).

It was quite late in this research before the lack of significant resistance was fully apparent by which time much of the other data in this thesis had been obtained. The significance of these data will be discussed more fully in the general discussion.

The plants identified as expressing the transgenes will be used for subcellular localisation and post-translational modification studies (Chapter 5).

Chapter five

5 Biochemical analysis of c-Myc:CF-2

5.1 Introduction

The *Cf* genes are predicted to encode plasma membrane (PM) bound, predominantly extra-cytoplasmic glycoproteins. Piedras *et al.* (2000), using a triple (3x) c-Myc epitope-tagged version of Cf-9, demonstrated it has a plasma membrane (PM) localisation, despite the presence of the endoplasmic reticulum (ER) retrieval-retention signal. Van der Hoorn *et al.* (2001a) mutated the ER retrieval, di-lysine, motif to reveal that it does not affect Cf-9 function nor localisation. Cf-2 does not carry the di-lysine repeat and like Cf-9 is predicted to be plasma membrane bound. In order to investigate this and its glycosylation status, an epitope-tagged version of Cf-2 has been engineered and stably transformed into both tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*). The epitope-tag chosen, due to availability, is the 3x c-Myc epitope.

The c-Myc epitope has been successfully employed in both animals and plants; Pang *et al.* (1999) demonstrated a plasma membrane localization of the human secretin receptor (SR). Secretin is a 27 amino acid peptide hormone that regulates pancreatic water, bicarbonate, enzymes and potassium ion secretion. This was revealed using a c-Myc epitope tagged version of the protein and confocal microscopy.

De Witt and Sussman (1995) engineered a c-Myc epitope-tagged version of the *Arabidopsis* proton pump *AHA3* and expressed it *in planta*. AHA3 was shown to be membrane bound by immunoblotting of microsomal membranes. Using both immunofluorescence and immunogold labeling with electron microscopy, the proton pump was shown to localize specifically to the PM of phloem companion cells. This demonstrated that it is possible to specifically label proteins encoded by individual genes of a gene family in differentiated cell types of higher plants (De Witt and Sussman, 1995).

The majority of extracellular and endomembrane bound plant proteins are glycosylated by N-linked oligosaccharides (Rayon *et al.*, 1998). Cf-2 carries 31 potential N-linked glycosylation sites and the second aim of this project was to determine the actual

level of glycosylation. The addition of carbohydrates to proteins serves many purposes, including; protecting the protein from degradation, targeting of the protein to the correct membrane and to aid correct folding of the protein (Helenius and Aebi, 2001). Pang *et al.* (1999) showed that mutations in the human secretin receptor at either amino acid 72 or 74 that resulted in the loss of a glycosylation site and hence the addition of a carbohydrate group, resulted in a reduction in secretin binding activity. This uggested that the addition of carbohydrate groups to a protein, in particular receptors, may affect their binding activity and perhaps specificity of that protein. It may be possible that the carbohydrate groups found on the Cf proteins play a role in determining their recognition specificity.

Both Pang *et al.* (1999) and De Witt and Sussman (1995) used microscopy to study the localization of their target proteins, an alternative would be to employ a fractionation method, i.e to separate the cell into its components. Pang *et al.* (1999) isolated total membranes (microsomal membranes) and used immunoblotting to confirm that the protein was membrane bound. This method can be extended and the membranes further separated. Due to the available equipment and expertise, it was decided to employ a cell fractionation technique to study the subcellular localization of Cf-2. The method used was two-phase aqueous partitioning and was adapted from Yoshida *et al.* (1983). Unlike density gradients, which separate membranes according to their size and density, two-phase aqueous partitioning separates membranes, using a two-polymer system, according to their surface properties. This is an extremely efficient method with a single partition yielding plasma membranes with greater than 85% purity, usually three partitions are formed resulting in over 90% purity (Yoshida *et al.*, 1983).

To ascertain the glycosylation status of Cf-2, the protein was treated with the endoglycosidase (PNGaseF). PNGaseF cleaves the bond between the asparagine residue of the protein and the *N*-acetlyglucosamine residue that links the carbohydrate to the protein (Figure 5.1). This liberates almost all oligosaccharides from glycoproteins. A shift in the mobility of the protein would be observed with PNGaseF treatment if the protein carries N-linked carbohydrate groups.

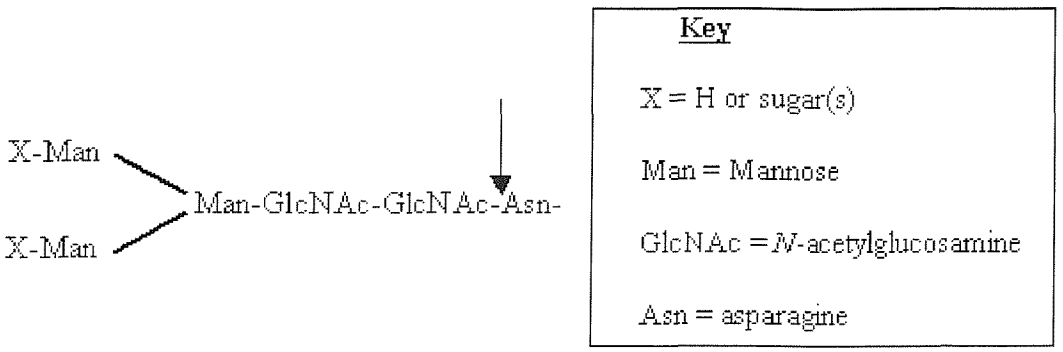


Figure 5.1: PNGaseF cleaves the bond between the asparagines of the protein and the *N*-acetylglucosamine of the carbohydrate.

The enzyme PNGaseF liberates most oligosaccharides from glycoproteins. The arrow indicates the bond broken by the glycoamidase, PNGase F. The bond broken is that between asparagines of the protein and the *N*-acetylglucosamine responsible for linking the carbohydrate to the protein.

5.2 Results

The first step towards determining the sub-cellular localisation of Cf-2 was to determine if it is membrane bound or cytosolic, by the separation of microsomal membranes from the cytosol. Two-phase aqueous partitioning was employed to produce a fraction enriched with plasma membrane vesicles to determine whether or not Cf-2 is plasma membrane bound. To investigate the level of glycosylation, microsomal membranes were incubated with glycoamidases.

5.2.1 Localisation of c-Myc:Cf-2

A homozygous line from the primary transformant designated T042 (Petit Gerard tobacco expressing the transgene *RP006,* Chapter 4) was used to establish the sub-cellular localisation of Cf-2. The first stage of this was to confirm that c-Myc:Cf-2 is indeed membrane bound. Crude membrane preparations (microsomal membranes) were isolated from an untransformed Petit Gerard plant and the Petit Gerard line expressing *RP006*

(described above). In addition to this the cytosolic fraction from the pRP006 line was kept. Several problems were encountered when preparing microsomal fractions with protease activity observed. This was overcome by the use of a cocktail of protease inhibitors and by performing the homogenisation and filtering stages in a cold room at 1^0C. Protein concentrations of each preparation were determined by a Bradford assay and an equal amount of each sample separated by SDS PAGE. This was subjected to western blot analysis, probed with a 1 in 750 dilution of anti-Myc polyclonal antibodies and detected by chemiluminescence. The analysis revealed that c-Myc:Cf-2 is found in the membrane and not soluble fraction of tobacco and that there are no cross-reactive bands in the untransformed negative control (Figure 5.2). Microsomal membrane and cytosolic preparations were also made from a cutting taken from the primary tomato transformant T010. Protein concentrations were determined by Bradford assays and the proteins subsequently separated by SDS-PAGE. Presence of c-Myc:Cf-2 was determined by western blot analysis (as above) and revealed that c-Myc:Cf-2 is membrane bound in tomato as in tobacco (Figure 5.3). Five times more total protein was loaded for the tomato preparations compared to the tobacco c-Myc:Cf-9 control. Despite this, a stronger signal was obtained from the tobacco sample. Figure 5.2 shows that c-Myc:Cf-2 and c-Myc:Cf-9 are over expressed in tobacco to a similar degree. Suggesting that expression levels of *RP006* in Cf0 tomatoes appears to be much lower in comparison to tobacco, however this is only one sample and may be due to positional effects.

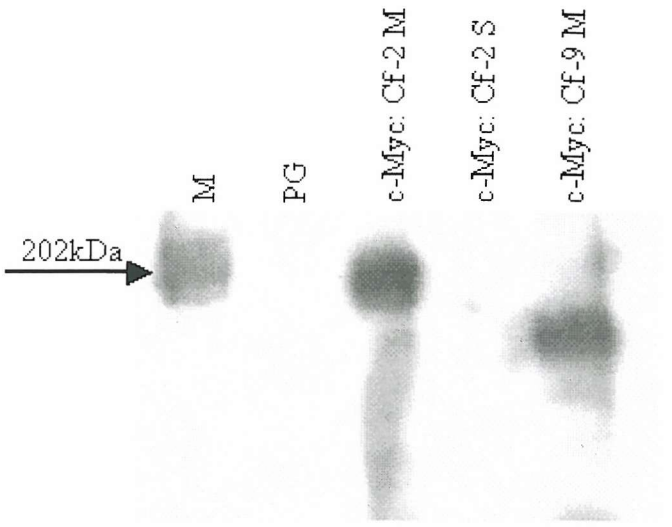


Figure 5.2: c-Myc: Cf-2 is found in the tobacco microsomal fraction.

40µg total protein has been loaded in each lane, separated on a 7.5% (w/v) polyacrylamide gel and subjected to western blot analysis. The membrane was incubated with a 1 in 750 dilution of polyclonal anti-c-Myc antibodies. PG indicates microsomes obtained from an untransformed Petit Gerard tobacco. c-Myc: Cf-2 M indicates the microsomal membranes purified from a pRP006 transformed Petit Gerard tobacco, c-Myc: Cf-2 S indicates the soluble (cytosolic) fraction obtained from the same plant. c-Myc:Cf-9 M indicates microsomal membranes obtained from a c-Myc Cf-9 tobacco (Piedras *et al.*, 2000) as a positive control. M indicates a molecular weight size marker (Kaleidoscope markers, BioRad, Hemel Hempstead, UK).

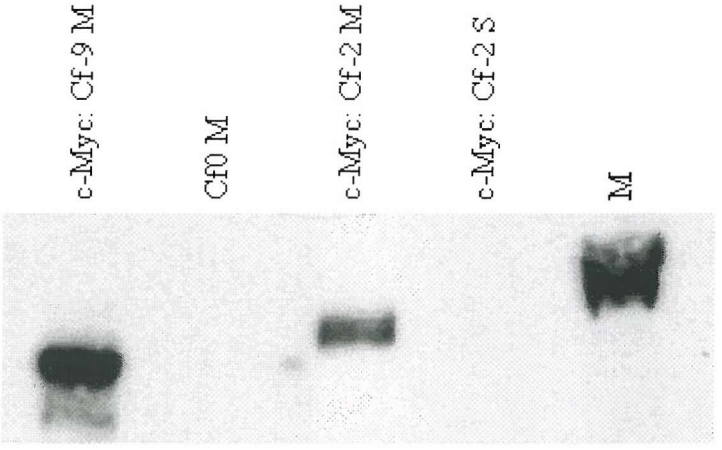


Figure 5. 3: c-Myc: Cf-2 is found in the tomato microsomal fraction.

100µg total c-Myc Cf-2 protein and 20µg total c-Myc: Cf-9 protein was loaded. C-Myc: Cf-9M indicates microsomal membranes purified from from a c-Myc Cf-9 tobacco (Piedras *et al.*, 2000). Cf0 M indicates microsomal membranes obtained from an untransformed tomato. C-myc: Cf-2 M and S indicate microsomal and soluble fractions respectively, isolated from a tomato (T010) expressing the gene *RP006* in a Cf0 background. Membranes were incubated with a 1 in 400 dilution of anti-c-Myc antibodies. M indicates a molecular weight size marker (kaleidoscope markers, BioRad, Hemel Hempstead, UK).

Two-phase aqueous partitioning was used to isolate an enriched fraction of plasma membranes. Two fractions were obtained upper 3 (U_3, plasma membrane enriched) and lower 3 (L_3). Equal amounts of U_3, L_3 and microsomal membranes were separated by SDS PAGE and subjected to western blot analysis. As a trial U_3, L_3 and microsomes were obtained from a 35S c-Myc: Cf-9 line (Piedras *et al.*, 2000). 20µg of total protein was used for each of the three preparations, which revealed that c-Myc: Cf-9 enriches with the plasma membrane phase (U_3) (Figure 5.4). These data were identical to that obtained by Piedras *et al.* (2000) and indicated the success of the procedure.

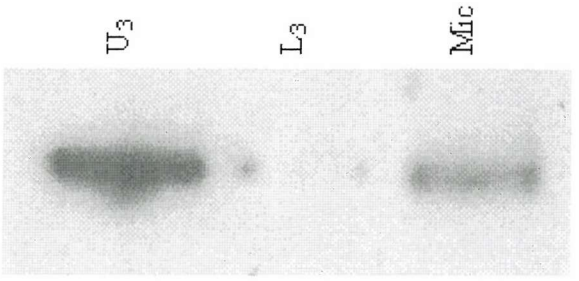


Figure 5.4: c-Myc: Cf-9 enriches with the tobacco plasma membrane fraction.

20µg total protein was loaded for plasma membrane fraction (U_3), lower fraction (L_3) and microsomal fraction (Mic) obtained from Petit Gerard expressing *RP006*. Samples were separated on a 7.5% (w/v) polyacrylmide gel. Western blots were performed and membranes incubated with a 1 in 750 dilution of polyclonal anti-c-Myc antibodies.

Plasma membrane enriched preparations were isolated from the homozygous T042 tobacco line (Chapter 4) and subjected to western blot analysis. This revealed that c-Myc:Cf-2 enriches with the U_3 phase (Figure 5.5 A). To determine the purity of the fractions the western blot was set up in triplicate and probed with antibodies raised against a plasma membrane aquaporin (PAQ) (Oshima *et al.*, 2001), and an ER marker, Binding protein (BiP) (donated by Prof. M. Chrispeels) (Figure 5.5 B & C respectively). In addition a cytochrome C oxidase assay was performed, as this enzyme is a marker for mitochondrial membranes. The western blots revealed that the U_3 phase was highly enriched for the plasma membrane while the L_3 phase contained very little plasma membrane (Table 5.1). However the enrichment of plasma membrane in the U_3 was greater than the enrichment of c-Myc:Cf-2 in the same phase. Both U_3 and L_3 phases contained an equivalent amount of ER membranes and virtually no mitochondrial membranes (although relative amounts of mitochondria is much higher in the upper and lower phases relative to the microsome, absolute enzyme activities were extremely low

(Table 5.1). This suggests that the majority of the c-Myc:Cf-2 enriches with the plasma membrane and is therefore PM bound.

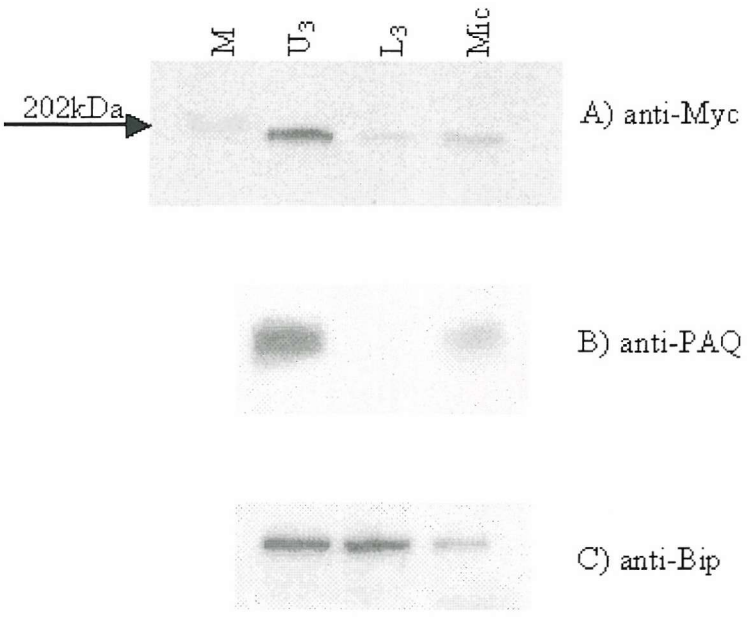


Figure 5.5: c-Myc:Cf-2 enriches with the tobacco plasma membrane.

10μg total protein was loaded for plasma membrane fraction (U_3), lower fraction (L_3) and microsomal fraction (Mic). Samples were separated on 7.5% (w/v) polyacrylamide gels and set up in triplicate. Western blots were performed and membranes probed with, A) 1 in 750 dilution of polyclonal anti-Myc antibodies, B) 1 in 4000 dilution of anti-PAQ antibodies a plasma membrane marker and C) 1 in 1000 dilution of anti-Bip antibodies, an ER marker. M indicates a molecular weight size marker (Kaleidoscope markers, Roche, Lewes, UK).

Table 5.1: c-Myc: Cf-2 enriches with the plasma membrane.

All values were normalised to the microsomal fraction. ER, PM and c-Myc:Cf-2 values were calculated as density readings from western blots using AlphaEase™ version 3.3b (Alpha Innotech, supplied by GRI, Braintree, UK). Anti-Bip, anti-PAQ and anti-Myc antibodies were used as markers of ER, PM and c-Myc:Cf-2 respectively. Cytochrome C oxidase assay was usedas a marker for mitochondria.

Phase	c-Myc: Cf-2	PM	ER	Mitochondria
U_3	1.76	1.51	1.67	2.35
L_3	0.57	0.27	1.57	7.41

This experiment was repeated using a homozygous line established from the primary transformant T065 (Petit Gerard tobacco expressing the transgene *RP006,* Chapter 4). Northern analysis indicates that expression levels of c-Myc:Cf-2 in this line is higher than that of the T042 homozygous line. As before c-Myc:Cf-2 was shown to enrich with the plasma membrane (Figure 5.6). In addition to the c-Myc:Cf-2 band, many more lower molecular weight bands were observed. These are likely to be breakdown products and so were included in calculations of relative protein concentrations. Separation of the plasma membrane from the lower fraction was shown to be even more successful, despite this c-Myc:Cf-2 was still found in the lower fraction and to a greater extent than observed for the T042 line (Figure 5.6 and Table 5.2). This indicates that as expression levels increase, so does the proportion of protein found in the ER, suggesting, a saturation of the secretory mechanisms in the cells.

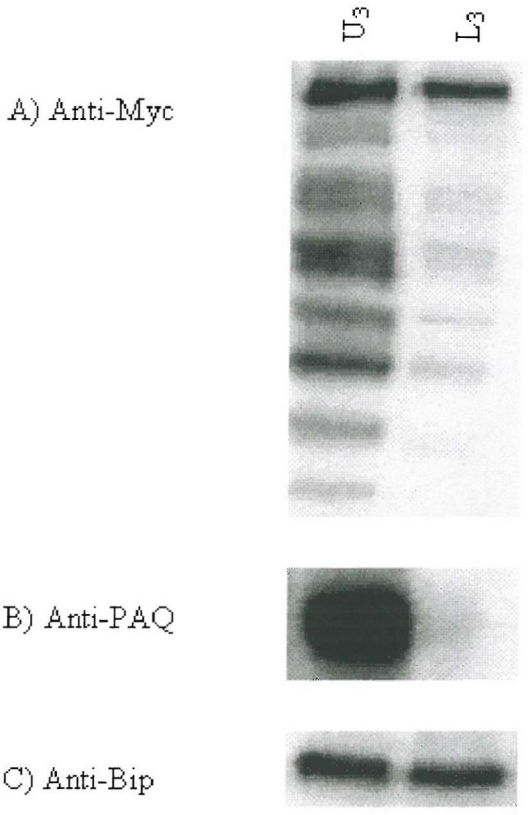


Figure 5.6: c-Myc: Cf-2 enriches with the tobacco plasma membrane.

10μg total protein was loaded for plasma membrane fraction (U_3) and lower fraction (L_3). Samples were separated on 7.5% (w/v) polyacrylamide gels and set up in triplicate. Western blots were performed and membranes probed with, A) 1 in 750 dilution of polyclonal anti-Myc antibodies, B) 1 in 4000 dilution of anti-PAQ antibodies a plasma membrane marker and C) 1 in 1000 dilution of anti-Bip antibodies, an ER marker.

Table 5.2: c-Myc: Cf-2 enriches with the plasma membrane.

The values for the lower phase (L_3) were set to 1. Values for the upper phase (U_3) are expressed relative to the lower phase. ER, PM and c-Myc: Cf-2 values were calculated as density readings from western blots using AlphaEase™ version 3.3b (Alpha Innotech corperation). Anti-Bip, anti-PAQ and anti-Myc antibodies were used as markers of ER, PM and c-Myc: Cf-2 respectively.

Phase	c-Myc: Cf-2	PM	ER
U_3	3.38	15.17	1.03
L_3	1.0	1.0	1.0

5.2.2 Post-translational modification of Cf-2

The sequence of Cf-2 predicts 31 N-linked glycosylation sites. To test the glycosylation state of Cf-2, microsomes obtained from the T042 homozygous line were treated with an endoglycosidase (PNGaseF).

Microsomes were first solubilised in a non-denaturing detergent and incubated with the enzyme for up to 1 hour. A pilot run indicated that the reaction had run to completion by this time. To gain adequate separation to detect any change in mobility due to the removal of carbohydrate groups, the SDS polyacrylamide gel was run so that all proteins smaller than 70kDa had been run off the bottom of the gel. Western blot analysis of the PNGaseF treated microsomes showed a considerable change in protein mass (Figure 5.7) confirming the presence of many N-linked carbohydrate groups. This too was confirmed in tomato with microsomes from T010 being treated in the same manner (Figure 5.8). Due to lack of suitable size markers, only rough estimations of the magnitude of the change in mass can be made. The change is approximately 20-30kDa, which is approximately equivalent to 10 carbohydrate groups, suggesting one third of the potential sites are glycosylated.

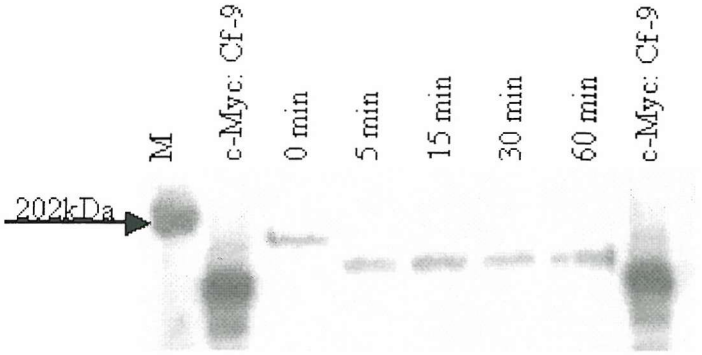


Figure 5.7: c-Myc: Cf-2 expressed in tobacco is glycosylated.

240µl (approximately 1.3mg total protein) of c-Myc: Cf-2 microsomes were solubilised with IGEPAL CA-630. The solubilised proteins were incubated with PNGase F and aliquots removed at the times indicated and separated on a denaturing 7.5% (w/v) polyacrylamide gel. Western blots were performed and membranes incubated with a 1 in 750 dilution of polyclonal anti-c-Myc primary antibodies. M indicates a molecular weight size marker (Kaleidoscope markers, BioRad, Hemel Hempstead, UK), c-Myc:Cf-9 microsomes were not treated with PNGase F and were included as a molecular weight reference.

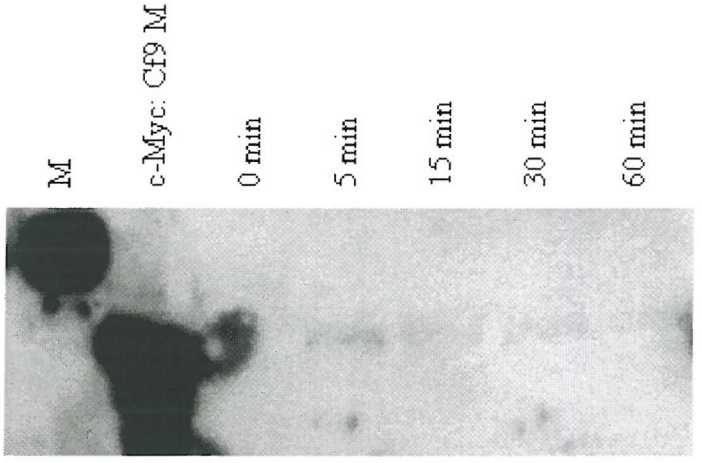


Figure 5.8: c-Myc: Cf-2 expressed in tomato is glycosylated.

240µl (approximately 2mg total protein) of tomato c-Myc: Cf-2 (T010) microsomes were solubilsed with IGEPAL CA-630. The solubilised proteins were incubated with PNGase F and aliquots removed at the times indicated and separated on a denaturing 7.5% (w/v) polyacrylamide gel. Western blots were performed and membranes incubated with a 1 in 400 dilution of polyclonal anti-c-Myc primary antibodies. M indicates a molecular weight size marker (Kaleidoscope markers, BioRad, Hemel Hempstead, UK), c-Myc: Cf-9 microsomes were not treated with PNGase F and included as a molecular weight reference.

In tobacco, c-Myc:Cf-2 enriches with the plasma membrane, it is predicted that the same is true for tomato. To test this a second endoglycosidase was employed. Endo H_f is an endoglycosidase that is unable to cleave N-linked carbohydrate groups from proteins that have passed through and left the ER. Therefore if the tomato c-Myc:Cf-2 is

insensitive to this enzyme it would indicate that it has passed through the ER to the plasma membrane. Microsomes obtained from the tomato T010 were solubilsed and denatured in 1x denaturation buffer (New England Biolabs, Hitchin, UK), omitting the initial solubilisation step employed with the PNGaseF assay. Microsomes were treated with Endo H_f for periods of up to 90 minutes, and resulting proteins separated by SDS-PAGE. Western blots revealed that the c-Myc:Cf-2 was sensitive to the endoglycosidase treatment (Figure 5.9). This was not expected if the protein is to be found in the plasma membrane. It was predicted that only a small portion of the protein should be sensitive to Endo H_f as the localisation study revealed that c-Myc:Cf-2 could be detected in the ER. The reduction in size by Endo H_f treatment was not as large as seen with PNGase F treatment. On closer inspection a second, less intense, band was detected of approximately 120kDa, the predicted size of Cf-2 lacking any post-translational modifications. It is possible that this was the portion of Cf-2, detected in the ER. However, it was possible that the 120kDa was a breakdown product rather than the completely de-glycosylated protein. To test this it was decided to translate *c-Myc:Cf-2 in vitro* using a system without glycosylation machinery. The system chosen was the rabbit reticulocyte lysate system.

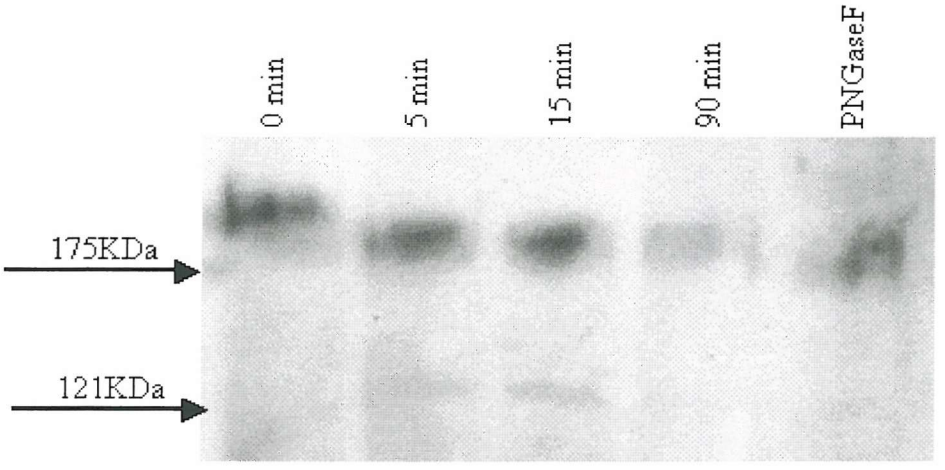


Figure 5.9: Cf-2::3x c-Myc is sensitive to the endoglycosidase EndoH$_f$.

Approximately 1mg total protein of microsomal membranes obtained from the tomato T010 were solubilised and denatured in 1x denaturing buffer (New England Biolabs, Hitchin, UK). Debris was pelleted at full speed in a microcentrifuge and the supernatant treated with 1000 U of EndoH$_f$. Length of treatment is aligned to relevant lanes. The proteins were separated on a denaturing 7.5% (w/v) polyacrylamide gel. Western blots were performed and membranes incubated with a 1 in 750 dilution of polyclonal anti-c-Myc primary antibodies. An aliquot (approximately 120µg) was treated with PNGase F as a positive control and one not treated at all as a negative control. Arrows indicate approxmate sizes as determined by alignment with size markers (Kaleidoscope markers, BioRad, Hemel Hempstead, UK) on the membrane.

The first step towards *in vitro* translation was to clone the open reading frame (ORF) of *c-Myc:Cf-2* into a suitable vector to enable transcription of the gene. The vector chosen was pGem3Zf+, as this allowed the use of the viral SP6 promoter. In the construct pRP006, a *Cla*I restriction site has been introduced at the very start of the ORF. This restriction site along with the *Bam*HI restriction site at the opposite end of the ORF were utilised to enable cloning of the coding region. A complete *Bam*HI restriction digest was performed, the DNA ethanol salt precipitated before the *Cla*I restriction digest was performed. A second *Cla*I site is present within the region that was to be cloned. To circumvent this problem a partial digest was performed. DNA fragments were separated by electrophoresis and the 4.3kb desired fragment was cut from the gel and purified using the Wizard kit (Promega, Sputhampton, UK). An additional *Cla*I site was present in the RP006 vector approximately 4.3kb upstream of the second *Cla*I site. Therefore this *Cla*I to *Cla*I vector fragment would co-migrate with the desired *Cla*I, *Bam*HI *Cf-2* fragment. This was not a problem as the vector, pGem3Zf+, was digested to completion with *Acc*I (which results in an overhang compatible with *Cla*I) and *Bam*HI and hence selecting for the desired fragment.

The ligation reaction products were used to transform *E.coli* DH5α using the calcium chloride method. Using blue/white selection approximately 30 putative positive colonies were identified. Plasmid DNA was prepared from 12 colonies and screened by restriction digest. Putative positives were further screened, by DNA sequencing of the insert ends. One clone was identified as being correct and designated pRP007. Due to the repetitive nature of the *Cf-2* insert it is possible deletions have occurred, to ensure the complete desired fragment is present test restriction digests were performed. The restriction digests revealed that pRP007 is indeed the desired clone and was used for *in vitro* transcription and translation.

The peptides produced by *in vitro* transcrition and subsequent translation were separated by SDS-PAGE and revealed the un-modified protein to be approximately 120KDa in size, as predicted by the DNA sequence (Figure 5.10). Many additional peptides of decreasing sizes were also produced; it was assumed these are either breakdown products produced by protease activity, or due to premature termination of transcription/ translation.

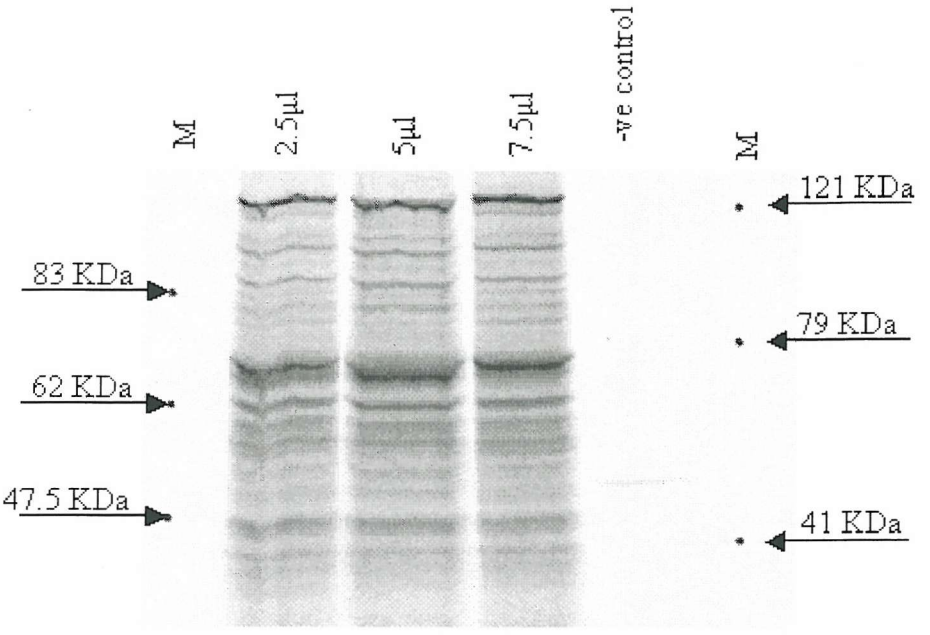


Figure 5.10: In vitro translation reveals the size of unglycosylated c-Myc Cf-2 to be approximately 120KDa.

Various amounts (as indicated by lane designations) of plasmid DNA (pRP007) was transcribed, purified and eluted in 50µl of RNase free water. 2µl of the transcription reaction was used in the translation reaction, the polypeptides produced were separated by SDS-PAGE using a 7.5% (w/v) polyacrylamide gel. After drying the gel was exposed to X-ray film, many products were produced that were specific to the construct pRP007. A no RNA control was also performed (designated, -ve control). M indicates where protein standards were loaded and used for alignment of the film and gel (Kaleidoscope markers, BioRad, Hemel Hempstead, UK and long range markers, Roche, Lewes, UK).

Further analysis of the glycosylation status of Cf-2 was performed by treating fractionated membranes, U_3 (plasma membranes) and L_3 from the tobacco T042 line, with PNGaseF and Endo H_f (Figure 5.11). Membranes were treated with the two enzymes for 10 minutes as previous experiments revealed the reaction to be complete by this time. Both PNGaseF and Endo H_f treatments gave very similar results, where two products were observed, one at approximately 175Kda (similar to Figures 5.7 and 5.8) and a smaller band at approximately 120KDa. Neither band was observable in the untreated samples.

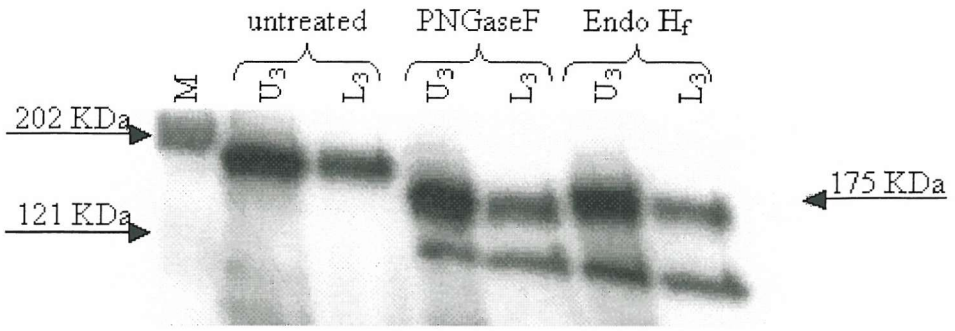


Figure 5.11: c-Myc:Cf-2 is highly glycosylated.

Upper (U3) and lower (L3) fractions, from the homozygous tobacco line T042, were treated with the endoglycosidases PNGaseF and Endo Hf for 10 minutes. Samples were loaded in 5µg (U3) and 10 µg (L3) alloquots. M indicates the protein size standards (Kaleidoscope markers, BioRad, Hemel Hempstead, UK).

5.3 Discussion

Initial analysis, the separation of membranes from the cytosol, confirmed that, consistent with the protein sequence of Cf-2, c-Myc:Cf-2 is membrane bound. Unlike Cf-9, Cf-2 does not carry the dilysine ER retrieval motif, suggesting a plasma membrane localisation. Further separation of the membranes, in particular the enrichment of the plasma membrane, revealed a likely plasma membrane localisation for Cf-2. This is consistent with the findings of Piedras *et al.* (2000) who demonstrated a plasma membrane localisation for Cf-9.

Some c-Myc:Cf-2 protein was detected in the lower fraction isolated from the T042 tobacco line during two-phase aqueous partitioning. Western blot analysis showed that little or no plasma membrane was in the lower fraction and certainly not enough to explain the observed levels of c-Myc:Cf-2. Therefore, it is proposed that a small proportion of c-Myc:Cf-2 detected is in other membranes e.g. the endoplasmic reticulum (ER). The plants analysed, express the c-Myc:Cf-2 protein at very high levels as the T-DNA uses the relatively strong CaMV 35S promoter. The high level of protein expression is likely to saturate the secretory machinery of the plant cell and result in a protein 'backlog' in the ER. If over-expression of a protein results in a backlog in the ER, it follows that the higher the level of expression, the greater the amount will be found in the ER. Indeed, in the tobacco line T065, which expresses the T-DNA to higher levels than the T042 line, as demonstrated by northern analysis, a greater proportion of the c-Myc:Cf-

2 protein was found in the lower fraction (ER). A PM localisation also seems likely in tomato, as tobacco is considered a suitable model for tomato. However, treatment with endoglycosidases could not confirm this. Microsomes prepared from the tomato line T010 (RP006/Cf0) were treated with two endoglycosidases (PNGaseF and Endo H_f). PNGaseF should cleave all N-linked carbohydrates from the protein. Endo H_f cannot cleave N-linked carbohydrates from proteins that have passed through the Golgi (and hence ER), for example proteins that are plasma membrane bound. Results of the treatment of the tomato microsomes with the two endoglycosidases were a little confusing with both enzymes cleaving carbohydrate groups from the transgenic protein. Proteins become insensitive to Endo H_f after the removal of mannose sugars in the Golgi apparatus. Plant oligosaccharide groups have a high mannose content relative to animals, which may go someway to explain the unexpected results (Rayon *et al.*, 1998). In mammals, the ER-mannosidase I is responsible for specifically removing mannose residues in the Golgi, no such enzyme has been detected in plants, although processed oligosaccharides are found (Rayon *et al.*, 1998). This additional potential difference between mammal and plant oligosaccharides may explain why enzymes, designed to work primarily in animal systems, do not behave in the predicted manner in plants.

Treatment of Cf-9 with PNGaseF revealed that, as predicted, it is highly glycosylated with carbohydrate groups totalling 55KDa (Piedras *et al.*, 2000). Without knowing the size of each carbohydrate group it is difficult to be certain of how many of the 22 potential sites are glycosylated. The c-Myc:Cf-2 protein when analysed by SDS-PAGE was shown to be approximately 200KDa, about 80KDa larger than the predicted size for the unglycosylated protein. Treatment of tobacco and tomato microsomes with the endoglycosidase PNGaseF resulted in a protein approximately 20-30KDa smaller than the glycosylated protein. This protein is substantially larger than the sequence predicts. This could be due to the enzyme not working to completion, the presence of additional carbohydrates groups that could not be cleaved by the enzyme, or that the Cf-2 protein does not co-migrate through the polyacrylamide gel with other proteins of a similar molecular weight. The Cf proteins are predicted to be extended coil-like structures rather than the typical globular type proteins, therefore it is feasible that they migrate at a different rate to the globular weight standards, even in SDS denaturing conditions. Treatment of tomato microsomes with Endo H_f, revealed a small portion of protein that migrated with a marker protein of 120KDa. It is possible that this was an artefact, or a

breakdown product of the tagged protein, such breakdown products were observed in some preparations. To test this *in vitro* transcription and translation of the ORF of *c-Myc:Cf-2* was performed using the rabbit reticulocyte lysate system. This system was chosen as it lacks glycosylation machinery and therefore would reveal the true size of c-Myc:Cf-2. A large number of peptides were produced; these were specific to the *RP007* gene and revealed that the likely size of the protein to be approximately 120KDa. The smaller peptides most likely arose due to protease activity and premature termination of transcription and/or translation. It is possible that correct folding of the protein did not occur and due to this, although being the same protein as seen after PNGaseF treatment of microsomes, migrates faster through the polyacrylamide gel.

To test this further the lower and upper phases from the T042 homozygous line were tested with PNGaseF and Endo H_f. Treatment of each fraction with each of the endoglycosidases resulted in the same pattern of de-glycosylated proteins. PNGaseF should be able to cleave all carbohydrate groups from all of the factions, whilst Endo H_f should cleave only the carbohydrates from a small portion of c-Myc:Cf-2 in the upper phase, due to ER contamination. Endo H_f should also cleave all of the carbohydrates from c-Myc:Cf-2 in the lower phase. Both enzymes cleaved a portion of carbohydrates from all the c-Myc:Cf-2 in each fraction, resulting in a protein that migrates at the same rate of the c-Myc:Cf-2 from microsomes treated with PNGaseF. In addition a second smaller protein was observed, in each treatment, which migrated at 120KDa. This observation in addition to the *in vitro* transcription translation results demonstrates the highly-glycosylated nature of Cf-2 with carbohydrates increasing the protein mass by approximately 80KDa. Assuming that the carbohydrate groups found on Cf-2 and Cf-9 are, on average, the same size then the proportion of the potential glycoylation sites that carry a carbohydrate group is the same. Why PNGaseF was unable to cleave all of the carbohydrate groups from c-Myc:Cf-2 in the microsomal fractions and why the endoglycosidases did not behave in the predicted manner on the purified membranes remains unclear.

Chapter six

6 Expression profiling of *Cf-2* and *Cf-9*

6.1 Introduction

Many genes are developmentally regulated, however, it might be expected that genes involved in resistance against pathogens to be expressed at all times. In particular, it might be expected that these genes be expressed from the earliest stages when a seedling is most vulnerable to pathogen attack. Indeed, mechanisms such as systemic acquired resistance (SAR) do not operate in many flowering or fruiting plants. When considering survival of the species, it may be a better strategy to invest energy in producing the next generation rather than fighting disease.

Transposon tagging was used to clone *Cf-9*, which involved crossing Cf9 plants carrying the transposable element to plants expressing *Avr9*. This produced F_1 seed carrying both Avr9 and Cf-9. The vast majority of seedlings died as the first true leaves emerged, presumably due to a massive defence response (Jones *et al.*, 1994). A key observation was that these F_1 seedlings appeared perfectly healthy until the first true leaves emerged, suggesting that at least some component of the *Cf-9*-mediated response is developmentally regulated (Jones *et al.*, 1994). Honee *et al.* (1998) tried to produce plants expressing both *Cf-9* and *Avr9* using a transgenic approach, by transforming Cf9 plants with *Avr9*. Normal callus formation was observed, however, transgenic plants were never recovered, lending further support to the idea that *Cf-9*-dependent defence is developmentally regulated.

In contrast with the early onset of *Cf-9*-dependent defences, a second developmentally regulated *Cf* gene, *Cf-9-B*, mediated defence is functional only in mature plants (Panter *et al.*, 2002). By performing Cf-9, Cf-9-B promoter/ coding region reciprocal swaps and by producing a Cf-9-B promoter GUS fusion, Panter et al. (2002) were able to demonstrate that the developmental regulation observed is probably not due to transcriptional control. These findings suggest the involvement of an additional gene or

genes in the regulation of *Cf-9* and *Cf-9-B* dependent resistance. As Avr2 had not been isolated until recently similar experiments have not been possible with *Cf-2*.

The aim of this study was to examine the expression profiles of *Cf-2* and *Cf-9*. This was achieved by the use of promoter GUS fusions stably transformed into susceptible (Cf0) tomatoes donated by the Jones laboratory, John Innes Centre, Norwich.

The initial experiment was designed to study the onset of expression of the *Cf* genes. It is known that Cf-9 is not functionally active in very young seedlings, using the promoter: GUS plants it was possible to test if the level of transcript in seedlings is responsible for the observed developmental regulation. In addition it was possible to investigate whether Cf-2 shows a similar profile. This expression profile was performed not only on seedlings but also on leaves of different ages.

The second experiment was designed to assess the effect of IF on *Cf* gene expression. It would seem plausible that *Cf–2* gene expression increases upon infection as levels of *Cf-2* in uninfected plants is very low (undetectable by northern analysis).

6.2 Results

It has been shown that *Cf-9* is developmentally regulated, being turned on only after the emergence of the first true leaves. To extend the analysis to include Cf-2 and examine whether this is due to expression levels or some down stream processing Cf-2 promoter: and Cf-9 promoter: GUS fusion tomato lines were used (Torres, Smoker and Jones, unpublised).

The *Cf-2* promoter: GUS fusion lines (8611 lines) were engineered, firstly, by subcloning the initial portion of the *Cf-2* gene. A *Cla*I restriction site was introduced, by oligo mtatgenesis, at the ATG translation START codon. All of the sequence upstream of the open reading frame (ORF) (*Xho*I-*Cla*I fragment, excised from minimal Cf-2.1, bases 1- 1684 GenBank accession U42444, Dixon et al., 1996) was cloned into *Eco*RV-*Cla*I restricted pBluescript ks+. The *Xho*I restricted *Cf-2* fragement was filled in and blunt end ligated with the *Eco*RV site in the vector. The *Cf-2* fragment was excised with the restriction enzymes *Eco*RI and *Cla*I and ligated into the vector pSLJ4KI that carries the

GUS reporter gene (Jones *et al.*, 1992). The *Cf-2* promoter: GUS fusion was excised with *Eco*RI and *Bam*HI and cloned into the multiple cloning site of the binary vector SLJ 7292 (http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/plasmidlist/s3d_plas.htm) (Torres, Smoker and Jones, unpublished).

The *Cf-9* promoter: GUS fusion lines (8642 lines) were engineered in a similar way (Torres, Smoker and Jones, unpublished). A *Cla*I restriction site was again introduced at the ATG translation START codon, an *Eco*RI, *Cla*I fragment was cloned into the vector SLJ4K1 (Jones et al., 1992). This fragment contained all of the sequence upstream of the start codon (a 1.75kb fragment starting from base 1, GenBank accession U15936) and along with the reporter gene were excised and ligated into the multiple cloning site of the binary vector SLJ 7292 (http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/plasmidlist/s3d_plas.htm). The contstructs were subsequently transformed by *Agrobacterium* mediated uptake into *L. esculentum* cv Money Maker. Single insert, homozygous lines were established by selfing transformants and screening of the progeny (Torres and Smoker, unpublished). Homozygous lines were selected and used for subsequent experiments.

Thirty to forty seeds, from selfed-plants homozygous for the promoter:GUS fusions, were sterilised and placed on seed germination media and allowed to grow (12hr light: 12 hr dark). Three Cf2 (8611C plant 3, 8611D plant 3 and 8611F plant 8) and three Cf9 lines (8642D plant 7, 8642L plant 11 and 8642P plant 5) were analysed for expression of the *Cf* genes. The seeds germinated at different rates allowing the harvesting of cotyledons at different stages. Three stages were chosen (Figure 6.1a), S1 (stage one) was the youngest and were identified as having no true leaves and no branching of the main root and were typically smaller than 10mm in length. S2 cotyledons were identified as coming from seedlings lacking true leaves with some branching of the root system and between 10 and 15mm in length. Finally S3 cotyledons (>15mm) were picked for having emerging true leaves (TL) and substantial branching of the root system, the true leaves were cut from these samples. The true leaves were assayed separately to gain an indication as to whether any patterns observed are due to age of the leaf or age of the plant. These experiments show that the expression levels of both *Cf-2* and *Cf-9* increase with age of the cotyledon (Figures 6.1 and 6.2). There is a clear difference between the S1 and S3 cotyledons with the S2 showing intermediate levels of expression. The experiment

was repeated for the lines 8611D plant 3 and 8642P plant 5 due to the large variation in activity observed. More replicates were employed and only S1, S3 (cotyledons and true leaves) samples were analysed. Again the same pattern of increased expression levels with increasing age of the leaf was observed (Figures 6.1 and 6.2).

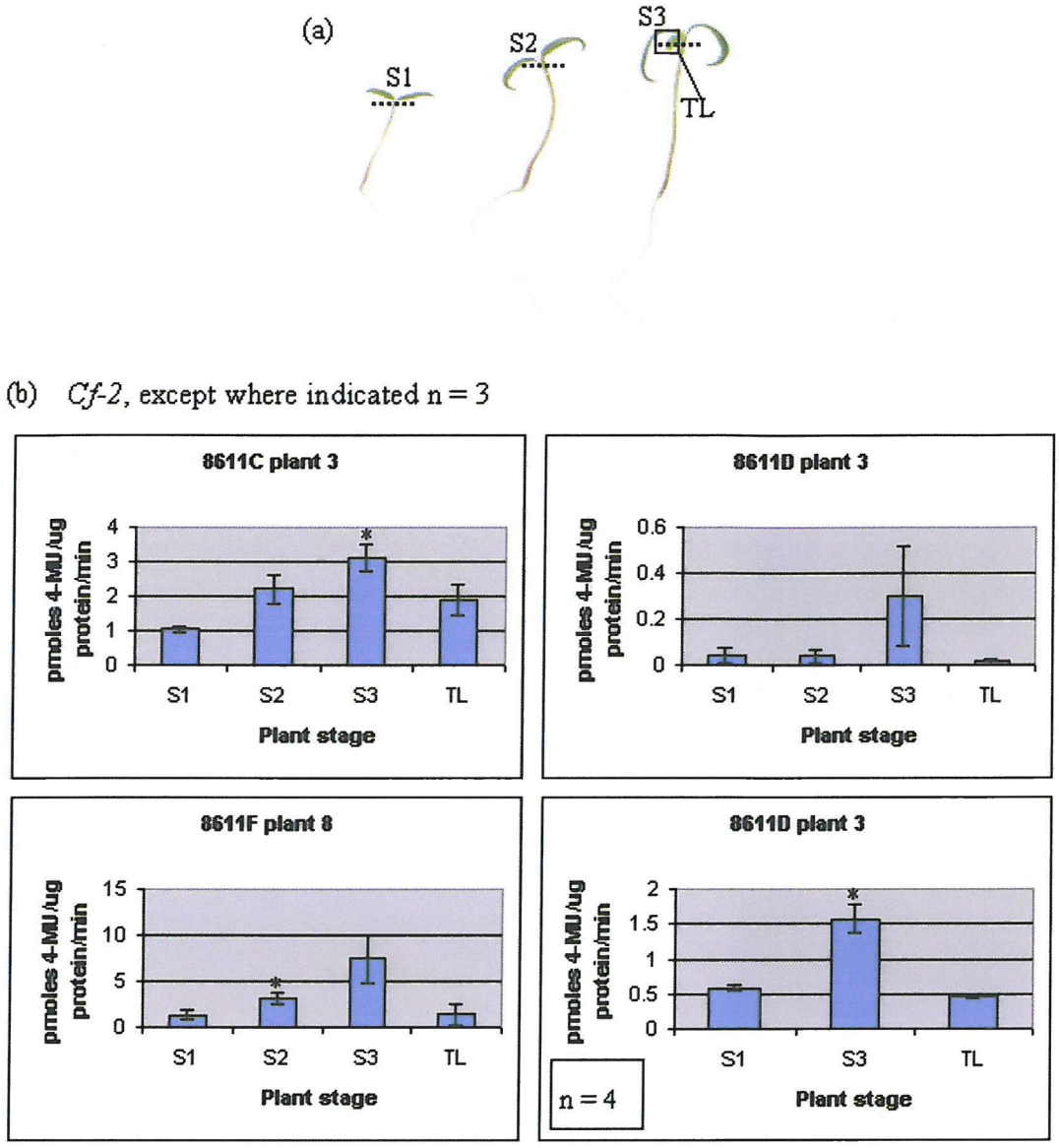
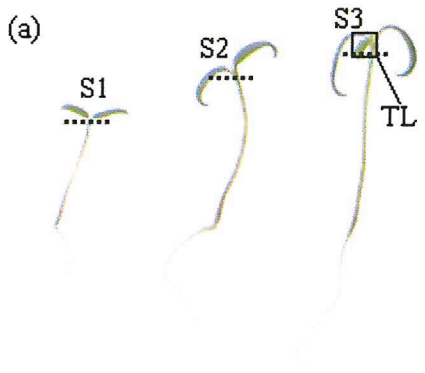


Figure 6.1: *Cf-2* expression increases with age of cotyledon and appears to follow the same pattern with true leaves.

Seeds were sterilised and spread over seed germination media. After 7-10 days, seedlings were harvested and analysed for GUS activity (b). The double cotyledons were cut from the stem as indicated by the doted line (a). Three double cotyledons were harvested from 3 individual seedlings at various stages (a) for each line. Stage 1 (S1) cotyledons were assayed just after they had opened and before the roots had begun to branch, typically <10mm. Stage 2 (S2) were harvested as the main root had begun to branch (10-15mm). Stage 3 (S3) cotyledons were harvested after the emergence of the first true leaves (typically >15mm), which were cut from the head of the seedling and assayed separately (TL). Error bars represent standard error of the mean, except where indicated n = 3. * Indicates statistically significant difference from S1 where P≤ 0.05 using a two-tailed t-test. A repeat of the line 8611D plant 3 was performed (b, bottom right) and did not include the S2 sample, 4 double cotyledons were harvested for each stage. Cf0 seedlings were also assayed for GUS activity, which did not exceed 0.06 pmoles 4-MU/µg protein/min, nor did it follow any pattern with age of the seedling.



(b) *Cf-9*, except where indicated n = 3

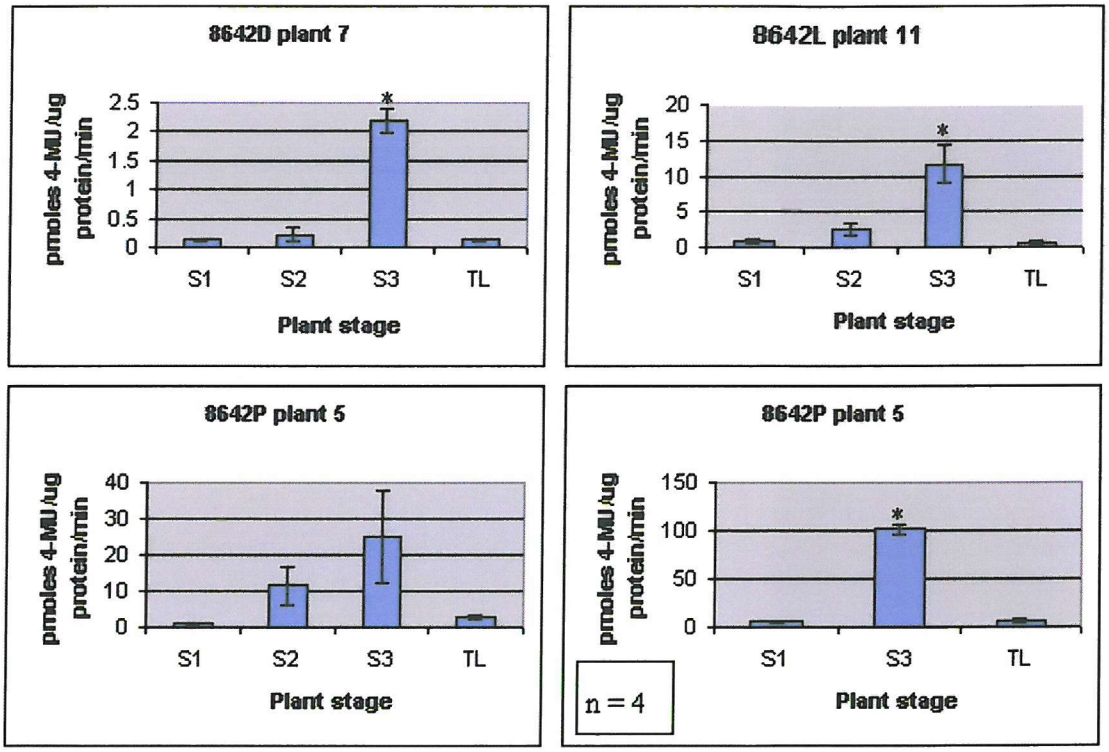


Figure 6.2: *Cf-9* expression increases with age of cotyledon and appears to follow the same pattern with true leaves.

Seeds were sterilised and spread over seed germination media. After 7-10 days, seedlings were harvested and analysed for GUS activity (b). The double cotyledons were cut from the stem as indicated by the doted line (a). Three double cotyledons were harvested from three individual seedlings at various stages (a) for each line. Stage 1 (S1) cotyledons were assayed just after they had opened and before the roots had begun to branch, typically <10mm. Stage 2 (S2) were harvested as the main root had begun to branch (10-15mm). Stage 3 (S3) cotyledons were harvested after the emergence of the first true leaves (typically >15mm), which were cut from the head of the seedling and assayed separately (TL). Error bars represent standard error of the mean, except where indicated n = 3. * Indicates statistically significant difference from S1 where P≤ 0.05 using a two-tailed t-test. A repeat of the line 8642P plant 5 was performed (b, bottom right) and did not include the S2 sample, 4 double cotyledons were harvested for each stage. Cf0 seedlings were also assayed for GUS activity, which never exceeded 0.06 pmoles 4-MU/µg protein/min, nor did it follow any pattern with age of the seedling.

To see if this pattern of increased expression with increasing age of the cotyledon holds for true leaves, three plants from each line were grown until the 13th node, counting from the bottom of the plant and not including cotyledons, was developing. Equivalent true leaves (second leaf in from the tip of the node) were harvested from each plant from the 13th (T1), 12th (T2), 6th (T3), and 2nd (T4) nodes. As with the cotyledons, the older leaves appear to have increased levels of expression of both *Cf-2* and *Cf-9* (Figures 6.3 and 6.4).

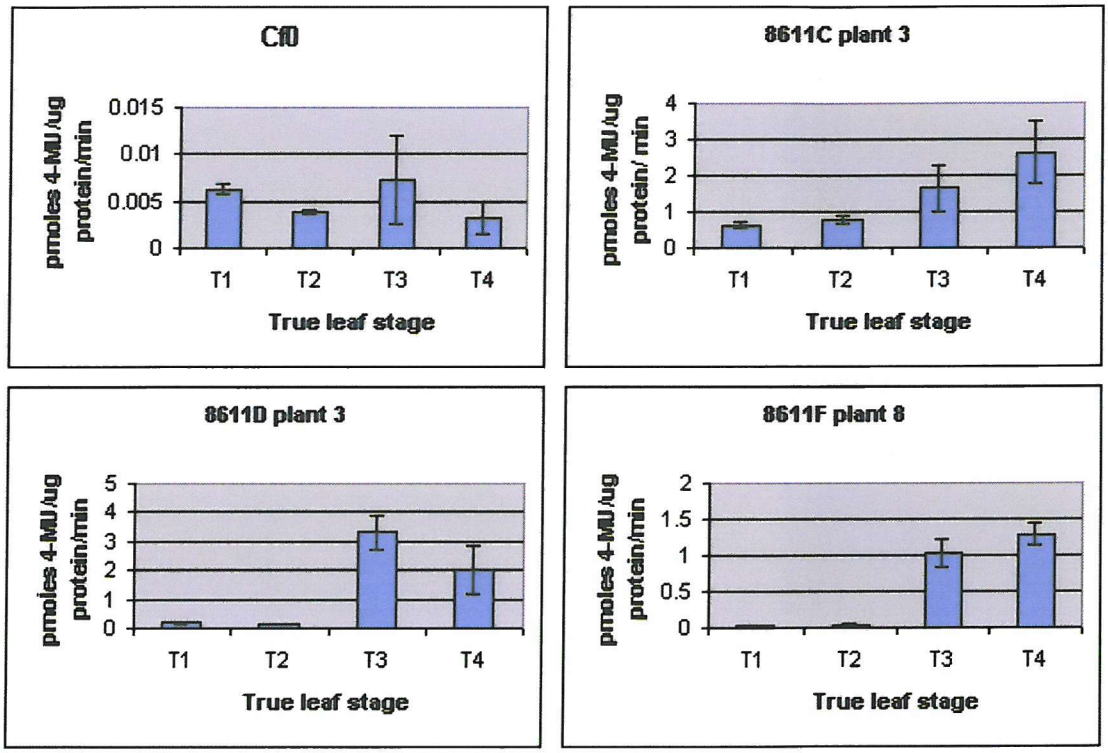


Figure 6.3: *Cf-2* expression levels increase with leaf age.

Plants were grown until the second flowers were just beginning to form (13th node). One true leaf was harvested from three individual plants of every line. Leaves were harvested from nodes 13 (T1 and youngest leaf on the plant), 12 (T2), 6 (T3), 2 (T4 and eldest leaves) and assayed for GUS activity. Error bars indicate the standard error of the mean, in each case n = 3.

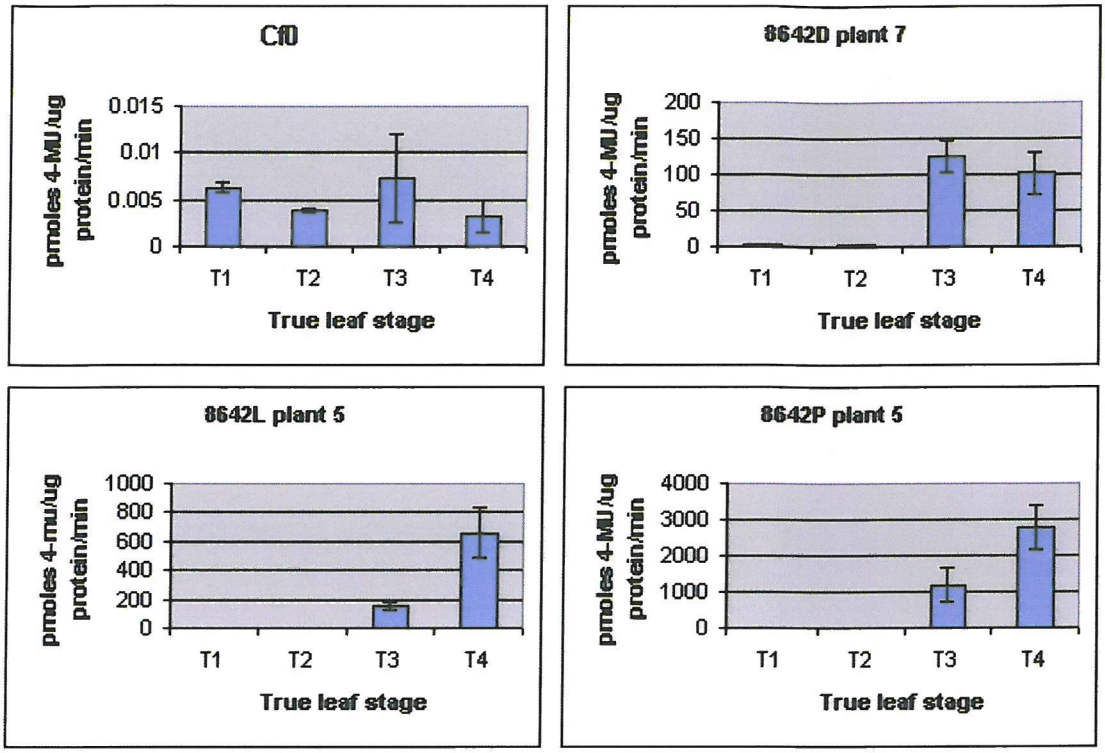


Figure 6.4: Cf-9 expression levels increase with leaf age.

Plants were grown until the second flowers were just beginning to form (13th node). One true leaf was harvested from each of the three plants of every line. Leaves were harvested from nodes 13 (T1 and youngest leaf on the plant), 12 (T2), 6 (T3), 2 (T4 and eldest leaves) and assayed for GUS activity. Error bars represent standard error of the mean, in each case n = 3.

To study the effect of fungal elicitors on *Cf* gene expression, all promoter: GUS lines were crossed with Cf0, Cf2 and Cf9 plants. As purified Avr2 was not available plants were infiltrated with IF (prepared from a *C. fulvum* race 5 infection on Cf0 plants). Cf0 crosses were performed as a negative control as these plants should not respond to the specific elicitors (Avr2 and Avr9). All lines were also crossed to both Cf2 and Cf9, to see if Cf-2/Avr2 responses affect *Cf-2* and *Cf-9* expression and similarly, if Cf-9/Avr9 responses affect *Cf-9* and *Cf-2* expression. Once crosses were obtained, plants were grown in a growth room in propagators. A pilot study was set up using one *Cf-2* promoter line (8611C plant 3) and one *Cf-9* promoter line (8642L plant 11). Equivalent leaves from three plants of each cross were infiltrated with water (as a negative control) and IF (diluted 1:1 v/v with water) using a syringe. At 48 hours a leaf disc was taken from the infiltrated leaves and assayed for GUS activity. Some tissue damage was observed and the leaves from Cf9 crosses that were infiltrated with IF were noticeably more flaccid in comparison to all other leaves. Infiltration with IF resulted in an increase in GUS activity, relative to

infiltration with water, in all plants crossed to Cf2 and Cf9. This increase was not observed with the Cf0 crosses (Figure 6.5). This experiment was extended to the remaining lines, with the exception of 8611D plant 3, as a complete set of crosses was not obtained. These data followed the same pattern as the preliminary study, with the plants crossed to Cf2 and Cf9 tomatoes experiencing an increase in GUS activity when infiltrated with IF relative to water (Figure 6.5). Comparisons of the IF treated leaves only, revealed a decrease in gene expression for the *Cf-2* promoter lines and an increase in the *Cf-9* promoter lines when comparing Cf2 and Cf9 crosses to the Cf0 cross (negative control).

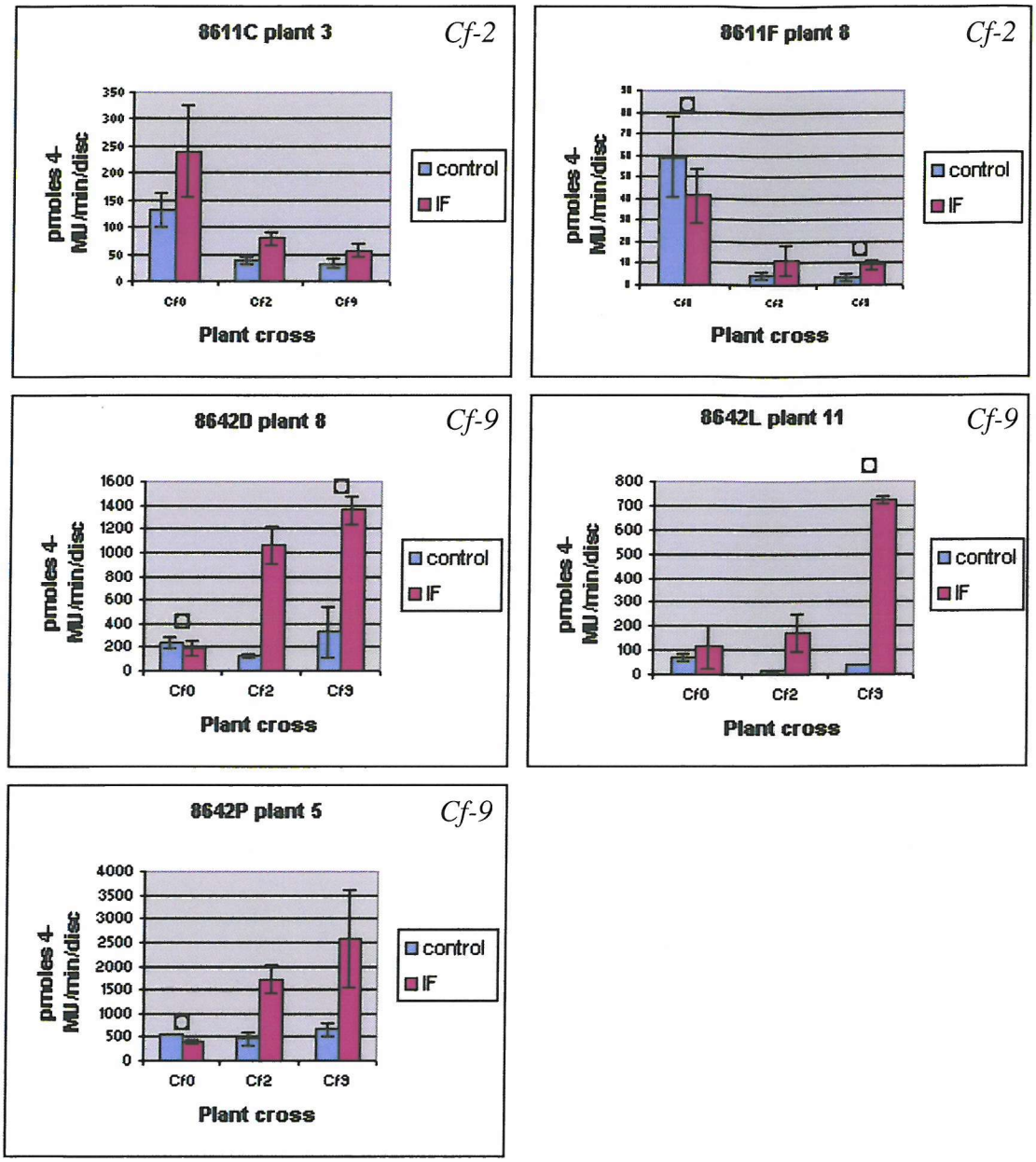


Figure 6.5: expression levels of Cf-2 and Cf-9 are increased after elicitation.

GUS lines (indicated by chart titles) were crossed to Cf0, Cf2 and Cf9 tomatoes. Equivalent leaves from three plants (▫ indicates where only 2 plants were used) of each cross were infiltrated with water (control) or IF (containing Avr2 and Avr9 and diluted 1:1 with water). After 48 hours a leaf disc was harvested using a number 2 cork borer, ground in GUS assay buffer and assayed for GUS activity per leaf disc. Error bars represent standard error of the mean, except where indicated n = 3 (▫ indicates where n = 2).

6.3 *Discussion*

An important consideration to be made when undertaking this study is that GUS is a very stable enzyme. Unlike most proteins it is not readily degraded (Jefferson *et al.*, 1986), therefore where an increase in expression is observed it is important to remember

this is an accumulation of what was already present plus any additional activities induced. In addition this makes it harder to notice any decrease in expression levels, as a significant level of GUS activity would remain from prior expression.

Cf-9-mediated defence is developmentally regulated, but this regulation was shown not to be due to transcriptional control (Honee *et al.*, 1998, Panter *et al.*, 2002). These data indicate that at the earliest stages, the developmental regulation of *Cf-9*-mediated defence may be under transcriptional control. In cotyledons, expression levels are extremely low until the emergence of the first true leaves, the point at which *Cf-9*-mediated defence becomes 'activated' (Honee *et al.*, 1998). It was shown that expression levels of *Cf-9* in the cotyledons prior to the emergence of the true leaves is higher than that observed in the emerging true leaves, indicating that expression levels are determined by the developmental stage of the leaf and not by the age of the plant. This was confirmed with analysis of mature plants, where young leaves exhibited low levels of expression with older plants exhibiting much higher expression levels. Despite exhibiting low expression levels, young leaves of mature plants carrying *Cf-9*, are resistant to pathogen challenge. This may suggest the involvement of at least a third interacting protein that is responsible for the regulation of the defence response.

Rcr3 is absolutely required for *Cf-2* mediated resistance and is developmentally regulated (Kruger *et al.*, 2002). Kruger *et al.* (2002) demonstrated that *Rcr3* expression increases with age of the plant irrespective of leaf age. A similar gene could be involved in the developmental regulation of *Cf-9* and/or *Cf-9-B*. Indeed, two *Rcr* genes (*Rcr1* and *Rcr2*) are required for full *Cf-9*-mediated resistance and were identified by mutagenesis (Hammond-Kosack *et al.*, 1994). Mutations in these genes resulted in only a weak phenotype, with a partial loss of function observed.

It is not known if *Cf-2* mediated defence can be activated in the youngest seedlings (pre-emergence of the first true leaves) however, it can certainly be activated in young and mature plants. The temporal expression pattern of *Cf-2* mirrors that of *Cf-9* and as with *Cf-9*, *Cf-2*-mediated defence may be regulated by an additional protein such as Rcr3.

The effects of IF infiltration on the expression of *Cf-2* and *Cf-9* was also studied. Consistently, IF treatment of the promoter: GUS fusion lines crossed with Cf2 and Cf9

resulted in an increase in GUS activity relative to the water control. This was not observed with the Cf0 crosses, where infiltration with IF and water made little difference to GUS activity. This suggests that changes in gene expression observed between water and IF infiltrated leaves is a likely result of Avr2 and Avr9 recognition. However, water is not the most suitable control and the IF contains many fungal proteins that may affect *Cf* gene expression. In addition, IF will contain 'particulates', not present in water, that could cause tissue damage which may affect gene expression. Therefore, only the IF treated samples should be compared to one another. Unfortunately, as in many cases only two samples were analysed, statistical analysis could not be performed. Studying the *Cf-2* promoter: GUS lines, it appears that elicitation reduces gene expression. Differences observed in GUS activity within these lines are relatively small (when compared with the Cf-9 promoter lines) and may be as a result of 'natural variation' and insufficient repetitions. On comparison of the IF treated *Cf-9* promoter: GUS lines, a large change in GUS activity is observed between the Cf0 crosses and the Cf2 or Cf9 crosses. This suggests that both Cf-2/Avr2 and Cf-9/Avr9 interactions have the ability to up-regulate Cf-9. There is little homology between the *Cf-2* and *Cf-9* promoters, so it might have been expected for any increase in promoter activity to be induced by only the corresponding Cf/Avr combination. The lack of homology may also suggest that different molecules affect the *Cf-2* and *Cf-9* promoters, although these data may indicate otherwise. One other explanation is that several molecules are up-regulated upon recognition of an Avr that in turn specifically up-regulates a *Cf* gene. If a tomato carries multiple *Cf* genes, up-regulation of them all may provide enhanced resistance if the race of fungus carries the corresponding Avr.

Some tissue damage was observed using the syringe infiltration method, which could affect the *Cf* expression levels. It was slightly easier to infiltrate the leaves with water relative to the IF, which increased the likelihood of tissue damage in the IF treated plants. Durrant *et al.* (2000) noted that the majority of genes up-regulated in Avr9 elicited *Cf-9* expressing tobacco cells were also up-regulated by wounding, although not to the same extent. Although wounding was observed in the infiltrated plants, after 48 hours plants infiltrated with IF did not appear any more damaged than those infiltrated with water. In addition, the plants crossed to Cf0 did not experience the changes in GUS activity as observed with the Cf2 and Cf9 crosses, suggesting that the changes seen were not entirely wound induced.

These data, in particular concerning the induction profiling IF, could be significantly improved. Due to the large variation that occurs, even within lines, a larger number of replicates should be used. In most cases only 3 biological replicates were used, in some cases this was reduced to two.

The induction experiments using intercellular fluid (IF), obtained from *C. fulvum* race 5 infection of Cf0 tomatoes, were crude and in need of modification. The first change should be the crosses. The pollen donors for the crosses should not come from Cf0, Cf2 and Cf9, as presented here. Instead the pollen should be from the GUS plants and the recipients Cf0, Cf2 and Cf9 plants, as confirmation of a successful cross can be obtained by staining for GUS activity, prior to treatment with IF. This would save time and IF.

The plants were infiltrated by forcing IF into the intercellularspaces using a syringe. The water control went in with relative ease, whilst slightly more resistance was experienced with the IF resulting in an increased likelihood of tissue damage. It is possible that wounding could affect gene expression, Durrant *et al* (2000) revealed that the majority of the cDNAs that increase in number after recognition by Avr9 are also up-regulated by wounding, although not to the same extent. In addition water is not the ideal negative control, as the IF would contain many proteins in addition to Avr2 and Avr9. Tobacco plants expressing Avr9 have been produced and IF can be produced from these plants which contains Avr9. Tobacco plants could be transformed to express Avr2, which could then be obtained by preparing IF from these plants. IF could then be obtained from non-transformed tobacco as a negative control. Using these IFs would provide better controls and there would be less variation between batches of IF. In addition, there would be more certainty that any observed changes in expression of *Cf-2* and *Cf-9* is due to the Avrs and not other non-specific elicitors that may be present in IF preparations made from infected tomatoes.

Due to the damage that occurs using this infiltration method, another less invasive method could be developed. For example, vacuum infiltration on whole plants may be used as a delivery method.

A more comprehensive study could be carried using these plant lines, for example, to study the effects of plant signalling molecules on Cf expression.

Signalling of the defence response involves many molecules and signalling pathways. There is to date no complete story of the events that occur between the perception of a pathogen and the onset of defence responses. As discussed in Chapter 1 many molecules have been implicated in the events that result in the defence response. One such molecule, clearly involved in local defence and activation of many *pathogenesis related* (*PR*) genes, is salicylic acid (SA). Following infiltration with intercellular fluid (IF) containing Avr2 and Avr9, Cf2 and Cf9 tomatoes respectively produce SA (Brading *et al.*, 2000). In addition to induction of its synthesis upon detection of the fungal Avr product, SA pre-treatment of Cf9 plants resulted in increased sensitivity to IF containing Avr9 (Brading *et al.*, 2000). Although SA is clearly involved in Cf mediated defence, neither Cf-2 nor Cf-9 require SA for resistance. This was demonstrated as Cf2 and Cf9 tomatoes expressing *NahG*, a gene that encodes salicylate hydroxylase and converts SA to the inactive catechol, were completely resistant to *C. fulvum* (Brading *et al.*, 2000). Although not required for resistance, SA appears to be important for necrosis, both Cf2 and Cf9 plants expressing *NahG* experience a delayed onset and completion of cell death. Indeed, the characteristic necrosis that results from Avr9 recognition by Cf-9 is completely prevented in the absence of SA and replaced by a Cf-2-like chlorosis (which is unaffected by the absence of SA) (Brading *et al.*, 2000). An additional observation of Brading *et al.* (2000), is that SA may even be an antagonist of *Cf-2*-mediated chlorosis, as the chlorotic regions observed in *Cf-2 NahG* tomatoes were greater than observed in control *Cf-2* plants. Jasmonic acid and ethylene are involved in SA-independent signalling pathways. Cross-talk between the SA-dependent and independent pathways is believed to have the ability to regulate the activation of defence mechanisms (Pieterse and van Loon, 1999). Durrant *et al.* (2000) identified proteins homologous to ethylene response element binding protein transcription factors and 1,3-lipoxygenase (an enzyme involved in the early stages of the pathway that results in Jasmonic acid production) that are rapidly and transiently induced in Cf9 expressing tobacco cells elicited with Avr9. This suggests a possible role for ethylene and Jasmonic acid in Cf-mediated defence signalling. In addition other proteins were observed to be up-regulated, including homolougues of the N resistance protein, a calcium binding protein and a RING-H2 zinc finger protein (Durrant *et al.*, 2000).

These promoter: GUS plants could be treated with salicylic acid, jasmonic acid or ethylene, for example. Plants could be treated with salicylic acid and jasmonic acid, by

immersion in a dilute solution or by spraying with a similar solution. Ethylene is a gas so plants could either be treated with ethylene in a contained environment or treated with the ethylene pre-cursor ACC (1-aminocyclopropane-1-carboxylic acid). Northern analysis could be employed as a control for the induction of a defence response by monitoring expression of *PR* genes.

Chapter seven

7 General Discussion

7.1 Introduction

The major aim of this project was to produce tobacco and tomato plants expressing a functionally active epitope-tagged version of Cf-2. These plants were then to be used to investigate the sub-cellular localisation and post-translational modification of Cf-2. The epitope-tag chosen was the triple myc (3x c-Myc) epitope used by Pierdras *et al.* (2000) to study the sub-cellular localisation of Cf-9. Two versions of the epitope-tagged construct were engineered and successfully expressed in tobacco and tomato. The first construct used the native Cf-2 promoter and the second the relatively strong CaMV 35S promoter.

7.2 Over-expression of epitope-tagged Cf-2.

No problems were predicted or encountered when over-expressing *Cf-2* (*RP006*) in tobacco, as *Cf-2*-dependent defence responses do not appear to function in *N. tabaccum.* However, over-expression of *Cf-2* in *L. esculentum* Cf0 (a completely susceptible tomato) was predicted to be difficult. Previous attempts to over-express *Cf-2* in tomato only produced only one transformant (Dixon, unpublished data). This was predicted to be due to auto-necrosis as a result of interaction with $Rcr3^{esc}$ (*L. esculentum* form of Rcr3) (Kruger *et al.*, 2002). Indeed, higher expression levels of the transgene were observed in the rcr3-3 line (an Rcr3 loss of function mutant) background relative to a Cf0 background. However, these data should be treated with caution as only one RP006/Cf0 plant was recovered. In addition it assumes that the epitope-tagged construct is functional. Intial analysis suggested that T010 (*RP006*/Cf0) was resistant to *C. fulvum* expressing *Avr*2. However, inoculation of second generation T010 plants revealed a loss of function.

Alternative epitope-tagged versions of Cf-2, with the epitope-tag in domain B, should be engineered. These constructs should be able to recognise Avr2 and elicit a defence response. These constructs could be over-expressed in both *L. esculentum* Cf0 and rcr3-3 backgrounds and the plants analysed phenotypically and by northern analysis.

Northern analysis would reveal if it were possible to over-express Cf-2 to a greater extent in the absence of a functional Rcr3. These plants should also be observed throughout their life cycle to investigate whether over-expression results in auto-necrosis at an earlier stage or to a greater extent than observed with plants carrying the same construct using the native promoter. These experiments could also be carried out using a non-epitope-tagged version of Cf-2 and would circumvent the problem of disruption of function due to the epitope-tag. However, producing transgenic plants over-expressing completely functional epitope-tagged constructs would create a valuable resource for further experimental work such as looking for interacting proteins using biochemical approaches.

7.3 Functionality of the epitope-tagged constructs.

The 3x c-Myc epitope-tag was inserted between the ultimate and penultimate amino acids of Cf-2. This site was chosen, as this portion of the protein is believed to serve no function other than as part of the membrane anchor. Piedras *et al.* (2000) and Van der Hoorn (2001a) using epitope-tagging and mutation analysis respectively revealed that, in their assays, the cytoplasmic tail of Cf-9 serves no function other than as a membrane anchor. The Cf proteins however, have no obvious signalling domain and it is possible that the cytoplasmic tail interacts with other proteins e.g. signalling molecules

Unlike Cf-9, it was not possible to assess the function of the transgenes in tobacco. *Avr2* was isolated only recently and so was not available for use in a transient assay as used to assess Cf-9 function. In addition it appears that a sufficiently homologous Rcr3 is not present in tobacco, without which Cf-2 cannot function (Jones, unpublished).

Inoculation of transgenic Cf0 tomatoes, carrying *RP005* and *RP006*, with *C. fulvum* race 4*GUS* revealed the transgene to be greatly reduced in function or perhaps completely non-functional. Using GUS activity it was possible to quantify levels of fungal growth, which should have indicated transgene effectiveness. However, this protocol relies upon an equivalent inoculum for each plant. These data presented here showed a great variation in fungal growth between putative transformants later revealed to lack the transgene. It would therefore, be useful to investigate inoculation protocols and growth conditions to produce a more reliable assay for quantitative analysis of *Cf* transgene function.

It was not possible to directly assess the function of the transgene (*RP006*) in an rcr3-3 background as *Cf-2*-mediated defence requires the presence of a functional *Rcr3* gene. This could be over come by crossing them to Cf0 tomatoes, which carry a functional Rcr3 and subsequent inoculation of F_1 individuals with *C. fulvum*. In addition this would generate Cf0 transgenic plants with higher expression levels of *RP006* than previously obtained. Avoiding the tissue culture phase and using rcr3-3 transformants may prove to be a more viable route to plants expressing *c-Myc:Cf-2* to higher levels. As rcr3-3 plants are homozygous for *Cf-2*, producing a plant that lacks a copy of both Cf-2 and Rcr3, but carries rcr3-3 and the epitope-tagged Cf-2, will require the use of marker-assisted selection. An alternative route would be to transform Cf0 plants that are homozygous for the rcr3-3 mutation. Crossing rcr3-3 plants with Cf0 plants and by subsequent selfing and screening of the F_2 progeny could produce Cf0/rcr3-3 plants. Once transformants in this background have been produced they could then be crossed to a Cf0 plant and screened for functionality of the epitope-tagged gene.

With the recent cloning of Rcr3 and Avr2, a transient assay is currently being developed to enable assessment of Cf-2 function (and epitope-tagged versions) in the *N. benthamiana*. Once established, the transient assay could be utilised to confirm the functionality of the epitope-tagged constructs, pRP005 and pRP006. Whether the insertion of the epitope-tag, into the cytoplasmic tail of Cf-2, severely disrupts or completely abolishes its function, a similar conclusion can be drawn. That is that the cytoplasmic tail of Cf-2 serves not only as a membrane anchor, but also has an additional function, possibly being involved in signalling.

7.4 Localisation of c-Myc:Cf-2

Here, using two-phase aqueous partitioning Cf-2 was shown to be plasma membrane bound in tobacco, with the majority of the c-Myc:Cf-2 purifying with the upper (plasma membrane) fraction. The proportion of c-Myc:Cf-2 found in the lower fraction correlated with increased expression levels of the T-DNA, probably due to a 'back-log' of protein in the ER and Golgi caused by saturation of the secretory machinery by over-expression. Although the c-Myc:Cf-2 protein was not functional it is unlikely that the change to the cytoplasmic tail affected the localisation of the protein, but cannot be ruled out completely. Localisation of c-Myc:Cf-2 was not demonstrated in tomato. Ideally

these experiments should be repeated with functionally active epitope-tagged versions of the protein in tobacco (for relative ease) and tomato, for absolute confirmation.

7.5 Post-translational modification of c-Myc:Cf-2

Treatment of Cf-9 with PNGaseF revealed that, as predicted, it is highly glycosylated with carbohydrate groups totalling 55KDa (Piedras *et al.*, 2000). Treatment of tomato microsomes with $EndoH_f$ and *in vitro* transcription and translation of *c-Myc:Cf-2*, revealed an unglycosylated protein of 120KDa, similar to the predicted size of Cf-2. These results obtained indicate that, like Cf-9, Cf-2 is highly glycosylated and probably to a similar degree.

7.6 Modelling Cf-2 mediated defence

Using data collected here with published data available, a model of Cf-2 action has been proposed (Figure 7.1). Extensive protein complexes are known to attach to many animal receptor molecules upon activation. It seems increasingly likely that a similar signalling complex is formed with the Cf-2 protein. This model predicts Cf-2 to be the 'guard', monitoring for the presence of an Rcr3/Avr2 complex. Upon detection of the fungus the complex is formed, the model here involves two interacting proteins. The first (Interactor 1) is possibly a membrane bound protein and the second (interactor 2) a cytoplasmic protein that interacts with the cytpolasmic tail of Cf-2. Interactor one has been proposed, as there is no evidence to date of a physical interaction between Avr2 and Cf-2, suggesting the involvement of an additional protein. It is predicted to be a membrane bound protein, like the HABS observed for Cf-9 (Kooman-Gersmann *et al.*, 1996). Interactor 1 could be responsible, either solely or incombination with Cf-2, for the recognition of the Rcr3/Cf-2 complex.

Unlike Cf-2 where a severe loss of function is observed, disruption of the Cf-9 cytoplasmic tail, by insertion of an epitope tag, only slightly reduced the response to Avr9 (Piedras *et al.*, 2000). This reduction in response to Avr9 indicates that this interactor may also function in *Cf-9*-mediated resistance, although is perhaps not absolutely required. Piedras *et al.* (2000) used plants over-expressing the epitope-tagged Cf-9 construct, which may mask the subtleties of complex protein interactions. It may be that over-expression of

Cf-9 means that interactor 1 alone is enough for resistance, but at lower expression levels you may require interactor 2 to increase the strength of the signal. This may be also true for Cf-2, as in the first generation T010 (*RP006*/Cf0 transformant) appeared resistant to *C. fulvum* challenge.

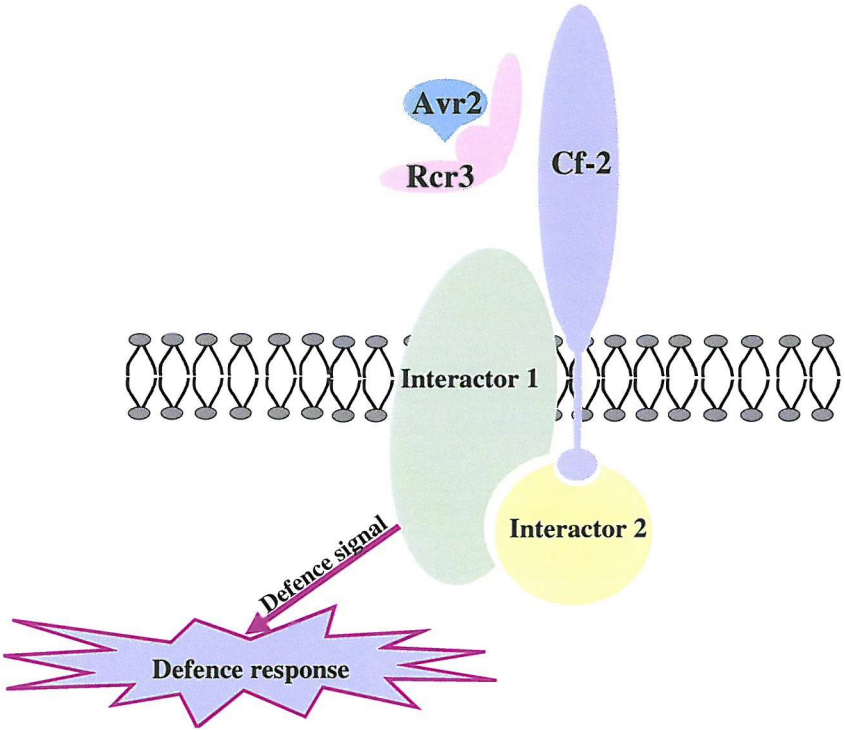


Figure 7.1: Model of Cf-2 function.

Krüger *et al.* (2002) proposed that Rcr3 is the virulence target of Avr2 and that the complex of Avr2 and Rcr3, or a product of that complex, is 'guarded' by Cf-2. Here it is proposed that interacting protein one aids in the recognition of this complex and that it also, along with interacor two, generates the signal for the defence response.

In animals there are proteins, which interact with small cytoplasmic tails of trans-membrane receptor proteins like that of Cf-2. One type of domain responsible for this interaction is called the PDZ (PSD-95, Discs large, ZO1) domain (Liu and Lengyel, 2000; Ikemoto et al., 2000). PDZ domains interact with bulky hydrophobic amino acids at the terminus of the cytoplasmic tails and interstingly, both Cf-2 and Cf-5 terminate with the bulky hydrophobic amino acid phenylalanine, whilst Cf-4 and Cf-9 terminate with tyrosine. It is possible that the C-terminus of Cf-2 interacts with a PDZ domain protein to form a signalling complex.

PDZ domains have quite an interrupted consensus sequence and thus any general search for homology would probably not reveal PDZ domain homology. Highly specific database searches have revealed PDZ domain containing proteins in plants (Dixon, unpublished). For example one protein in *A. thaliana* is closely related to the chaperon protein Deg P from *E. coli*. It is therefore proposed that the second interacting protein in this model (Figure 7.1) may carry a PDZ domain.

It is possible that interactor 2 is continuously bound to the C-terminus of Cf-2 and that it is responsible for the recruitment of interactor 1, which interacts with the final 8.5 LRRs of Cf-2. A conformational change may occur to interactor 1 upon binding of the Rcr3/Avr2 complex that results in binding to Cf-2. As the last 8.5 LRRs are the most conserved between all Cf proteins, they have been proposed to be involved in interacting with signalling molecules i.e. interactor 1. In this case interactor 2 could also act to stabilise the complex.

There is no obvious signalling domain present in the Cf proteins, therefore it is likely that at least one of the proteins in the complex has a domain capable of generating a signal. As it appears *Cf-9*-mediated resistance still functions despite disruption of its interaction with interactor 2, interactor 1 is likely to have the ability to generate a signal. Other proteins will function downstream of interactors one and two to carry the defence signal.

One obvious next step in the research of Cf-mediated resistance is to investigate the interaction between Cf-2, Avr2 and Rcr3. This could be achieved by epitope-tagging of each of the proteins and using immune precipitation to obtain protein complexes. This would be made simpler with the development of the previously mentioned transient assay and could be used to detect difference between elicited (with Avr2) and non-elicited (without Avr2) plants.

To search for candidate interactor 2 type proteins, a yeast-2-hybrid approach could be employed. This is likely to produce additional non-specific interactions, but these can be accounted for with appropriate controls. Both protocols may also identify other, yet to be determined, interacting proteins.

Several groups studying animal receptors with short cytoplasmic tails have successfully employed affinity purification using fusion proteins as bait (Ikemoto *et al.*, 2000). In the early stages of this project, the possibility of the cytoplasmic tail interacting with other proteins was discussed. A Cf-2 cytoplasmic tail: glutathione-S-transferease (GST) fusion construct was built. Using the vector pGex-5x-1 (Amersham Pharmacia, Little Chalfont, UK), the cytoplasmic tail of Cf-2 (obtained by PCR using the primers RP5 and RP6, Appendix I) was fused to a gene encoding GST, with expression of the fusion protein inducible by IPTG. The fusion construct, designated pRP003 (Appendix II), was expressed in *E.coli*. Sonication of the bacteria and analysis by SDS-PAGE revealed at least a portion of the protein to be soluble. This protein can be bound to a column of glutathione beads and used as bait for various plant fractions. This project was abandoned at this stage, as the transgenes *RP005* and *RP006* appeared initially to be functional. Due to the highly basic nature of the cytoplasmic tail it is likely that non-specific interaction will occur, these can be reduced by increasingly stringent washes. In addition, as epitope-tagging of the cytoplasmic tail disrupts Cf-2 function, possibly by preventing protein-protein interactions. An epitope-tagged tail: GST fusion could be used as a negative control to identify specific interactions. If enough interacting protein is obtained it may them be possible to sequence and identify it.

Other interesting avenues for investigation include studying the importance of the glycoslyation sites. It has been shown for other proteins (specifically the human secretin receptor) that the addition of carbohydrates to a protein can determine its binding capability (Pang *et al.*, (1999). The N-linked glycosylation sites could, in turn and in combination, be mutated so they can no longer be glycosylated. By comparing the sizes of each mutant, it would be possible to determine which of the sites are glycosylated on the native protein. In addition, it would be possible to determine if any of the glysosylated sites are essential for Cf-2 function and perhaps Avr2 recognition. Due to the large number of potential glycosylation sites this would be a big undertaking that would be made easier by the development of a working transient assay.

Cf-2 is predominantly comprised of LRRs with cysteine residues at either end of the LRR region, these have the potential to form disulphide bridges (Dixon *et al.*, 1996). Di Matteo *et al.* (2003) determined the crystal structure of a polygalacturonase-inhibiting protein (PGIP), a more appropriate model for Cf protein structure than previously

described structures (Kajava, 1998). In this model, the LRR structure is capped by cysteine residues, forming disulphide bridges, which are predicted to maintain the integrity of the structure. By mutating the cysteine residues of Cf-2 it would be possible to investigate their importance in Cf-2-mediated resistance and potentially Cf-2 structure.

Here it has been demonstrated that the majority of Cf-2 is found in the plasma membrane and that, like Cf-9, is highly glycosylated. An unexpected, yet interesting, finding is that the cytoplasmic tail of Cf-2 appears to be involved in protein-protein interaction, presenting a potentially exciting route for future research.

References

Agrios, George N., 1997. *Plant Pathology.* fourth edition, Academic Press. New York, USA.

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J., Staskawicz, B.J. 1994. *RPS2* of *Arabidopsis Thaliana*- a leucine rich repeat class of plant disease resistance genes. *Science.* **265:** 1856-1860.

Benghezeal, M., Wasteneys, G.O., Jones, D.A. 2000. The C-terminal dilysine motif confers endoplasmic reticulum localization to type I membrane proetins in plants. *Plant Cell.* **12:** 1179-1201.

Blatt, M.R., Brearley, J., Hammond-Kosack, K., Jones, J.D.G. 1999. K+ channels of Cf-9 transgenic tobacco guard cells as targets for *Cladosporium fulvum* Avr9 elicitor-dependent signal transduction. *Plant Journal.* **19:** 453-462.

Blumwald, E., Aharon, G.S., Lam, B.C. 1998. Early signal transduction pathways in plant-pathogen interactions. *Trends in Plant Science.* **3:** 343-346.

Botella, M.A., Coleman, M.J., Hughes, D.E., Nishimura, M.T., Jones, J.D.G., Somerville, S.C. 1997. Map positions of 47 *Arabidopsis* sequences with sequence similarity to disease resistance genes. *Plant Journal.* **12**: 1197-1211.

Botella, M.A., Parker, J.E., Frost, L.N., Bittner-Eddy, P.D., Beynon, J.L., Daniels, M.J. Holub, E.B., Jones, J.D.G. 1998. Three genes of the *Arabidopsis RPP1* complex resistance locus recognise distinct *Peronospora parasitica* avirulence determinants. *Plant Cell.* **10:** 1847-1860.

Brading, P.A., Hammond-Kosack, K.E., Parr, A., Jones, J.D.G. 2000. Salicylic acid is not required for *Cf-2*- and *Cf-9*-dependent resistance of tomato to *Cladosporium fulvum. The Plant journal.* **23:** 305-318.

Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. 1976. *Analytical Biochemistry.* **72:** 248-254.

Church, G.M., Gilbert, W. 1984. Genomic sequencing. *Proceedings of the National Academy of Sciences of the United States of America.* **81**: 1991-1995.

Citovsky, V., Warnick, D., Zambryski, P. 1994. Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. *Proceedings of the National Academy of Sciences of the United States of America.* **91**: 3210-3214.

Clarke, A., Desikan, R., Hurst, R.D., Hancock, J.T., Neill, S.J. 2000. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant Journal.* **24**: 667-677.

de Jong, C.F., Takken, F.L.W, Cai, X.H., de Wit P.J.G.M, Joosten, M.H.A.J. 2002. Attenuation of Cf-mediated defence responses at elevated temperatures correlates with a decrease in elicitor-binding sites. *Molecular Plant-Microbe Interactions.* **15**: 1040-1049.

DeWitt, N.D., Sussman, M.R. 1995. Immunocytological localization of an epitope-tagged plasma membrane proton pump (H^+-ATPase) in phloem companion cells. *The Plant Cell.* **7**: 2053-2067.

Di Matteo, A., Federici, L., Slavi, G., Johnson, K.A., Savino, C., De Lorenzo, G., Tsernoglou, D. 2003. The crystal structure of the polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein involved in plant defense. *Proceedings of the National Aacademy of Sciences.* **100**: 10124-10128.

Dineshkumar, S.P., Whitham, S., Choi, D., Hehl, R., Corr, C., Baker, B. 1995. Transponson tagging of tabacco mosaic virus resistance gene *N*- its possible role in the TMV-N-mediated signal transduction pathway. *Proceedings of the National Academy of Sciences of the United States of America.* **92:** 4175-4180.

Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas,C.M., Harrison, K., Jones, J.D.G. 1996. The tomato *Cf-2* disease resitance locus comprises two functional genes encoding leucine rich repeat proteins. *Cell.* **84:** 451-459.

Dixon, M.S., Hatzixanthis, K., Jones, D.A., Harrison, K., Jones, J.D.G. 1998. The tomato *Cf-5* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. *The Plant Cell.* **10:** 1915-1925.

Dixon, M.S., Golstein, C., Thomas, C.M., van der Beizen, E.A., Jones, J.D.G. 2000. Genetic complexity of pathogen perception by plants: The example of *Rcr3*, a tomato gene required specifically by Cf-2. *Proceedings of the National Academy of Sciences of the United States of America.* **97:** 8807.

Durner, J., Wendehenne, D., Klessig, D.F. 1998. Defence gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP-ribose. *Proceedings of the National Academy of Sciences of the United States of America.* **95**: 10328-10333.

Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E., Jones, J.D.G. 2000. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell.* **12**: 963-977.

Fields, S., Sternglanz, R. 1994. The 2-hybrid system: An assay for protein-protein interactions. *Trends in Genetics.* **10**: 286-292.

Flor, H.H. 1971. Current status of the gene-for-gene concept. *Annual Review of Phytopathology.* **9:** 275-296.

Friedman, A.M., Long, S.R., Brown, S.E, Buikema, W.J., Ausubel, F.M. 1982. Cloning of a briad host range cosmid cloning vector and its use in the genetic analysis of Rhizobium mutants. *Gene.* **18**: 289-296.

Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R.W., Dangl, J.L. 1995. Structure of the Arabidopsis *RPM1* gene enabling dual specificity disease resistance. *Science.* **269**: 843-846.

Haanstra, J.P.W., Lauge, R., Meijer-Dekens, F., Bonnema, G., de Wit, P.J.G.M., Lindhout, P. 1999.The *Cf-ECP2* gene is linked to, but not part of, the *Cf-4/Cf-9* cluster on the short arm of chromosome one in tomato. *Molecular and General Genetics.* **262:** 839-845.

Hammond-Kosack, K.E., Jones, D.A., Jones, J.D.G. 1994. Identification of two genes required in tomato for full *Cf-9*-dependent resistance to *Cladosporium fulvum. The Plant Cell.* **6:** 361-374.

Hammond-Kosack, K.E., Jones, J.G.D. 1994. Incomplete dominance of tomato *Cf* genes for resistance to *Cladosporium fulvum. Molecular Plant-Microbe Interaction.* **7:** 58-70

Hammond-Kosack, K.E., Jones, J.D.G. 1996. Resistance gene dependent plant defense responses. *The Plant Cell.* **8:** 1773-1791.

Hammond-Kosack, K.E., Silverman, P,. Raskin, I., Jones, J.D.G. 1996. Race-specific elicitors of *Cladosporium fulvum* induce changes in cell morphology and the synthesis of ethylene and salycylic acid in tomato plants carrying the corresponding *Cf* disease resistance gene. *Plant Physiology.* **110:** 1381-1394.

Hammond-Kosack, K.E., Jones, J.D.G. 1997. Plant disease resistance genes. *Annual Review of Plant physiology and Plant Molecular Biology.* **48:** 575-607.

Hancock, J.T., Desikan, R., Clarke, A., Hurst, R.D., Neill, S.J. 2002. Cell signalling following plant-pathogen interactions involves the generation of reactive oxygen and relative nitrogen species. *Plant Physiology and Biochemistry.* **40**: 611-617.

Helenius, A., Aebi. 2001. Intracellular function of N-linked glycans. *Science.* **291** 2364-2369.

Honee, G., Buitink, J., Jabs, T., de Kloe, J., Sijbolts, F., Apotheker, M., Weide, R., Sijen, T., Stuvier, M., de Wit, P.J.G.M. 1998. Induction of defense related responses in Cf-9 tomato cells by the Avr9 elicitor peptide of *Cladosporium fulvum* is developmentally regulated. *Plant physiology.* **117:** 809-820.

Hookyaas, P.J.J., Beijersbergen, A.G.M. 1994. The virulence system of *Agrobacterium tumefaciens*. *Annual Review of Phytopatholgy*. **32**: 157-179.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eicholtz, D., Rogers, S.G., Fraley, R.T. 1985. A simple method for transferring genes into plants. *Science*. **227:** 1229-1231.

Hu, W., Li, F., Yang, X., Li, Z., Xai, H., Li, G., Wang, Y., Zhang, Z. 2004. A flexible peptide linker enhances the immunoreactivity of two copies HbsAg PreS1 (21-47) fusion protein. *Journal of Biotechnology*. **107**: 83-90.

Hulbert, S.H. 1997. Structure and evolution of the rp1 complex conferring rust resistance in maize. *Annual Review of Phytopathology*. **35:** 293-310.

Ikemoto, M., Arai, H., Feng, D., Tanaka, K., Aoki, J., Dohmae, N., Takio, K., Adachi, H., Tsujimoto, M., Inoue, K. 2000. Identification of a PDZ-domain-containing protein that interacts with the scavenger receptor class B type I. *Proceedings of the National Academy of Sciences of the United States of America*. **97:** 6538-9543.

Jarvik, J.W., Telmer, C.A. 1998. Epitope tagging. *Annual Review of Genetics*. **32**: 601-618.

Jefferson, R.A., Burgess, S.M., Hirsh, D. 1986. Beta-Glucoronidase from *Escherichia coli* as a Gene-Fusion Marker. *Proceedings of the National Academy of Sciences of the United States of America*. **83**: 8447-8451.

Jeong, S., Trotochaud, A.E., Clark, S.E. 1999. The *Arabidopsis CLAVATA2* gene encodes a receptor like protein required for the stability of the CLAVATA1 receptor like kinase. *The Plant Cell*. **11:** 1925-1933.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *The EMBO journal*. **19:** 4004-4014.

Johal, G.S., Briggs, S.P. 1992. Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science*. **258:** 985-987.

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J., Jones. J.D.G. 1994. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transponson tagging. *Science*. **266:** 789-792.

Jones, D.A., Jones, J.D.G. 1997. The role of leucine rich repeat proteins in plant defenses. *Advances in Botanical Research*. **24:** 89-167.

Jones, J.D.G., Shlumukov, L., Harland, F., English, J., Scofiels, S.R., Bishop, G.J., Harrison, K. 1992. Effect vectors for transformation and expression of heterologous genes and assaying transposon excision in transgenic plants. *Transgenic Research*. **1**: 285-297.

Jones, J.D.G. 1996. Plant disease resistance genes: Structure, function, and evolution. *Current Oppinion in Biotechnology*. **7**: 155-160.

Joosten, M.H.A.J., Cozijnsen, T.J., de Wit, P.J.G.M. 1994. Host-resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature*. **367:** 384-386.

Joosten, M.H.A.J., de Wit, P.J.G.M. 1999. The tomato–*Cladosporium fulvum* interaction: A versatile experimental system to study plant–pathogen interactions. *Annual Review of Phytopathology*. **37:** 335-367.

Kajava, A.V. 1998. Structural diversity of leucine-rich repeat proteins. *Journal of Molecular Biology*. **277:** 519-527.

Kircher, S., Gil, P., Kozma-Bognar, L., Fejes, E., Speth, V., Husselstein-Muller, T., Bauer, D., Adam, E., Schafer, E., Nagy, F. 2002. Nucleocytoplasmic partitioning of the plant photoreceptors phytochrome A, B, C, D and E is regulated differentially by light and exhibits diurnal rhythm. *The Plant Cell*. **14** 1541-1555

Kobe, B., Deisenhofer, J. 1994. The leucine rich repeat: a versatile binding motif. *TIBS*. 415-421.

Kooman Gersmann, M., Honee, G., Bonnema, G., de Wit, P.J.G.M. 1996. A high affinity binding site for the Avr9 peptide elicitor of *Cladosporium fulvum* is present on plasma membranes of tomato and other solanaceous plants. *Plant Cell.* **8:** 929-938.

Kooman-Gersmann, M., Vogelsang, R., Vossen, P., van den Hooven, H.W., Mahe, E, Honee, G., de Witt, P.J.G.M. 1998. Correlation between binding affinity and necrosis-inducing activity of mutant AVR9 peptide elicitors. *Plant Physiology.* **117:** 609-618.

Krüger, J., Thomas, C.M., Goldstein, C., Dixon, M.S., Smoker, M., Tang, S.K., Mulder, L., Jones, J.D.G. 2002. A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. *Science.* **296**: 744-747.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature.* **277**: 680-685.

Lam, C.H.B., Xing, T., Higgins, V.J., Blumwald, E. 1998. Efect of race specific elicitors of *Cladosporium fulvum* on the tomato plasma calcium ATPase. *Physiological and Molecular Plant Pathology.* **52:** 309-321.

Langford, A.N. 1948. Autogenous necrosis in tomatoes immune from *Cladosporium fulvum* cooke. *Canadian Journal of Research.* **26:** 35-64.

Lauge, R., Joosten, M.H.A.J., van den Akerweken, G.F.J.M., van den Broek, H.W.J., de wit, P.J.G.M. 1997. The in planta-produced extracellular proteins ECP1 and ECP2 of *Cladosporium fulvum* are virulence factors. *Molecular Plant-Microbe interactions.* **10:** 725-734.

Lauge, R., Joosten, M.H.A.J., Haanstra, J.P.W., Goodwin, P.H., Lindhout, P., de Wit, P.J.G.M. 1998a. Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen. *Proceedings of the National Academy of Sciences of the United States of America.* **95:** 9014-9018.

Lauge, R., de Wit, P.J.G.M. 1998b. Fungal avirulence genes: Structure and possible functions. *Fungal Genetics and Biology.* **24:** 285-297.

Leonard, K.J. 1994. Stability of equilibria in a gene-for-gene coevolution model of host-parasite interactions. *Phytopathology*. **84:** 70-77.

Ling, H.D., Kriseleit, D., Ganal, M.W. 1998. Effect of ticarcillin/potassium clavulnate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). *Plant Cell Reports*. **17:** 843-847.

Liu, X., Lengyel, J.A. 2000. *Drosophila arc* encodes a novel adherens junction-associated PDZ domain protein required for wing and eye development. *Developmental Biology*. **221:** 419-434.

Lohman, K.N., Gan, S., John, M.C., Amasino, R.M. 1994. Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiol. Plant.* **92**: 322-328.

Luderer, R., Rivas, S., Nürnberger, T., Mattei, B., Van den Hooven, H.W., Van der Hoorn, R.A.L., Romeis, T., Wehrfritz, J.M., Blume, B., Nennstiel, D., Zuidema, D., Vervoort, J., De Lorenzo, G., Jones., J.D.G., de Witt, P.G.J.M., Joosten, M.H.A.J. 2001a. No evidence of binding between resistance gene product Cf9 of tomato and avirulence gene product Avr9 of *Cladosporium fulvum*. *Molecular plant microbe interactions*. **14:** 867-876.

Luderer, R., Joosten, M.H.A.J. 2001b. Avirulence proteins of plant pathogens: determinants of victory and defeat. *Molecular Plant Pathology*. 2**:** 355-364.

Luderer, R., Takken, F.L.W, de Wit, P.J.G.M, Joosten, M.H.A.J. 2002a. *Cladosporium fulvum* overcomes Cf-2-mediated resistance by producing truncated AVR2 elicitor proteins. *Molecular Microbiology*. **45**: 875-884.

Luderer, R., De Kock, M.J.D., Dees, R, H, L., De Wit, P.J.G.M., Joosten, M.H.A.J. 2002b. Functional analysis of cysteine residues of ECP elicitor proteins of the fungal tomato pathogen *Cladosporium fulvum*. *Molecular Plant Pathology*. **3**: 91-95.

Marmeisse, R., van den Akerveken, G.F.J.M., Goosten, T., de Wit, P.J.G.M., van den Broek, H.W.J. 1993. Disruption of the Avirulence gene AVR9 in 2 races of the tomato

pathogen *Cladosporium fulvum* causes virulence on tomato genotypes with the complementary resistance gene *Cf-9*. *Molecular Plant-Microbe interactions.* **6:** 412-417.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., Tanksley, S.D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*. **262:** 1432-1435.

McDowell, J.M., Dhandaydham, M., Long, T.A., Aarts, M.G.M., Goff, S., Holub, E.B, Dangl, J.L. 1998. Intragenic recombination and diversifying selection contribute to the evolution of Downy Mildew resistance at the *RPP8* locus of *Arabidopsis*. *The Plant Cell.* **10:** 1861-1874.

Meyers, B.C., Shen, K.A., Rohani, P., Gaut, B.S., Michelmore, R.W. 1998. Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. *The Plant Cell.* **11:** 1833-1846.

Meyers, B.C., Dickerman, A.W., Michelmore, R.W., Sivaramakrishnan, S., Sobral, B.W., Young, N.D. 1999. Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide binding superfamily. *Plant Journal.* **20:** 317-332.

Monro, S., Pelham, H.R.B. 1984. Use of peptide tagging to detect proteins expressed from cloned genes: Deletion mapping functional domains of *Drosophila* hsp70. *EMBO Journal.* **3**: 3087-3093.

Murray, V. 1989. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Res.* **17:** 8889.

Noel, L., Moores, T.L., van der Beizen, E.A., Parniske, M., Daniels, M.J., Parker, J.E., Jones, J.D.G. 1999. Pronounced intraspecific haplotype divergence at the *RPP5* complex disease resistance locus of *Arabidopsis*. *Plant Cell.* **11:** 2099-2111.

Oldroyd, G.E.D., Staskawicz, B.J. 1998. Genetically engineered broad-spectrum disease resistance in tomato. *Proceedings of the National Academy of Sciences of the United States of America.* **95**: 103000-10305.

Oliver, R.P., Farman, M.L., Jones J.D.G., Hammond-Kosack, K.E. 1993. Use of fungal transformants expressing beta-glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. *Molecular Plant-Microbe Interactions.* **6**: 521-525.

Oshima, Y., Iwasaki, I., Suga, S., Murakami, M., Inoue, K., Maeshima, M. 2001. Low aquaporin content and low osmotic water permeability of the plasma and vaculolar membranes of a CAM plant *Graptopetalum paraguayense*: comparisom with radish. *Plant Cell Physiology.* **42:** 1119-1129.

Pang, R,T., Sai-Ming, S, N.G., Cheng, C, H., Holtman, M.H., Miller, L.J., Chow, B,K. 1999. Role of N-linked glycosylation on the function and expression of the human secretin receptor. *Endocrinology.* **140**: 5102-5111.

Pansegrau, W., Schoumacher, F., Hohn, B., Lanka, E. 1993. Site-specific cleavage and joining of single-stranded-DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: Analogy to bacterial conjugation. *Proceedings of the National Academy of Sciences of the United States of America.* **90**: 11538-11542.

Panter, S.N., Hammond-Kosack, K.E., Jones, J.D.G., Jones, D.A. 2002. Developmental control of promoter activity is not responsible for mature onset of Cf9-B-mediated resistance to leaf mould in tomato. *Molecular Plant-Microbe Interactions.* **15**: 1099-1107.

Parniske, M., Hammond-Kosack, K.E., Golstein, C., Thomas,C.M., Jones, D.A., Harrison, K., Wulff, B.B.H., Jones, J.D.G. 1997. Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. *Cell.* **91:** 821-832.

Parniske, M., Wulff, B.B.H., Bonnema, G., Thomas, C.M., Jones, D.A., Jones, J.D.G. 1998. Homologues of the *Cf-9* disease resistance gene (*Hcr9s*) are present at multiple loci

on the short arm of tomato chromosome 1. *Molecular Plant-Microbe Interactions.* **12:** 93-102.

Parniske, M., Jones, J.D.G. 1999. Recombination between diverged clusters of the tomato *Cf-9* plant disease resistance gene family. *Proceedings of the National Academy of Sciences of the United States of America.* **96:** 5850-5855.

Piedras, P., Hammond-Kosack, K.E., Harrison, K., Jones, J.D.G. 1998. Rapid, Cf-9 and Avr9-dependent production of active oxygen species in tobacco suspension cultures. *Molecular Plant-Microbe interactions.* **11:** 1155-1166.

Piedras, P., Rivas, S., Droge, S., Hillmer, S., Jones, J.D.G. 2000. Functional, c-myc-tagged *Cf-9* resistance gene products are plasma membrane localised and glycosylated. *Plant Journal.* **21:** 529-236.

Pieterse, C.M.J., van Loon, L.C. 1999. Salicylic acid-independent plant defence pathways. *Trends in Plant Science.* **4**: 52-58.

Rayon, C., Lerouge, P., Faye, L. 1998. The protein N-glycosylation in plants. *Journal of experimental Botany.* **49**: 1463-1472.

Rivas, S., Mucyn, T., van den Burg, H.A., Vervoort, J., Jones, J.D.G. 2002a. A similar to 400KDa membrane-associated complex that contains one molecule of the resistance protein Cf-4. *Plant Journal.* **29**: 783-796.

Rivas, S., Romeis, T., Jones, J.D.G. 2002b. The Cf-9 disease resistance protein is present in an similar to 420-KDa heteromultimeric membrane-associated complex at one molecule per complex. *Plant Journal.* **29**: 689-702.

Romeis, T., Piedras, P., Zhang, S.Q., Klessig, D.F., Hirt, H., Jones, J.D.G. 1999. Rapid Avr9 and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. *Plant Cell.* **11:** 273-287.

Romeis, T., Piedras, P., Jones, J.D.G. 2000. Resistance gene-dependent activation of a calcium-dependent protein kinase in the defence response. *Plant Cell.* **12**: 803-815.

Romeis, T., Ludwig, A.A., Martin, R., Jones, J.D.G. 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. *EMBO Journal.* **20**: 5556-5567.

Salmeron, J.M., Barker, S.J., Carland, F.M., Mehta, A.Y., Staskawicz, B.J. 1994. Tomato mutants altered in bacterial disease resistance provide evidence for a new locus controlling pathogen recognition. *The Plant Cell.* **6:** 511-520.

Salmeron, J.M., Oldroyd, G.E.D, Rommens, C.M.T., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D., Staskawicz, B.J. 1996. Tomato *Prf* is a member of the leucine rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell.* **86:** 123-133.

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular cloning: A laboratory manual, second edition. Cold Spring Harbour Laboratory Press. New York, USA.

Sanger, F., Nicklen, S., Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America.* **74:** 5463-5467.

Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W, Staskawicz, B.J. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science.* **274**: 2063-2065.

Schaller, G.E., De Wit, N.D. 1995. Analysis of the H+-ATPase and other proteins of the *Arabidopsis* plasma membrane. *Methods in Cell Biology.* **50**: 129-148.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Ly, P.I., Holsten, T., Gardener, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C., Ronald, P. 1995. A receptor kinase like protein encoded by the rice disease resistance gene *Xa21*. *Science.* **270:** 1804-1806.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology.* **98**: 503-517.

Spanu, P. 1997. HCf-1, a hydrophobin from the tomato pathogen *Cladosporium fulvum*. *Gene*. **193:** 89-96.

Swiderski, M.R., Innes, R.W. 2001. The Arabidopsis *PBS1* resistance gene encodes a member of a novel protein kinase subfamily. *Plant Journal*. **26**: 101-112.

Takken, F.L.W., Thomas, C.M., Joosten, M.H.A.J., Golstein, C., Westerink, N., Hhille, J., Nijkamp, H.J.J., de Wit, P.J.G.M., Jones, J.D.G. 1999. A second gene at the tomato *Cf-4* locus confers resistance to *Cladosporium fulvum* through recognition of a novel avirulnce determinant. *The Plant Journal*. **20:** 279-288.

Takken, F.L.W., Luderer, R., Gabriels, S.H.E.J., Westerink, N., Lu, R., de Wit, P.J.G.M., Joosten, M.H.A.J. 2000. A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. *Plant Journal*. **24**: 275-283.

Tameling, W.I.L., Elzinga, S.D.J., Darmin, P.S., Vossen, J.H., Takken, F.L.W., Haring, M.A., Cornelissen, B.J.C. (2002). The tomato *R* gene products *I-2* and *Mi-1* are functional ATP binding proteins with ATPase activity. *The Plant Cell*. **14:** 2929-2939.

Thara, V.K., Tang, X.Y., Gu, Y.Q., Martin, G.B., Zhou, J.M. 1999. *Pseudomanas syringae* pv tomato induces the expression of tomato EREBP-like genes *Pti4* and *Pti5* independent of ethylene, salicylate and jasmonate. *Plant Journal*. **20:** 475-483.

Tigchelaar, E.C. 1984. Collections of isogenic tomato stocks. *Reproductive Tomato Genetics Coop*. **34**: 55-57.

Thomas, C.M., Jones, D.A., Parniske, M., Harrison, K., Blaint-Kurti, P.J., Hatzixanthis, K., Jones, J.D.G. 1997. Characterisation of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. *Plant Cell*. **9:** 2209-2224.

Thomas, C.M., Dixon, M.S., Parniske, M., Golstein, C., Jones, J.D.G. 1998. Genetic and molecular analysis of tomato *Cf* genes for resistance to *Cladosporium fulvum*.

Philosophical transactions of the Royal Society of London Series B- Biological Sciences. **353:** 1413-1424.

Thomas, C.W., Tang, S., Hammond-Kosack, K., Jones, J.D.G. (2000). Comparison of the hypersensitive response induced by the tomato *Cf-4* and *Cf-9* genes in *Nicotiana* spp. *Molecular Plant Microbe Interactions.* **13**: 465-469.

Trotochaud, A.E., Hao, T., Wu, G., Yang, Z., Clark, S.E. 1999. The CLAVATA1 receptor like kinase requires CLAVATA3 for its assembly into a signalling complex that includes KAPP and a Rho-related protein. *The Plant Cell.* **11:** 393-405.

Van den Akerveken, G.F.J.M., Vankan, J.A.L., Joosten, M.H.A.J., Muisers, J.M., Verbakel, H.M., de wit, P.J.G.M. 1993. Characterisation of 2 putative pathogenicity genes of the fungal tomato pathogen *Cladosporium fulvum. Molecular Plant-Microbe interactions.* **6:** 210-215.

Van den Hooven, H.W., Appleman, A.W., Zey, T., De Wit, P.J.G.M., Vervoort, J. 1999. Folding and conformational analysis of Avr9 peptide elicitors of the fungal tomato pathogen *Cladosporium fulvum. European Journal of Biochemistry.* **264**: 9-18.

Van der Beizen, E.A., Jones, J.D.G. 1998. Plant disease resistance proteins and the gene for-gene-concept. *Trends in Biochemical Sciences.* 454-456.

Van Camp, W., van Montagu, M., Inze, D. 1998. H_2O_2 and NO: redox signals in disease resistance. *Trends in Plant Science.* **3**: 330-334.

Van der Hoorn, R.A.L., Van der Ploeg, A., de Witt, P.G.J.M., Joosten, M.H.A.J. 2001a. The C-terminal dilysine motif for targeting to the endoplasmic reticulum is not required for Cf-9 function. *Molecular plant microbe interactions.* **14:** 412-415.

Van der Hoorn, R.A.L., Roth, R., de Witt, P.J.G.M. 2001b. Identification of distinct specificity determinants in resistance protein Cf-4 allows construction of a Cf-9 mutant that confers recognition of avirulence protein Avr4. *Plant cell.* **13:** 273-285.

Van der Hoorn, R.A.L., Kruijt, M., Roth, R., Brandwagt, B.F., Joosten, M.H.A.J., de Witt, P.G.J.M. 2001c. Intragenic recombination generated two distinct *Cf* genes that mediate Avr9 recognition in the natural population of *Lycopersicon pimpinellifolium. Proceedings of the natural academy of sciences.* **98:** 10493-10498.

Vankan, J.A.L., van den Akerveken, G.F.J.M., de Wit, P.J.G.M. 1991. Cloning and characterisation of cDNA of avirulence gene *AVR9* of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mould. *Molecuar Plant-Microbe interactions.* **4:** 52-59.

Warren, R.F., Henk, A., Mowrey, P., Holub, E., Innes, R.W. 1998. A mutation within the leucine-rich repeat domain of the *Arabidopsis* disease resistance genes RPS5 partially supresses multiple bacterial and downy mildew resistance genes. *Plant Cell.* **10:**1439-1452.

Westerink, N., Roth, R., van den Burg, H.A., de Wit, P.J.G.M, Joosten, M.H.A.J. 2002. The Avr4 elicitor protein of Cladosporium fulvum binds to fungal components with high affinity. *Molecular Plant-Microbe Interactions.* **15**: 1219-1227.

Wulff, B.B.H., Thomas, C.M., Smoker, M., Grant, M., Jones, J.D.G. 2001. Domain swapping and gene shuffling identify sequences required for induction of an Avr-dependent hypersensitive response by the tomato Cf-4 and Cf-9 proteins. *Plant cell.* **13:** 255-272.

Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M., Turner, G.J. 2001. Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by *RPW8. Science.* **291:** 118-120.

Xing, T., Higgins, V.J., Blumwald, E. 1996. Regulation of plant defense response to fungal pathogens: two types of protein kinases in the reversible phosphorylation of the host plasma membrane H+ ATPase. *The Plant Cell.* **8:** 555-564.

Xing, T., Higgins, V.J., Blumwald, E. 1997a. Identification of G proteins mediating fungal elicitor induced dephosphorylation of host plasma membrane H+ ATPase. *Journal of Experimental Botany*. **48:** 229-237.

Xing, T., Higgins, V.J., Blumwald, E. 1997b. Race specific elicitors of *Cladosporium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells. *Plant Cell.* **9:** 249-259.

Yoshida, S., Uemura, M., Niki, T., Sakai, A., Gusta, L.V. 1983. Partition of membrane particles in aqueous two-polymer phase system and its practical use for purification of plasma membranes from plants. *Plant Physiology.* **72**: 105-114.

Zambryski, P.C. 1992. Chronicles from the Agrobacterium-plant-cell-DNA transfer story. *Annual Review of Plant Physiology.* **43**: 465-490

Zhou, J., Loh, Y., Bressan, R.A., Martin, G.B. 1995. The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell.* **83:** 925-935.

Zhou, J., Tang, X., Martin, G.B. 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis related genes. *The EMBO Journal.* **16:** 3207-3218.

Zou, H., Henzel, W.J., Lui, X., Lutsch, A., Wang, X. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell.* **90:** 405-413.

Zupan, J.R., Zambryski, P. 1997. The *Agrobacterium* DNA transfer complex. *Critical Reviews in Plant Sciences.* **16**: 279-295.

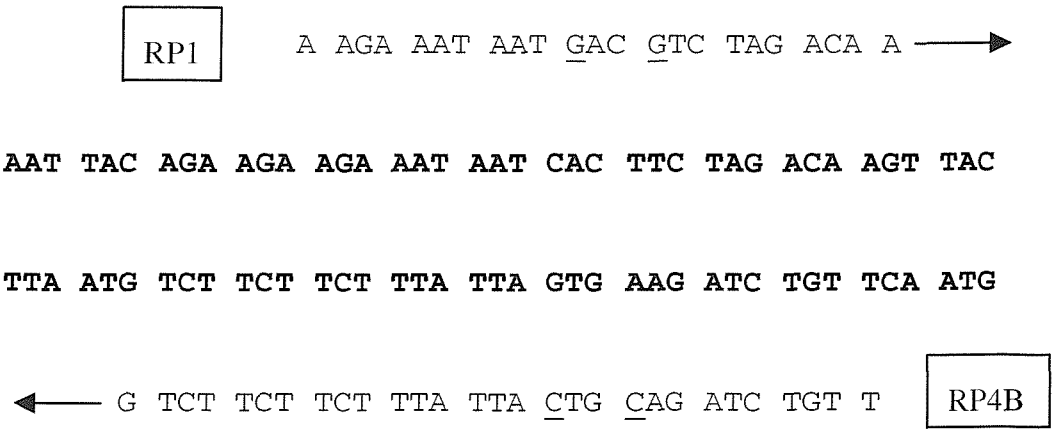
Zupan, J.R., Ward, D., Zambryski, P. 1998. Assembly of the VirB transport complex for DNA transfer from *Agarobacterium tumefaciens* to plant cells. *Current opinion in micribiology.* **1:** 649-65.

Appendix I

Designing primers

To introduce an *Aat*II restriction site into the C terminal end of *Cf-2*, the mutagenic primers RP1 and RP4B were designed. They follow the sequence of the *Cf-2* gene, in the place where the *Aat*II site is to be introduced there are two mis-matches. RP1 and RP4B were used with either the universal forward or universal reverse primers, depending upon the orientation of the insert in the vector (figure one).

Figure one. An alignment of the two mutagenic primers RP1 and RP4B with the *Cf-2* sequence.



The bold text indicates the *Cf-2* sequence. Underlined text denotes the bases, which do not match the template sequence, these changes introduce an *Aat*II restriction site (GAC GTC).

The two mis-matches greatly reduces the annealing temperature that could be used so; relatively lengthy primers were designed to counteract this.

PCR cycle employed:

94^0C for 2 minutes

94^0C for 30 seconds

40^0C (RP1), 42^0C (RP4B) for 30 seconds

72^0C for 1 minute

} 30 cycles

72^0C for 5minutes

14^0C hold

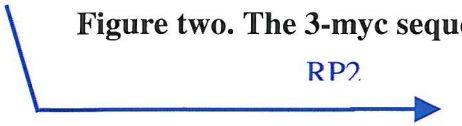
The primers RP2 and RP3 were designed amplify the 3-myc sequence from the plasmid LB2339 (figure two), so it could be inserted into the *Cf-2* sequence (Dixon, unpublished). The primers were designed to introduce an *Aat*II site at either end of the sequence. When designing these primers it was important to consider the restriction efficiency when the restriction site is at the end of a linearised piece of DNA like a PCR product. For >90% cleavage efficiency an *Aat*II restriction site should have at least 2 bases either side of the sequence (New England Biolabs catalogue). A second important consideration that the restriction sites had to follow the correct open reading frame. The primer sequences are as follows:

RP2 5' ATG (**GAC GTC**) GGT GAA CAA AAG TTG 3'

***Aat*II**

RP3 5' ATG (**GAC GTC**) TCC GTT CAA GTC TTC TTC 3'

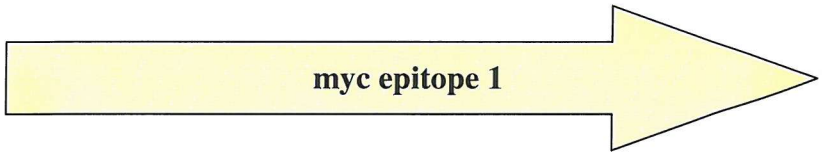
Figure two. The 3-myc sequence, and the alignment of the primers RP2 and 3.



GGT GAA CAA AAG TTG ATT TCT GAA GAA GAT TTG AAC GCT

CCA CTT GTT TTC AAC TAA AGA CTT CTT CTA AAC TTG CGA

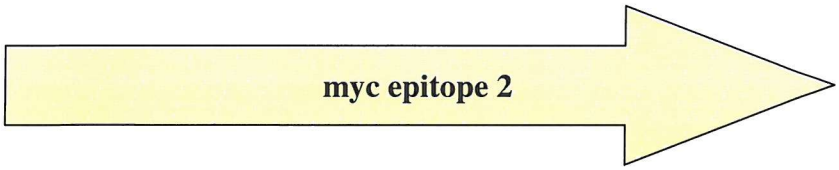
Gly Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly



GAA CAA AAG CTA ATC TCC GAG GAA GAC TTG AAC GGT

CTT GTT TTC GAT TAG AGG CTC CTT CTG AAC TTG CCA

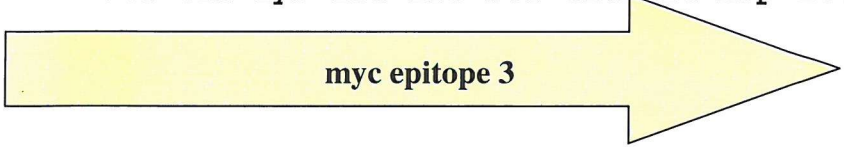
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly



CAA CAA AA TTA ATC TCA GAA GAA GAC TTG AA

GTT GTT TTT AAT TAG AGT CTT CTT CTG AAC TT

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn



The annealing temperature is calculated using only the primers that anneal to the DNA sequence (i.e. only those to the 3' side of the *Aat*II resriction site). The presence of bases in the primer that do not anneal to the template DNA have the effect of reducing the optimal annealing temperature. The PCR cycle used with the primers RP2 and RP3 is as follows:

94^0C for 2 minutes

94^0C for 30 seconds

38^0C for 30 seconds

72^0C for 30 seconds

35 cycles

72^0C for 5 minutes

14^0C hold

As with the RP2 and 3 primers RP5 and RP6 are designed to introduce restriction sites at either end of the stretch of DNA, which they are designed to amplify. RP5 and 6 were designed to amplify the DNA encoding the cytoplasmic tail of Cf-2. They were designed to fit the open reading frame of the *Cf-2* and pGEX-5x-1 vector sequence. The two restriction sites chosen in the multiple cloning site of the pGex vector were *Xho*I and *Eco*RI. The *Eco*RI site was introduced at the N terminal end of the insert by RP5 and the *Xho*I site at the C termini by RP6. For >90% cleavage to occur at an *Eco*RI site there needs to be only one base either side of the sequence (New England Biolabs catalogue). For >75% cleavage efficiency at an *Xho*I site there has to be at least 3 bases either side of the site (New England Biolabs catalogue). The primer sequences were as follows:

RP5 5' CG(**G AAT TC**)G GAA ATC TAA GAT GGC TT 3'

(*Eco*RI)

RP6 5' CCG (**CTC GAG**) CTA GAA GTG ATT ATT TCT TCT 3'

(*Xho*I)

The PCR cycle used:

94^0C for 2 minutes

94^0C for 30 seconds

48^0C for 30 seconds } 35

72^0C for 30 seconds

72^0C for 5 minutes

14^0C hold

To ampllify the most variable regions the primers V1F and V2R2 (Dixon, unpublished) were employed. The product of which was from base 2110 (V1F) to base 3719 (V2R2) (1.69kb) of *Cf-2*. The primer sequences are as follows;

V1F 5' TTC AGA TCA TCC GCA TAT TTC 3'

V2R2 5' GTT TCT CGG CAT ATA CAA CAC 3'

The PCR cycle used:

94^0C for 2 minutes

35 cycles {

94^0C for 30 seconds

$52\text{-}58^0$C for 30 seconds (depended upon the PCR machine and exact DNA that was used)

}

72^0C for 1 minute 30 seconds

72^0C for 5 minutes

14^0C hold.

The primers CF2R1 and MD20 were used to amplify the conserved region of *Cf-2*. They amplify a fragment from base 3872 (MD20) to 5285 (CF2R1) (1413bp) of *Cf-2*. The primer sequences are as follows;

MD20 5' GGG AGC AAT ACC ACA ATG TTT TGG 3'

CF2R15' AGC AAA TCC TTT ACA CCA GCG 3'

MD8 is another primer used in combination with CF2R1 to produce a 330bp fragment of the C terminal region of the conserved region of *Cf-2*.

PCR primer sequence;

MD8 5' GCA AAG GAG AAA GAA GCA GCG AGG 3'

The same PCR cycle is used, except the extension time is reduced to 30 seconds.

The PCR cycle used

94^0C for 2 minutes

35 cycles {

94^0C for 30 seconds

54^0C for 30 seconds

72^0C for 1 minute 30 seconds

}

72^0C for 5 minutes

14^0C hold.

Miscellaneous primers

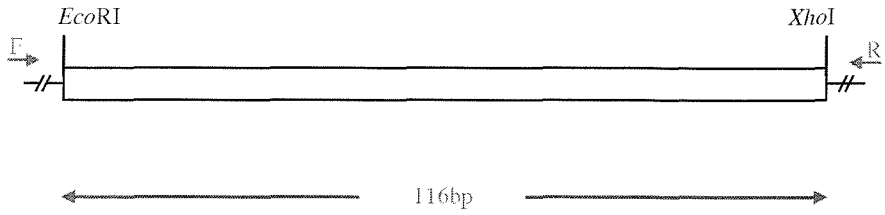
The Primer V3F (Dixon, unpublished) was used in conjunction with V2R2 for the production a Southern and Northern probe for *Cf-2*.

V3F 5' ACA TGT GAG AGA AGA CAT TAC G

The primer Cf5F1 (Dixon, unpublished) was designed for amplification of products from *Cf-5* but can be used on *Cf-2* as there is an identical match. This primer amplifies from base 4655.

Cf5F1 5' TCA AGG ACC TCA ATT CCG

Appendix II



A schematic representation of pRP003. The 116bp insert was produced by PCR using the primers RP5 and RP6 on minimal Cf-2 (Dixon *et al.*, 1996) template DNA. The primers introduced *Eco*RI and *XhoI* sites at either end of the Cf-2 tail encoding sequence. The PCR fragment was cloned into the vector pGex-5x-1 to produce a GST fusion construct. (Not to scale)