Molecular aspects of resistance to late blight disease in potato (solanum tuberosum L.)

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Molecular Aspects of Resistance to Late Blight Disease in Potato (*Solanum tuberosum* L.)

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ABSTRACT

Diseases caused by micro-organisms are still a major threat to the agro-industry worldwide. Diseases not only have negative effects on crop yields, but also they can affect the quality of crops post-harvest. Genetic engineering is one of several strategies that have been developed to control plant diseases and to enhance plant disease resistance to pathogens. Although some genetic strategies have provided plants with enhanced disease resistance, some pathogens can easily overcome this resistance by rapid evolution resulting in a lack of durability in the field.

The oomycete *Phytophthora infestans*, the causal pathogen of late blight disease of potato, is an example of a crop pathogen that causes a major problem in one of the most important crops worldwide. Many efforts have been made trying to control this pathogen including chemical controls and genetic engineering, but unfortunately it remains a severe problem and the control measures are rarely very successful. Due to the complexity of this pathogen, and to limit the need for chemical control, breeding programmes to incorporate durable forms of genetic resistance are crucially needed. Although, this type of resistance is believed to be effective against all known races of P. infestans and provide in additional some level of general resistance, until now the genetic bases of this type of resistance is still unknown and the molecular mechanisms poorly understood.

This project set out to isolate and identify gene sequences that are induced during the compatible interaction between cultured potato plants and *P. infestans*, specifically those leading to the establishment of durable resistance.

It was demonstrated that the potato variety Stirling is capable of developing this type of resistance as judged by the development of resistant shoots during the interaction with *Phytophthora*. These shoots showed very strong resistance not only to *Phytophthora* but also to other potato pathogens (*R. solani* and *F. sulphureum*) even after two generations of culturing the plants in the absence of the pathogen.

The fast production of ROS and the tight deposition of callose surrounding the hypersensitive cells, which deprive the pathogen of nutrients and limit pathogen growth to a small region of the plant, may be important factors in the success of the durable plants in defending themselves against the pathogen attack.

cDNA subtracted libraries were constructed from Stirling plants treated with *P. infestans* and untreated control plants. In order to collect plant samples free of pathogen and cover the whole period from the start of infection until the establishment of durable resistance a preliminary testing of the progress of infection was carried out. The suppression subtractive hybridisation was employed successfully to enrich the low abundance differentially expressed sequences induced during the potato-*P. infestans* interactions.

528 clones were randomly selected, grown and used in a screening exercise. Based on the hybridisation with the forward subtracted probe but not with the reverse subtracted probe, a selection of at least sixty of these clones have been sequenced and subjected to bioinformatics analyses. A database search of the sequenced cDNAs revealed that these sequences have homology to diverse classes of genes, and thus were organised into categories according to their putative functions. Some sequences may warrant inclusion in more than one category. As expected the largest category (37 sequences; compress 63%) was stress- and defence-response related sequences. Among these sequences, six were homologous to ESTs isolated in similar stress situations (elicitor or fungal treated potato or tomato plants) but with unknown function. Moreover, 9 sequences categorised as unknown function as they had homology to gene sequences published in the databases but with unknown function. Among the sixty sequenced cDNAs, eighteen (30%) were novel potato gene sequences.

Preliminary attempts were carried out to describe the gene expression associated with the establishment of resistance to *P. infestans* using appropriate gene expression assays (DNA array technology), but unfortunately the results were unsatisfactory and the approach requires further development.

DECLARATION

I confirm that no part of the material presented has previously been submitted for a degree in this or in any other university. If material has been generated through joint work, my independent contribution has been clearly indicated. In all other cases material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

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ABBREVIATIONS

µg	microgram
µl	microliter
Avr-gene	avirulence gene
C_2H_4	ethylene
cDNA	complementry deoxyribonucleic acid
Cf genes	*Cladosporium fulvum* resistance genes
Cy3	Cyanine 3
Cy5	Cyanine 5
DAB	3,3'-diaminobenzidine
DEPC	diethyl pyrocarbonate
DF	dilution factor
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid
ESTs	expressed sequence tags
EtBr	ethidium bromide
g	gram
GM	Genetic Modified
H_2O_2	hydrogen peroxide
HR	hypersensitive response
HRGPs	Hydroxyproline-rich glycoproteins
L	litre
LB-medium	Luria-Bertani – medium
LRR	leucine-rich repeat
M	marker
MES	2-(n-morpholino) ethanesulphonic acid
mRNA	messenger ribonucleic acid
MS-medium	Murashige and Skoog – medium
NADH	nicotinamide adinine dinucleotide
NBS	nucleotide-binding site
NBT	nitroblue tetrazolium

O_2^-	superoxide anion
°C	degree centigrade
OH^-	hydroxyl radical
PA	phosphatidic acid
PAL	phenylalanine ammonialyase
PCD	programmed cell death
PCR	polymerase chain reaction
PGI	Phytophthora Genome Initiative
PGPR	plant growth promoting rhizobacteria
PK	protein kinase
poly (A^+)	RNA polyadenylic ribonucleic acid
PPO	Polyphenol oxidase
PR- proteins	pathogenesis-related proteins
R-genes	resistance genes
RIP	ribosome inactivating proteins
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	round per minute
rRNA	ribosomal ribonucleic acid
SA	salicylic acid
SAR	systemic acquired resistance
SCRI	Scottish Crop Research Institute
SOD	superoxide dismutase
ssDNA	single stranded deoxyribonucleic acid
Taq	Thermus aquaticus
TM	transmembrane region
Tris	tris (hydroxymethyl) aminoethane
UV	ultraviolet
v/v	volume/volume
v/w	volume/weight
var	variety
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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1 Introduction

1.1 An overview of plant-pathogen interactions and plant defence mechanisms

Plants constitute the largest and most important group of autotrophic life forms on the earth. Their abundant organic material serves as a nutritional source for all heterotrophic organisms including animals, insects, and microbes. Plants, like animals, are continually exposed to pathogen attack. The range of pathogenic organisms that attack plants is diverse and each has a unique mode of pathogenicity. Despite the vast array of potential pathogens, plants have evolved natural resistance to microbial attack (non-host resistance). Therefore, resistance is the rule and susceptibility is the exception (Hammond-Kosack and Jones, 1996). Non-host resistance is the consequence of either the inability of a parasite to infect a plant or the ability of a plant to successfully recognise a parasite and rapidly activate its defence mechanisms, leading to the resistance phenotype (incompatible interaction). In contrast, relatively few pathogens have evolved the means to successfully colonise a plant host and establish basic compatibility by escaping recognition, and thus avoiding induction of host defence responses, by damaging or weakening the plant cells with toxins, or by inhibiting host defence mechanisms.

Generally, pathogens deploy one of three main strategies to attack plants: necrotrophy, biotrophy, or hemibiotrophy. Necrotrophs are pathogens that produce toxic enzymes and metabolites that kill the cells directly upon invasion then metabolise their contents. Some have a broad host range, and cell death is often induced by toxins and/or enzymes (Walton, 1996). Other necrotrophs produce host-selective toxins that are effective over a very narrow range of plant species. One of the best examples of host-selective toxicity

is that of T toxin from *Cochliobolus heterostrophus* race T, which binds to a protein of the inner mitochondrial membrane of the host (Levings and Siedow, 1992). In contrast, biotrophs and hemibiotrophs initially feed on plants parasitically, keeping the cells in infected plant tissue alive for a significant fraction of the pathogen's life cycle; this is followed by a more necrotrophic existence during the later stages of infection by hemibiotrophic pathogens. Plant defences must be adapted to combat these three different types of pathogenesis.

Because plants lack a circulatory system and antibodies, they have evolved complex and sophisticated defence systems that are different from the vertebrate immune system to survive a variety of pathogens that attack them. Their modes of defensive action may be to (i) kill the pathogen directly, (ii) block the action of pathogen enzymes required for infection, or (iii) create barriers to pathogen growth. In contrast to animal cells, each plant cell is capable of defending itself by means of a combination of pre-formed and induced defence mechanisms. In spite of the recent focus on inducible defensive responses in plants, there is considerable evidence that pre-formed defences are a major component of non-host resistance. These pre-formed defence lines include cell walls, wax layers and chemical barriers such as preformed peptides, proteins, and non-proteinaceous secondary metabolites which confer general resistance to a wide variety of pathogens (Broekaert *et al.*, 1995; Heath, 2000a; Osbourn, 1996). Plants produce a large number of secondary metabolites, many of which have antifungal activity. Some of these compounds, such as saponins, exist in healthy plants in their biologically active forms (constitutive compounds). Others, such as cyanogenic glycosides and glucosinolates, occur as inactive precursors and are activated in response to pathogen attack or tissue damage. This activation often involves plant enzymes, which are

released, mixed with the precursors which are converted to active forms as a consequence of plant cells breakdown. Compounds belonging to the latter category are still regarded as constitutive because they are derived, immediately, from preexisting precursors rather than complete *de novo* synthesis (Osbourn, 1996). The term "phytoanticipin" has been proposed to distinguish between these preformed antimicrobial compounds and phytoalexins, which are synthesised from remote precursors in response to pathogen attack or environmental stresses, as a consequence of *de novo* synthesis of plant enzymes (Vanetten *et al.*, 1994).

If a pathogen overcomes these first lines of plant defences (i.e. constitutive defence lines), there is a second line of weapons, which is mounted by proteins encoded by specific resistance plant genes (*R*-genes). At this level at least three basically different resistance mechanisms can be recognised including:

The *R*-gene product mediates specific recognition of a complementary product of the corresponding pathogen avirulence gene (*Avr*-gene) (the gene-for-gene model) as proposed by Flor in the 1940s (Flor, 1947). In resistant plants, the specific recognition between a pathogen *Avr*-gene product, the so-called elicitor, and a host receptor, the product of the *R*-gene, causes induction of various defence responses often involving ion fluxes, generation of reactive oxygen species (ROS), protein phosphorylation and other signals (Hammond-Kosack and Jones, 1996; Somssich and Hahlbrock, 1998). These signals subsequently trigger transcription of plant defence genes encoding proteins such as pathogenesis-related (PR) proteins (e.g. proteinase inhibitors, PR-1, chitinases and 1,3-β-glucanases), glutathione S-transferases and enzymes involved in secondary metabolism. In addition, plant cells, which are in the immediate vicinity of, or in direct contact with the invading pathogen die. This phenomenon is called the

hypersensitive response (HR), which is a main feature of gene-for-gene-based resistance (see section 1.2 and the subsections therein).

The *R*-gene product inactivates a toxin, which is produced by the necrotrophic pathogens to kill the plant cells directly upon invasion then metabolise their content or to inhibit the induction of active defence responses. The *Hm1* gene from maize, the first *R*-gene to be isolated, is the best example of this mechanism. The *Hm1* gene (*Helminthosporium maydis*; race1) encodes a NADPH-dependent HC-toxin reductase that detoxify the HC-toxin produced by the leaf spot fungus *Cochliobolus carbonum*, which induces disease symptoms on susceptible plants by inhibiting histone deacetylase (Johal and Briggs, 1992; Walton, 1996).

The *R*-gene product primes the plant defence responses. An example of this resistant mechanism is the *Mlo* gene of barley, which provides resistance against the fungal pathogen, *Erysiphe graminis f. sp. hordei*. The *Mlo* gene product might act as a negative regulator of plant cell death and other plant defence responses. Mutagenesis of susceptible plants carrying *Mlo* alleles, confers a leaf lesion phenotype and a broad spectrum resistance against a broad spectrum of fungal isolates (Buschges *et al.*, 1997).

1.2 Plant active defence mechanisms

As mentioned earlier, plants have evolved a wide array of defence mechanisms against pathogen attack. Resistance or susceptibility to a particular pathogen depends on various factors, including pathogen recognition, activation of host plant signal transduction pathways and induction of active defence molecules. These defence mechanisms can be very complex and sophisticated (see figure1-1; Hammond-Kosack and Jones, 1996), so inevitably in this short introduction I will only discuss some of

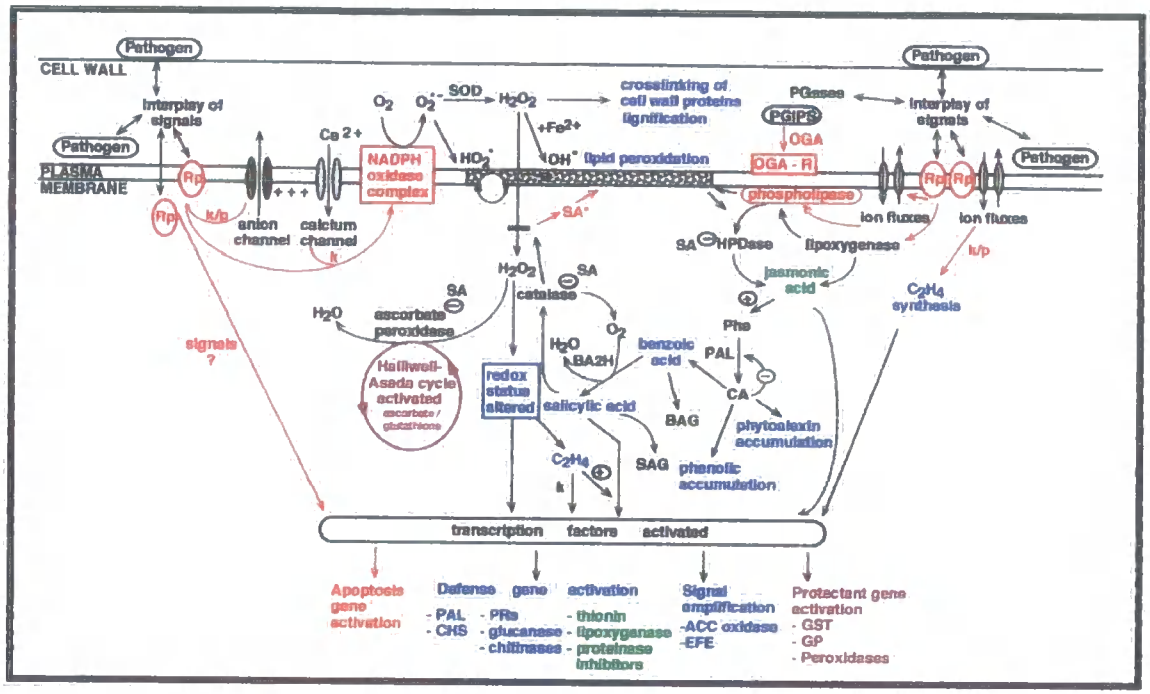


Figure 1-1 Complexity of signalling events controlling activation of defence responses. Plant receptor proteins (Rp) act as receptor to detect the pathogen *Avr*-dependent signal and thus initiate downstream signalling. The immediately downstream signalling events involve kinases, phosphatases, G-proteins, and ion fluxes. Several distinct and rapidly activated outcomes are recognized, including the production of reactive oxygen species, direct induction of defence gene transcription, jasmonic acid biosynthesis, and/or ethylene biosynthesis. Once the earliest defence responses have been activated, the complexity of the biochemical pathways within the responding cell increase enormously as new signal molecules are generated. The alterations of cellular redox status and/or cellular damage will activate preformed cell protection mechanisms and induce genes encoding various cell protectants. Considerable amplification of specific defence responses then occurs, via either positive feedback or signal cross-talk.

(+) indicates positive and (-) indicates negative interactions. Components and arrows indicated in red are only postulated to be present in plant cells, whereas those in blue indicate known plant defence responses; green indicates plant defence responses also activated by JA, and purple indicates plant protection mechanisms, ACC oxidase, 1-aminocyclopropane-l-carboxylate oxidase; BAG, benzoic acid glucoside; BA2H, benzoic acid-2-hydroxylase; CA, cinnamic acid; CHS, chalcone synthase; EFE, ethylene-forming enzyme; $HO_2^{\cdot}$, hydroperoxyl radical; HPDase, hydroxyperoxide dehydrase; GP, glutathione peroxidase; GST, glutathione S-transferase; k, kinase; $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxylradical; OGA and OGA-R, oligalacturonide fragments and receptor; p, phosphatase; PAL, phenylalanine ammonia-lyase; PGases, polygalacturonases; PUPS, plant polygalacturonic acid inhibitor proteins; Phe, phenylalanine; PR, pathogenesis related; Rp, plant receptor protein; SA and SAG, salicylic acid and salicylic acid glucoside; $SA^{\cdot}$, and SOD, superoxide dismutase. (Adapted from Hammond-Kosack and Jones, 1996).

these response mechanisms thought to be involved in resistance. Several recent published reviews, which cover many of these defence mechanisms in more detail, can be consulted for further information (e.g. Dangl and Jones, 2001; Heath, 2000b; Morris, 2001; Romeis, 2001; Takken and Joosten, 2000).

1.2.1 Hypersensitive response (HR)

The terms "hypersensitive response" (HR) and "hypersensitivity" describe the localized and rapid death of one or a few host plant cells in response to invasion by an avirulent pathogen. In plant-pathogen interactions, if a plant contains a disease-resistance gene (*R*-gene) product (receptor) that can recognize the matching avirulence gene (*Avr*-gene) product (elicitor) from a pathogen, the HR is triggered and the pathogen is contained within the infected tissue and the result is a defence response (incompatible interaction). Whereas, in the absence of a functional resistance gene or an avirulence gene product, no recognition occurs and the interaction between plant and pathogen results in disease (compatible interaction).

Recent evidence suggests that the HR responses can occur in both incompatible and compatible plant-pathogen interactions by two mechanisms (i) as a result of a switch in cell metabolism to biochemical pathways that produce an array of compounds or free radicals that are toxic to both the pathogen and the plant cell, thus causing the latter to die rapidly, or (ii) it may be the outcome of pathogen recognition activating an internal pathway for plant cell suicide - programmed cell death (PCD) (Dangl *et al.*, 1996; Mittler *et al.*, 1997; Mittler and Lam, 1996).

The hypersensitive response has been described as the most powerful defence system that plants have. It is a highly intensive, complex defence mechanism that involves a

rapid loss of membrane integrity in the infected host cells, local cell death, local accumulation of large quantities of phenolic compounds and cell-wall fortification in cells surrounding the area of cell death, and, more importantly, distal activation of general defence in uninfected parts of the plant, which prevents further infections on parts of the plant distant from the infection site, the so-called systemic acquired resistance (SAR) (see section 1.2.5) (Goodman and Novacky, 1994; Kamoun, 2001).

The HR effect can be phenotypically diverse, depending on the genotypes of the interacting plant and pathogen, ranging from HR in a single or a few host plant cells to more spreading necrotic areas limiting the pathogen from further growth (Kamoun, 2001). The HR has been proposed to play a central role in disease resistance. It plays an effective defence against biotrophic pathogens that require living host cells for nutrition, because plant cell death strips the pathogen of access to further nutrients thereby confining pathogen growth to a small region of the plant. The role of HR is less clear in interactions involving hemibiotrophic and necrotrophic pathogens, because these pathogens can obtain nutrients from dead plant cells. Nevertheless, cellular degradation may lead to the release of harmful preformed substances that are stored in the vacuole (Osbourn, 1996). On the other hand, the levels of induced phytoalexins (see section 1.2.4), which are usually rapidly accumulated in plant cells during the interaction with pathogens may accumulate to inhibitory concentrations because they are no longer metabolized.

Although the HR is a common feature of many resistance reactions, several studies have demonstrated that HR is not an essential component in disease resistance. For example, under high humidity, the Cf genes of tomato confer resistance to particular pathotypes of the fungus *Cladosporium fulvum* without invoking a visible HR (Hammond-Kosack

et al., 1996). By contrast, induction of cell death may be used by necrotrophic pathogens for their pathogenicity to aid in the invasion of the plant. For example, (Govrin and Levine, 2000) demonstrated that hypersensitive cell death does not protect *Arabidopsis* plants against infection by the necrotrophic fungal pathogens *Botrytis cinerea* that attacks over 200 different plant species and *Sclerotinia sclerotiorum*. By contrast, *B. cinerea* triggered HR, which facilitated its colonization of plants.

Although the role of cell death in plant defence is not clear in all cases, it does seem to assist in slowing down the pathogen growth within the host cells. A better understanding of the control of cell death during various types of plant–pathogen interactions should help elucidate the role that this process plays in either host resistance or pathogen proliferation.

1.2.2 Reactive oxygen species (ROS) and oxidative enzymes

The production of reactive oxygen species (ROS), often referred to as the 'oxidative burst', plays a key role in plant defence. The oxidative burst is one of the earliest aspects of plant defence responses in the incompatible interaction with a pathogen. It has been suggested that the ROS produced in the oxidative burst could serve not only as protectants against invading pathogen, but could also be the signals activating further plant defence reactions, including the HR of infected cells (e.g. Baker and Orlandi, 1995; Wojtaszek, 1997a). The oxidation of phenolic compounds as cells undergo the HR suggests that there is an increase in phenol oxidizing enzymes activity and the production of reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide anions (O_2^-). These ROS result from the successive one-electron steps in the reduction of molecular oxygen (see figure 1-2) (Goodman and

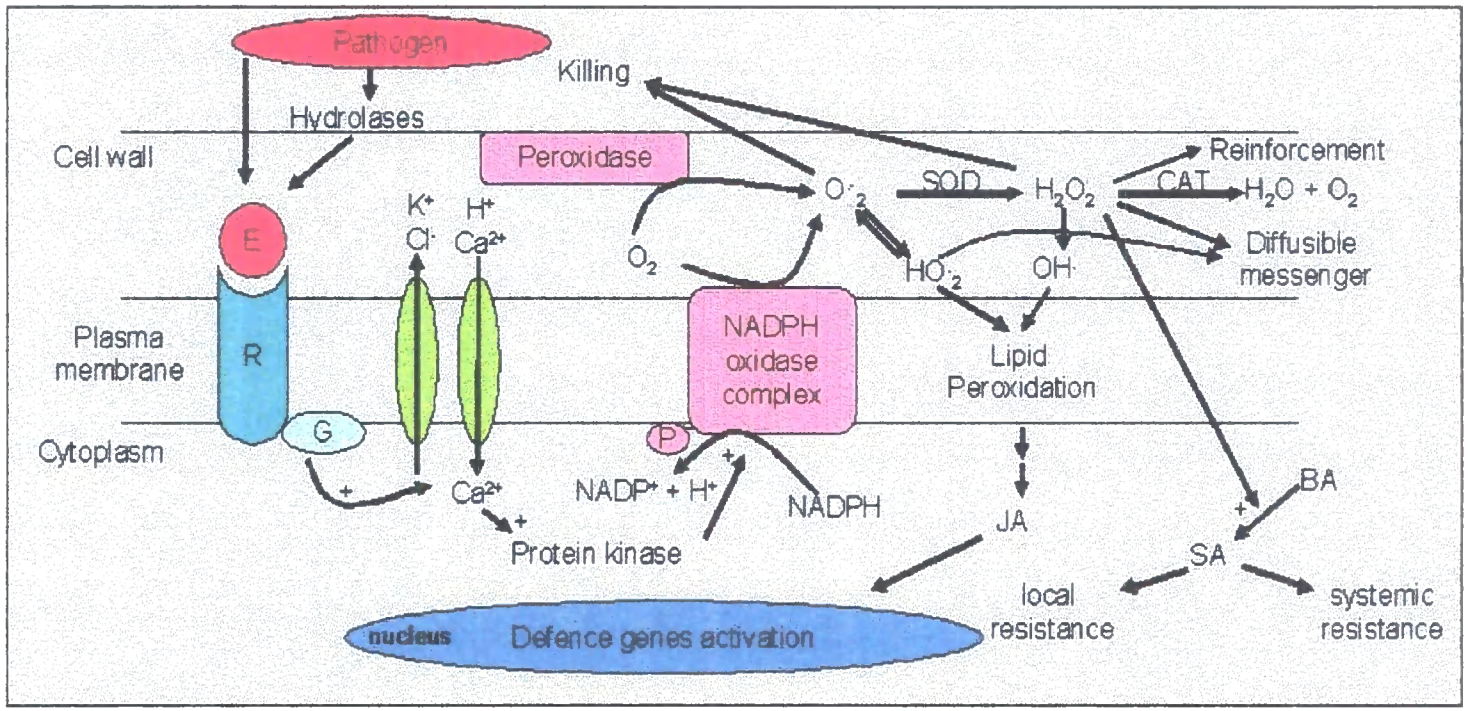


Figure 1-2 Possible origin of ROS generation from molecular oxygen and roles of ROS in plant defence: binding of elicitor (E) to a plasma membrane receptor (R) initiates a signalling cascade via G-proteins (G) leading to ion channels opening, and activation of a protein kinase that activates a membrane-bound NADPH oxidase by phosphorylation. Alternatively, alkalinization of the extra cellular matrix (apoplast and cell wall) as a result of ion movement activates a pH-dependent cell wall peroxidase. These events lead to the synthesis of superoxide anions (O^-_2), which dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) and then to H_2O via catalase (CAT) activity. (Figure prepared for this thesis; refer to the text for more details about the roles of ROS in disease resistance).

Novacky, 1994).

Several possible mechanisms have been proposed for the generation of ROS during the plant defence response including: (i) the activation of NADPH oxidase, which reduces molecular oxygen to a superoxide anions O_2^- that rapidly dismutate via superoxide dismutase (SOD), to hydrogen peroxide (H_2O_2). (ii) the pH-dependent cell wall peroxidases that produce H_2O_2 during the alkalinisation of the apoplast (Bolwell *et al.*, 1995). (iii) the germin-like oxalate oxidase system that can produce H_2O_2 from oxalic acid (Zhang *et al.*, 1995); although this system seems to be limited to interactions with pathogens involving cereals, some evidence suggested the present of germin-like protein in dicotyledoneouns plants (Wojtaszek, 1997b), (IV) Copper-containing amine oxidases can also catalyse the oxidation of various amines and polyamines, compounds that form during wounding and pathogenesis yielding NH_3 and H_2O_2 (Bolwell and Wojtaszek, 1997), and (v) lipoxygenase, which is also proposed as a possible source of ROS in the oxidative burst, but in most systems studied ROS production have been shown to precede lipoxygenase activity (Baker and Orlandi, 1995). The first two mechanisms have received the most attention as ROS generating systems during the pathogen infection.

Several roles for ROS in plant defence responses during pathogen infection have been proposed: as direct antimicrobial agents against the invading pathogen; as agents for strengthening of plant cell walls by cross-linking structural proteins in the cell wall to limit pathogen invasion; as activators of defence genes and as stimulators of the hypersensitive response (HR), salicylic acid production, and systemic acquired resistance (SAR) (Guo and Li, 2000; Vranova *et al.*, 2002) (see figure 1-2). For example, Peng and Kuc (1992) demonstrated that the concentration of H_2O_2 produced

by elicited plants is sufficient to significantly retard microbial growth, and in addition H_2O_2 has been found to participate in reactions that strengthen the structure of plant cell walls by cross-linking soluble proteins into the matrix of the plant cell wall. Bolwell *et al.*, 1995 have indicated that H_2O_2 is essential for the formation of lignin polymer precursors via peroxidase activity. It has also been demonstrated that hydroxyproline and proline-rich cell wall glycoproteins were rapidly oxidatively cross-linked in cell walls after fungal elicitor treatment. This protein cross-linking rapidly makes the plant cell wall more resistant to microbial penetration and enzymatic degradation (Brisson *et al.*, 1994).

H_2O_2 has also been shown to activate some protection mechanisms. For example, it has been reported that H_2O_2 from the oxidative burst not only promotes the cross-linking of cell wall structural proteins, but also functions as a local trigger of programmed death in challenged cells and as a diffusible signal for the induction of genes encoding cell-protectant enzymes such as glutathione peroxidase and glutathione S-transferase in adjacent cells. Glutathione peroxidase and other peroxidative enzymes destroy ROS and block oxidant-mediated programmed cell death (PCD). While glutathione S-transferase detoxifies lipid hydroperoxides generated by ROS. Levine *et al.*, 1994 have shown that inoculation of soybean cells with avirulent *P. syringae* pv. *Glycinea* induces cell death only in the challenged cells and not in adjacent cells separated from the challenged cells by two dialysis membranes. As little as 2mM H_2O_2 was found to induce transcription of glutathione S-transferase and glutathione peroxidase in the second, uninfected set of cells. Since both enzymes participate in the detoxification of H_2O_2, their expression may help host plant cells to escape the damage effect of the generated ROS.

A signalling role for some ROS has also been proposed. Leon *et al.*, (1995) have demonstrated that infiltration of tobacco leaves with H_2O_2 increased benzoic acid-2-hydroxylase activity, an enzyme that is required for salicylic acid (SA) biosynthesis. Salicylic acid is an important signalling molecule involved in both locally and systemically induced disease resistance responses (Metraux, 2001). Furthermore, the lipid peroxides formed as a consequence of action of ROS on plasma membrane fatty acid may also have a direct signalling role in SA accumulation (Leon *et al.*, 1995).

Wu *et al.*, (1995) provided convincing evidence that H_2O_2 generation is involved in conferring disease resistance. The constitutive expression of an H_2O_2-generating glucose oxidase in transgenic potato plants resulted in good resistance to the bacterial soft rot pathogen *Erwinia carotovora* sp *carotovora* and enhanced resistance to the fungal pathogen *Pytophthora infestans*. More recently, (Hückelhoven *et al.*, 2001) reported that the non-host resistance of barley to *Blumeria graminis* f.sp. *tritici* is associated with H_2O_2 accumulation in papillae that form as potential barriers at the point of attempted infection by fungal hyphae (see section 1.2.3).

Expression of resistance is often accompanied by the activation and/or *de novo* synthesis of the phenol-oxidizing enzymes peroxidase and polyphenoloxidase and the lipid peroxidizing enzyme lipoxygenase (Goodman and Novacky, 1994). Peroxidase activity often increases in response to infection and this enzyme may function in defence through production of antimicrobial quantities of hydrogen peroxide as well as in more traditional cell wall lignification and crosslinking (Rasmussen *et al.*, 1995). Phenoloxidases may also contribute to plant defence. For example, (Lazarovits and Ward, 1982) indicated that an increase in activity (but apparently not in *de novo* synthesis) of phenoloxidase has been correlated with the onset of the hypersensitive

response in soybean and the interaction of phenoloxidase with endogenous phenols in the dying cell could be a major cause of the brown pigmentations observed in the responding cells. Lipoxygenase, may also contribute to the hypersensitive response via disruption of cell membrane lipids (Goodman and Novacky, 1994), and in a direct defence response through the formation of toxic lipid oxidation products such as trans-2-hexenal and cis-3-hexenol, which appeared to be highly bactericidal (Croft *et al.*, 1993).

1.2.3 Cell wall modification

The first barrier that most pathogens encounter before establishing an infection is the physical barriers that protect all plant cells, namely the cuticle and the cell wall. Most pathogens, particularly bacteria and viruses, depend on wounds or natural openings, such as stomata, to enter the plant cells. Other pathogens such as fungi have developed sophisticated ways to physically penetrate the cuticle with an appressorium form, which is an infection peg that extends into the plant cells. Others secrete extracellular hydrolytic enzymes such as cutinase, an esterase specific for cutin, which has been proposed as an essential enzyme for some pathogens to infect a plant (Schafer, 1994). After breaching the cuticle, fungal and bacterial pathogens secrete a large number of hydrolytic enzymes capable of digesting plant cell wall polymers. These enzymes include cellulases, pectinases, xylanases and proteases (Salmond, 1994; Walton, 1994). It is not surprising that plants have evolved means to perceive and respond defensively to the physical or chemical events associated with such penetration. Plant cells may respond quickly to infection by modifying cell walls in such a way that the walls become more effective barriers to pathogen ingress into and through tissues. Alterations in the structure of plant cell walls may contribute to resistance, either by stopping

pathogen entry directly or by slowing down the penetration process, thus allowing the plant time to activate further defence mechanisms including *de novo* synthesis of enzymes and antimicrobial compounds.

There are several types of cell wall modification that have been correlated with resistance. The formation of cell wall appositions (papillae) is an example of cell wall modification that occurs rapidly in response to fungal invasion at sites of attempted penetration. Papillae often form immediately beneath the penetration peg and are heterogeneous in composition. They are thought to physically block fungal penetration of host cells. They consist mainly of callose and silicon oxide. Callose deposition is also frequently associated with point of pathogen invasion. An important role for callose in the expression of resistance is supported by the *Arabidopsis lsd* (lesion simulating disease) resistant mutants, which show a stronger deposition of callose at the site of pathogen penetration than susceptible wild-type plants (Dietrich *et al.*, 1994).

Lignification and similar phenolic compound deposition have also been correlated with resistance (Nicholson and Hammerschmidt, 1992). Lignin is formed by polymerization of precursors produced in the phenylpropanoid pathway. The first step in this pathway is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL). PAL provides precursors for lignin and for several other phenylpropanoid-derived secondary plant products involved in resistance. Examples are furanocoumarin and isoflavonoid phytoalexins in parsley and legumes, respectively, as well as salicylic acid (SA) (Ward *et al.*, 1991). These deposits of lignin are often highly localized and appear to block the progression of the fungal hyphae (Stein *et al.*, 1993). If lignification occurs after cell wall penetration, the entire cell may lignify, thus potentially trapping the pathogen within a lignified chamber (Hammerschmidt *et al.*, 1985). Stein *et al.*, (1993) observed

that the same process may also aid in resistance by direct lignification of the pathogen cell walls. Lignification of fungal cell walls makes them more rigid and impermeable, thus hindering further growth of the pathogen as well as reducing the uptake of water and nutrients from the host cells. This has been observed for hyphae of *Colletotrichum orbiculare* in cucumber leaf tissue (Stein *et al.*, 1993). Thus, this lignification process may also function to contain the pathogen in one place until other defences come into effect.

After infection, strengthening of the cell wall can also occur by peroxidase-catalyzed cross-linking of hydroxyproline-rich structural cell wall glycoproteins (Bradley *et al.*, 1992). Hydroxyproline-rich glycoproteins (HRGPs) are thought to play an important role in the organization of primary cell wall architecture and in the initiation of lignin polymerization (Bolwell *et al.*, 1995; Showalter, 1993). Bradley *et al.*, (1992) indicated that the rapid oxidative crosslinking of preformed HRGPs may constitute one of the earliest defence responses associated with the oxidative burst. Quite frequently, more than one type of cell wall modification may occur in a plant actively restricting infection.

In conclusion, modifying the plant cell wall can increase resistance in various ways. For example increasing the mechanical strength of the cell walls may (i) decrease the susceptibility to cell wall degrading enzymes, (ii) constitute a diffusion barrier preventing free nutrient movement (i.e. cytoplasmic contents) to the extracellular pathogen, and therefore help to reduce nutrient availability and starve the pathogen. (iii) retard the diffusion of toxins and degrading enzymes secreted by necrotrophic pathogen to sensitive plant cells. In addition, the low molecular weight phenolic precursors of lignin and the free radicals produced during polymerization reactions themselves might

exert a toxic effect on pathogens, inactivate pathogen enzymes or toxins or, by binding to fungal cell walls, make them more rigid, resistant to hydrolases and impermeable, thus hindering further growth or uptake of water and nutrients.

1.2.4 Phytoalexins

Phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plants as a result of infection or stress. Phytoalexins have been characterised from at least twenty different families including monocots and dicots. Depending on the plant species examined, the groups of compounds identified as phytoalexins include phenylpropanoid derivatives, flavonoid- and isoflavonoid-derived phytoalexins, diterpenes, sesquiterpenes, polyketides and more others. Phytoalexins represent a chemically diverse group of compounds derived from a number of different metabolic pathways. The major biosynthetic pathways, including shikimate, acetate-mevalonate and acetate-malonate, that provide phytoalexin precursors are common in all plants. Phytoalexin precursors can be derived from one of these three biosynthetic pathways or a combination of two or three of them (figure 1-3). For example, polyketide phytoalexins such as 6-methoxymellein and sesquiterpene phytoalexins such as rishitin are derived from the acetate-malonate and acetate-mevalonate pathways, respectively, while phenylpropanoid phytoalexins such as chlorogenic acid is produced from phenylalanine, a product of the shikimic acid pathway. Other phytoalexins, like the pterocarpan pisatin, is derived from products of the shikimic acid and acetate-malonate pathways. Phytoalexins like the kievitone and glyceollins are biosynthesised using precursors from three primary metabolic pathways (the shikimic acid, acetate-malonate, and acetate-mevalonate pathways). The production of phytoalexins after infection

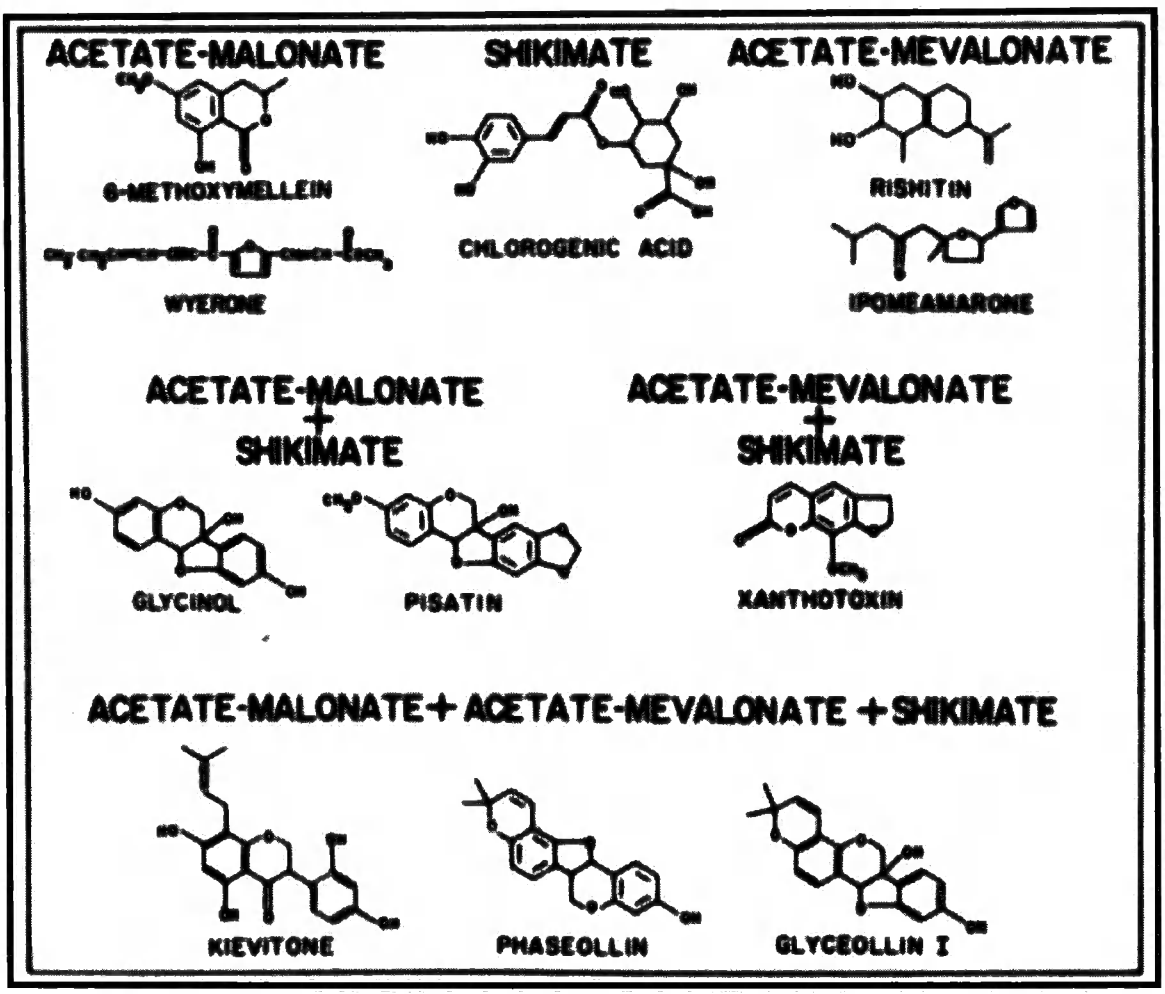


Figure 1-3 Examples of phytoalexins representing a diversity of biosynthetic pathways (Taken from **Kuc, 1995**).

suggests that a product of the pathogen or the host-pathogen interaction is involved in triggering phytoalexin biosynthesis (Hammerschmidt, 1999; Kuc, 1995).

The current definition of phytoalexins does not include any criteria that would allow discrimination between a primary role for phytoalexins in defence versus just a response as a consequences of infection. However, several lines of evidence support a role for phytoalexins in disease resistance. This evidence comprises data documenting: (I) localisation and timing of phytoalexin accumulation at or near the infection site and to a concentration inhibitory to the pathogen; (II) strong positive correlation between the degree of incompatibility and the phytoalexin content followed pathogen challenge of particular cultivars; (III) association of rapid phytoalexin accumulation with resistance genes that condition rapid restriction of pathogen development; (IV) a positive relationship between pathogen virulence and tolerance to phytoalexins; (V) an increase of plant tissue resistance by stimulation of phytoalexin production prior to inoculation (reviewed in Hammerschmidt, 1999; Smith, 1996).

Associated with the synthesis and accumulation of phytoalexin is the induction of genes that involve in the biosynthesis of these compounds. For example infection of legumes with incompatible pathogens, or treatment of tissues with elicitors resulted in the rapid induction of phenylalanine ammonia-lyase and chalcone synthase, which are co-ordinately regulate the phenylpropanoid and flavonoid pathways (Dixon and Paiva, 1995), while in solanaceous plants, genes that regulate terpenoid phytoalexins biosynthesis (e.g. hydroxymethyl gluteryle CoA reductase) were induced (Kuc, 1995).

1.2.5 Systemic acquired resistance

Systemic acquired resistance (SAR), which is characterised by activation of a long-

lasting systemic resistance against a broad-spectrum of pathogens, is one of the most important components of the inducible complex defence resistance mechanisms that plants have developed to defend themselves against pathogens. It was shown that inoculation of plants with a pathogen induced protection against subsequent infections with the same pathogen as well as to other pathogens including fungi, bacteria and viruses. The wide range of pathogen protection and the associated changes in gene expression, distinguish SAR from other disease resistance responses.

Most necrotizing pathogens, which cause tissue necrosis, can induce resistance against a subsequent infection with widely different pathogens. This resistance is expressed locally at the site of pathogen invasion as well as systemically in distal uninfected parts of the plant. The induction of resistance in parts of the plant distant from the site of primary infection is believed to result from the translocation of a systemic signal produced at the site of primary infection, transported though the plant to uninfected tissues. This signal triggers the plant defence responses against further pathogen attack. The defence responses involved in SAR include a combination of physical changes such as cell wall lignification and callose deposition, and induction of various pathogenesis-relate proteins (for reviews see; Mauch-Mani and Metraux, 1998; Metraux, 2001; Ryals *et al.*, 1996 and Sticher *et al.*, 1997).

The importance of salicylic acid (SA), as a signalling molecule in SAR was documented by experiments using transgenic plants, inhibitors of the biosynthetic pathway of SA and *in vitro* SA-labelling (reviewed in Ryals *et al.*, 1996). However, a number of reports have indicated that SAR can be induced in plants independently of SA. For example, Pieterse *et al.*, (1996) demonstrated that plant growth promoting rhizobacteria (PGPR), root-colonizing bacteria, triggered a systemic resistance response in transgenic

Arabidopsis plants unable to accumulate SA due to overexpression of salicylic acid hydrolase that degrades SA to catechol.

1.2.6 Pathogenesis-related proteins and antimicrobial peptides

Among the most frequently observed biochemical events that follow plant infection by pathogens is the induction and accumulation of novel families of proteins collectively known as pathogenesis-related proteins (PR proteins). These PR proteins are defined as proteins coded for by the host plant but induced specifically in pathological or related stress situations (Van Loon *et al.*, 1994). These proteins do not only accumulate locally in the infected leaf, but are also induced systemically, as part of the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses (section 1.2.5).

PR proteins were initially identified in soluble extracts of tobacco leaves reacting hypersensitively to tobacco mosaic virus infection. Since then they have been found in a wide variety of infected plant species belonging to various families, suggestive of a universal role for these proteins in adaptation to biotic stress conditions. Originally, the 10 major acidic PR proteins, which were isolated from the infected tobacco leaves, were grouped into five families (PR-1 to PR-5) on the bases of their relative mobility on native polyacrylamide gels and on their serological relationships (Van Loon *et al.*, 1987). Then, in 1994 a unifying nomenclature for PR- proteins was proposed based on their grouping into families sharing amino acid sequences, their serological relationship, and/or enzymatic or biological activity. By then another six groups of protein induced by pathogens have been recommended for inclusion as PR proteins bringing the total to 11 families (PR-1 to PR-11) (Van Loon *et al.*, 1994) (see table 1-1). Different members

Table 1-1 The families of pathogenesis-related proteins in plants*

Family	Type member	Properties	Target in pathogen
PR-1	Tobacco PR-1a	Unknown	Membrane
PR-2	Tobacco PR-2	β-1,3-glucanase	Cell wall glucan
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	Cell wall chitin
PR-4	Tobacco R	Chitinase type I, II	Cell wall chitin
PR-5	Tobacco S	Thaumatin-like	Membrane
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	Proteinase
PR-7	Tomato P_{69}	Endoproteinase	Unknown
PR-8	Cucumber chitinase	Chitinase type III	Cell wall chitin
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase	**
PR-10	Parsley `PR1'	Ribonuclease-like	Unknown
PR-11	Tobacco class V chitinase	Chitinase, type I	Cell wall chitin
PR-12	Radish Rs-AFP3	Defensin	Membrane
PR-13	Arabidopsis THI2.1	Thionin	Membrane
PR-14	Barley LTP4	Lipid-transfer protein	Membrane

* Adapted from Fritig *et al.*, 1998; Van Loon and Van Strien, 1999.

** Peroxidase has indirect antimicrobial activity by catalysing oxidative crosslinking of proteins and phenolics in the plant cell wall, and thus protects the host from degradation by pathogen's hydrolytic enzymes.

within each family are assigned letters according to the order in which they are described. Thus, the same designation for a PR-protein in different plant species does not necessarily mean that they are the same protein. They must belong to the same PR family (number), but the lettering only reflects how many proteins of this family had been identified within those plant species before their discovery. Within one family, several members may share similar biological activities but differ significantly in other properties such as subcellular localisation, substrate specificities or physicochemical properties (Fritig *et al.*, 1998). For example, each of the original five classical groups of PR proteins has two subclasses: a basic subclass found in the plant cell vacuole and an acidic subclass usually found in the extracellular space (Kitajima and Sato, 1999).

Inclusion of three additional families of PR proteins was proposed in 1998 following discussions at the 5th International Workshop on Pathogenesis-related Proteins in Plants, held at Aussois, France (Van Loon and Van Strien, 1999). These include the pathogen-induced plant defensins (PR-12), thionins (PR-13) and lipid transfer proteins (LTPs) (PR-14) (table 1-1). These groups are families of peptides or low molecular mass proteins with antimicrobial activities and their induction has been observed in a number of plant species upon infection of the leaves by pathogens (Broekaert *et al.*, 1997).

Most PR proteins have a damaging effect on the structure of pathogen cells (see table 1-1). The mode of action of some of the PR proteins, as antimicrobial proteins, has been clearly identified such as PR-2 and PR-3 families. PR-2 family members have β-1,3 glucanase activity which hydrolyses the structure β-1,3 glucan present in the pathogen cell wall resulting in weakened the cell wall. This weakened cell wall results in cell lysis and death. Also, PR-3, PR-4, PR-8 and PR-11 families (endochitinases) cleave pathogen cell wall chitin polymer (a linear homopolymer of β-1,4 *N*-acetylglucosamine) also

resulting in a weakened cell wall. Chitinases can also display lysozyme activity and hydrolyse bacterial peptidoglycan. Also, PR-1 and PR-5 family members interact with the pathogen plasma membrane.

Although the antimicrobial activities of the other groups have been demonstrated and the overexpression of their genes in transgenic plants has been shown to mediate host plant-pathogen resistance (for a review see Punja, 2001; Selitrennikoff, 2001), their mode of action is still unclear.

1.3 Oomycetes and potato late blight disease

Oomycetes morphologically and physiologically look like fungi. However, modern studies of metabolism and rRNA sequence analyses have shown that the oomycetes are taxonomically distant from fungi and are more closely phylogenetic relatives of brown algae, within the kingdom Stramenopiles (Cooke *et al.*, 2000; Tyler, 2001). The oomycetes include many destructive pathogens of plants, animals and humans, and many of the strategies that have been developed for protection against fungal diseases fail when applied to oomycete diseases. Oomycetes have a physiology and biochemistry distinct from fungi, thus many of the most effective fungicides fail against them. For example, the azole fungicides block ergosterol biosynthesis, which is an essential metabolic pathway in fungi, by inactivating the key enzyme in this pathway (cytochrome P450 sterol 14-demethylase). The blocking of this enzyme decreases the conversion of 14-alpha-methylsterols to ergosterol, thus accumulation of C14-sterols leads to changes in membrane fluidity and function. Although these fungicides have been used extensively in agriculture and medicine against fungi, it does not affect the oomycetes because they do not synthesize sterols but acquire them from their host

plants. Moreover, as oomycetes contain no membrane sterols, the target for the toxic plant saponins, they can penetrate the epidermal cells of host and nonhost plants (see below).

A further complication is that oomycetes reproduce sexually and asexually, thereby increasing genetic flexibility that enables them to adapt rapidly to and overcome chemical control measures and genetic resistance bred into plant hosts. Isolates resistant to previously effective chemicals in several oomycete species including *Phytophthora infestans* have been discovered. One such example is metalaxyl, the most effective fungicide against *Phytophthora* that was used extensively throughout Europe to combat potato late blight. Metalaxyl, a systemic fungicide, proved extremely effective and was often adopted as the only means of control. Even at low concentrations, metalaxyl immediately stops the development of mycelium and prevents sporulation of the pathogen upon entering the plant (Gisi and Cohen, 1996). In the mid 1990s, metalaxyl was lost as an effective chemical for the control of late blight in the USA and Canada, as metalaxyl-sensitive strains of *P. infestans* were displaced by resistant genotypes (Goodwin *et al.*, 1998).

The oomycetes comprise a large number of economically important and highly destructive plant pathogens and include the *Phytophthora* species (meaning "plant destroyer"; Birch and Whisson, 2001), which are an extremely broad host-range group of plant pathogenic organisms affecting potatoes, tomatoes, soybeans, peppers, and more than two hundred other plant species worldwide. *Phytophthora* species cause some of the most destructive plant diseases in the world. For example, *Phytophthora infestans*, the causal pathogen of the late blight disease of potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*), is reckoned to be the most damaging microbial

pest of potato crops worldwide. It attacks both tubers and foliage during any stage of crop development. When conditions are favourable, the fungus can spread rapidly through the foliage and if no controls are implemented, entire fields can be destroyed. In the mid-1800s, late blight caused widespread potato crop failures throughout Northern Europe, including Ireland where it was responsible for the Irish Potato Famine (one of the most awful tragedies recorded in human history). During that period, Ireland lost more than two million of its population (more than a quarter of its population) due to starvation and emigration, as a direct consequence of late blight disease, making this disease one of the most important crop diseases in history (Birch and Whisson, 2001).

Late blight is controlled worldwide by the application of fungicides. The disadvantage of protection by chemicals is that the quantities used and frequencies of application, in practice, are often higher than necessary. This means financial and environmental consequences, and more importantly it leads to the evolution of resistance and more virulent fungal isolates (see below). Because of the recent spread of more virulent forms of *P. infestans* and the economic importance of potato, as the fourth most important food crop worldwide after wheat, maize, and rice, development of resistance to this pathogen is currently badly needed and one of the highest objectives in potato breeding programs worldwide (Ewing *et al.*, 2000; Gebhardt and Valkonen, 2001).

Several major resistance genes (nineteen *R*-genes) to viruses, fungi, bacteria and nematodes have been identified in potato. Eleven of these genes play an important role in potato resistance to *P. infestans*. Unfortunately, resistance to late blight associated with the presence of some of these genes (race-specific or vertical resistance) is only effective against certain races of the pathogen and can be easily overcome by rapid evolution of the pathogen resulting in a lack of durability in the field. In contrast, race-

non-specific or horizontal resistance so called "durable resistance", which means resistance that remains effective in a widely grown cultivar for a long period of time in an environment favourable to the disease (Johnson, 1993), is believed to be effective against all known races of the pathogen and provide a general resistance independent of the virulence of the pathogen. Therefore, this type of resistance, which is believed to be controlled by an unknown number of minor genes, is required (Gebhardt and Valkonen, 2001; Swiezynski *et al.*, 2000; Vleeshouwers *et al.*, 2000a). Until now the genetics of durable resistance is still unknown and such genes have not been identified. In fact, it is very difficult to define criteria suitable for the identification of such genes, and as a result it is not easy to predict which genes will provide durable resistance. Johnson (1993), who introduced the term "durable resistance", indicated that no single genetic model is appropriate to distinguish between durable and non-durable resistance.

Although the genetics and physiology of *P. infestans* and its interaction with potato have been intensively studied (Freytag *et al.*, 1994; Kamoun *et al.*, 1999), progress in understanding the molecular events involved in infection and resistance response to *P. infestans* attack is still limited. Nevertheless, it is clear that the outcome of *Phytophthora*-potato interactions, compatible or incompatible, is decided after the pathogen penetration of the host epidermal cells. Using a diverse set of wild *Solanum* species and potato cultivars with various levels of resistance to late blight as well as non-host plants, Vleeshouwers *et al.*, (2000b) demonstrated that *P. infestans* is able to penetrate epidermal cells of many different plant species, including nonhost plants, which indicates that the host defence responses mainly occur post-penetration. Freytag *et al.*, (1994) have shown that in the early phases of interaction there are only small differences between the compatible and incompatible reactions. Early reactions at the

penetration site include rearrangement of the cytoplasm, rapid apposition of callose and hypersensitive response (HR) in the penetrated cell. Once the infecting hyphae reach the mesophyll cells differences in the plant's reaction are visible. In the compatible interaction, some cells undergo HR, but the hyphae can escape this response and continue to colonize the tissue, while in the incompatible interaction, the pathogen is contained in the cells undergo HR and dies (figure 1-4) (Cuypers and Hahlbrock, 1988; Freytag *et al.*, 1994).

During the interaction between potato and *Phytophthora*, specific genes have to be regulated as part of the infection and the defence mechanisms. Several studies have demonstrated that the infection of *P. infestans* leads to transcriptional activation of various genes in potato (e.g. Avrova *et al.*, 1999; Beyer *et al.*, 2001; Birch *et al.*, 1999; Zhu *et al.*, 1995a). An inclusive analysis of such genes that are induced during potato-*Phytophthora* interactions, especially in systems leading to long-lasting "durable" resistance, might lead to a better understanding of the molecular processes involved in durable resistance, which might potentially lead to the development of biotechnological strategies for the fight against this destructive pathogen.

In the last 150 years, enormous efforts have been made in order to control losses in potato yields due to late blight disease including: the use of certified seed programmes, crop rotation, use of fungicides, disease-forecasting and grower education. More recently, fungicides, which are particularly effective against *P. infestans*, and plant breeding programmes, which select for late blight resistance in host plants, have made great advances in controlling late blight in potatoes. Unfortunately, despite all these achievements late blight is still a damaging disease. As mentioned earlier, isolates of *P. infestans* resistant to metalaxyl, such as US-8 that destroyed many potato crops in the

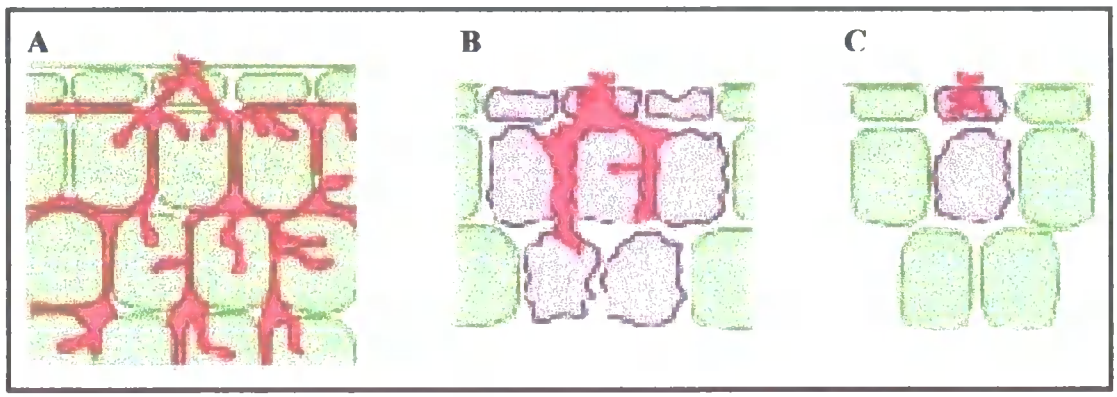


Figure 1-4 Schematic view of early infection events during susceptible and resistant interactions between *Phytophthora infestans* and plants. Penetration of plant tissue is observed on all plants. (A) In susceptible plants, no visible defence responses occur. Secondary hyphae grow into the intercellular space, form haustoria (digit-like feeding structures) inside mesophyll cells, and rapidly colonize the mesophyll tissue. (B) In resistant plants, cells display the HR. The infecting hyphae of the pathogen are contained within a group of dead plant cells or (C) within the penetrated epidermal cell, depending on the genotypes of the interacting plant and pathogen. Macroscopically, the HR lesions can be visible as brownish-black spots on leaves or may not be visible. In several nonhost plants, the HR is induced extremely quickly and is usually localized to one or two plant cells (Adapted from Kamoun, 2001).

United States in 1994, have been found. Moreover, recently, Groves and Ristaino, (2000) indicated that a wide range of chemically diverse fungicides can induce normally heterothallic metalaxyl-resistant isolates of *P. infestans* to form oospores *in vitro* after short exposures to the fungicides. This means fungicides can induce phenotypic changes in *P. infestans* and they have a non-target effect on the reproductive biology of the pathogen. Annually losses due to late blight and control measures are estimated to exceed $5 billion. Therefore, *P. infestans* is regarded as a serious problem to potato crop worldwide (Birch and Whisson, 2001; Tyler, 2001).

Recently, the destructiveness of oomycete diseases, especially *Phytophthora* species, and the difficulty of controlling them, has led to an intensive effort to develop molecular genetic tools to investigate these organisms and the genetic bases of their pathogenicity. *Phytophthora* researchers around the world have set up the *Phytophthora* Genome Initiative (PGI) database (http://www.ncgr.org/pgc/pgi/index.html) to organise and support genome-scale studies of *P. infestans* and *P. sojae* (a soybean pathogen). Several thousand (approximately 2,000-3,000) expressed sequence tags (ESTs) of *P. infestans* and *P. sojae* are available in this public database (http://www.ncgr.org/pgc). Also, the US Department of Agriculture (Initiative for Future Agriculture and Farming Systems) funded a project to sequence 41,000 additional *P. sojae* ESTs and 14,000 *P. infestans* ESTs, two years ago (2000). In addition, 35,000 *P. infestans* ESTs developed by an international consortium funded by Syngenta are expected to become available to the public in 2003 (Birch and Whisson, 2001; Tyler, 2001).

1.4 An overview of engineering pathogen resistance in crops

1.4.1 Introduction

Diseases caused by micro-organisms are currently some of the major factors limiting crop production worldwide. Diseases not only have negative effects on crops yield, but also they can affect the quality of crops post-harvest. Since farmers started to cultivate plants, fungal diseases have been one of the main causes of considerable crop losses. Several disease control measures have been developed to control plant diseases and pests. These control measures are mainly based on chemical, biological, genetic and cultural methods. Currently, for reasons of cost, effectiveness and ecological considerations, much research is aimed at transgenic expression of genes that can confer significant levels of disease resistance to provide an environmentally friendly alternative to traditional control measures.

In the early 1980's, with the beginning of the molecular era of plant biotechnology and with the improvements in transformation techniques and advanced molecular techniques for plant breeding, a major area of research has been to identify, characterise and clone various genes involved in disease resistance. Consequently, many resistance mechanisms that plants have evolved to respond to pathogen attack, have been elucidated and many genes involved in these responses and whose encoded products have antimicrobial activity or are involved in the synthesis of products with such activities have been identified. The identification of many of these genes has made it possible to subsequently evaluate their specific roles and importance in disease resistant using transgenic plants. These include: (I) genes that encode proteins, peptides, or antimicrobial compounds that are directly toxic to pathogens or that reduce their

growth. Examples of these include pathogenesis-related proteins (PR proteins) such as hydrolytic enzymes (chitinases, glucanases), antifungal proteins (osmotin and thaumatin-like), antimicrobial peptides (thionins, defensins, lectins), ribosome inactivating proteins (RIP), and enzymes involved in the generation of phytoalexins (see sections 1.4.3 and 1.4.4); (II) genes that directly or indirectly activate general plant defence responses pathways. These include the production of specific elicitors, salicylic acid (SA), hydrogen peroxide (H_2O_2), and ethylene (C_2H_4) (see section 1.4.2); (III) resistance genes involved in the interactions with avirulence factors and in the hypersensitive response (see sections 1.2.5 and 1.2.6); (IV) gene products that directly inhibit pathogen virulence products such as polygalacturonase and oxalic acid, and (V) gene products that enhance plant structural defences. These include elevated levels of peroxidase and lignin. Table 1-2 shows examples of genetically engineered plants, particularly emphasising potato and tobacco, to enhance resistance to fungal diseases. Success in these approaches is by no means assured and there are numerous papers describing failed strategies (e.g. table 1-2). Figure 1-5 illustrates some of the genes (from both plant and non-plant sources) that have been used to enhance fungal disease resistance in crop plants.

This short introduction will try to mention some of the strategies used and advances made to enhance disease resistance against fungal pathogens, and will try to address some of the problems encountered these strategies. There are many recent reviews on the subject of genetic engineering for disease resistance and the reader is directed to them for more details in this area (e.g. Dempsey *et al.*, 1998; Honee, 1999; Melchers and Stuiver, 2000; Punja, 2001; Shah, 1997; Stuiver and Custers, 2001).

Table 1-2 Some examples of genetically modified plants to enhance resistance to fungal diseases (adapted from Melchers and Stuiver, 2000; Punja, 2001).

Strategy used and plant species engineered	Expressed gene product	Effect on disease development	Reference
Expression of hydrolytic enzymes			
Potato (*Solanum tuberosum* L.)	*Tricoderma harzianum* endochitinase	Lower lesion numbers and size due to *Alternaria solani*; reduce mortality due to *Rhizoctonia solani*	Lorito *et al.*, 1998
Tobacco (*Nicotiana tabacum* L.)	*Tricoderma harzianum* endochitinase	Reduced symptoms due to *Alternaria alternata, Botrytis cinerea,* and *R. solani*	Lorito *et al.*, 1998
Expression of PR-proteins			
Potato (*Solanum tuberosum* L.)	Tobacco osmotin	Delayed onset and rate of disease due to *P. infestans*	Liu *et al.*, 1994
Expression of antimicrobial peptides			
Potato (*Solanum tuberosum* L.)	Alfalfa defensin	Enhanced resistance to *Verticillium dahliae*	Gao *et al.*, 2000
Expression of phytoalexins			
Tobacco (*Nicotiana tabacum* L.)	Grape resveratrol synthase	Reduced colonization by *Botrytis cinerea*	Hain *et al.*, 1993
Tomato (*Lycopersicon esculentum*)	Grape resveratrol synthase	Reduced lesion development by *P. infestans*; no effect on or *Botrytis cinerea*	Thomzik *et al.*, 1997
Alteration of structural component			
Potato (*Solanum tuberosum* L.)	Cucumber peroxidase	No effect on disease due to *Fusarium sambucinum* and *P. infestans*	Ray *et al.*, 1998
Regulation of plant defence responses			
Potato (*Solanum tuberosum* L.)	*Aspergillus niger* glucose oxidase	Delayed lesion development due to *P. infestans*; reduced disease development due to *A. solani* and *Verticillium dahliae*	Wu *et al.*, 1995
Expression of combined gene products			
Tobacco (*Nicotiana tabacum* L.)	Barely chitinase and β-1,3-glucanase	Reduce disease severity due to *Rhizoctonia solani*	Jach *et al.*, 1995

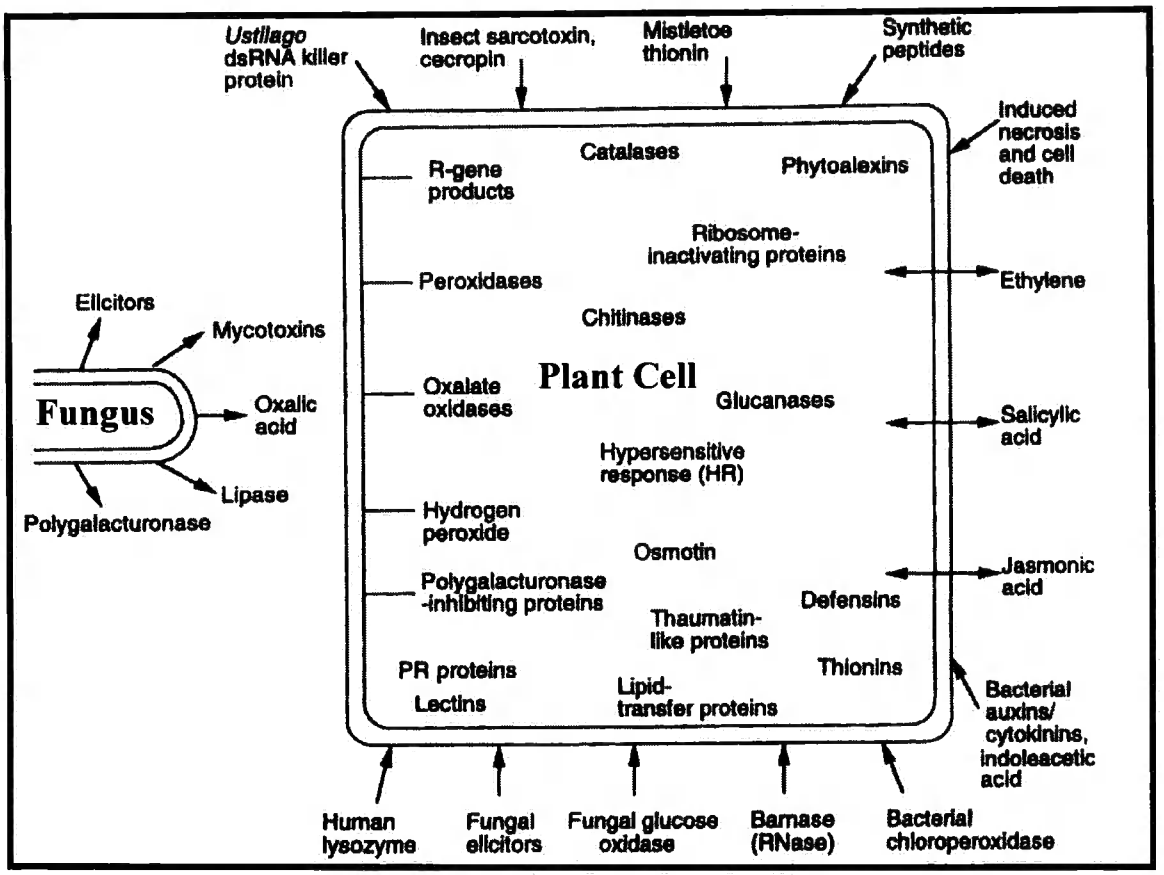


Figure 1-5 Some of the genes that have been used to enhance fungal disease resistance in crop plants. Transgenic plants with enhanced disease resistance have been engineered to express gene products to counter fungal virulence products (from hypha on left), enhanced expression of plant-derived gene products (inside of cell) or through expression of gene products from non-plant sources (outside of cell) (Adapted from Punja, 2001). See Punja, (2001) for details of references to individual strategies.

1.4.2 Using molecules involved in signal transduction pathways as activators for plant defence responses

Plants use several different defence pathways against different pathogens. Generally, these pathways are characterised by the signalling molecules that play an important role in the regulation of expression of defence proteins. Salicylic acid is the best known signalling molecule that induces expression of plant defence genes, and plays a crucial role in triggering systemic acquired resistance (SAR) to further infection by a broad range of pathogens (see section 1.2.5). Jasmonic acid and ethylene have also been shown to play an important role as a signalling molecules in activating other defence pathways. Activation of either of these pathways also leads to resistance, but to a distinct group of pathogens from those associated with salicylic acid induced resistance (Thomma *et al.*, 1998). In addition to these three pathways, evidence suggests that other signalling molecules such as reactive oxygen species (ROS) might be involved in pathways leading to the activation of a diverse set of defence mechanisms resulting in the establishment of plant disease resistance (Grant *et al.*, 2000).

Treatment of plants with one or more of these signalling molecules causes the induction of plant defence responses. Therefore, an increase in these signalling molecules was seen as a possible strategy for engineering plants to enhance their resistance against pathogens. For example, Verberne *et al.*, (2000) demonstrated that the overexpression of SA in tobacco plants transformed with two bacterial genes coding for enzymes that convert chorismate into SA, enhanced PR-protein production and provided resistance to viral and fungal infection resembling SAR in nontransgenic plants. Also, Yu *et al.*, (1999) demonstrated that expression of tobacco class II catalase (Cat2NT), an enzyme with SA-binding activity, in transgenic potato enhanced defence gene expression

leading to SAR and enhanced tolerance to *P. infestans*. Moreover, overexpression of the *NPR1* gene (non-expresser of PR genes; also called SAI1 for salicylic acid-insensitivity), which was identified as a key regulator in transducing the SA signal leading to general acquired resistance responses, in transgenic *Arabidopsis* increased the level of PR proteins during infection and enhanced resistance to *Pseudomonas syringae* and *Peronospora parasitica* (Cao *et al.*, 1998). Furthermore, transgenic potato plants constitutively overexpressing H_2O_2-generating glucose oxidase from *Aspergillus niger*, resulted in enhanced resistance to *P. infestans* and *Alternaria solani* as a result of constitutively elevated levels of H_2O_2, which were sufficient to activate an array of host defence mechanisms (Wu *et al.*, 1997).

There are some disadvantages for engineering resistance through the use of these signalling molecules. Most mutants possessing constitutive expression of a defence pathway show reduced yield or plant vigour, and there seems to be antagonism between the different defence pathways, which leads to increased susceptibility to other pathogens. For example, Doares *et al.*, (1995) have shown that salicylic acid (SA) and acetylsalicylic acid (ASA) are potent inhibitors of systemin- and jasmonic acid (JA)-induced synthesis of proteinase inhibitor mRNAs and proteins.

1.4.3 Antimicrobial proteins

One of the well established and widely used strategies in the engineering of pathogen resistance is the overexpression of antipathogenic proteins such as chitinases and glucanases, which belong to the PR proteins and have been shown to exhibit antifungal activity *in vitro*. The antifungal activity of plant glucanases (PR-2) and chitinases (PR-3) is thought to occur by hydrolysing the structural (β-1-3) glucan and chitin present in

fungal cell wall resulting in weakened cell wall and rendering fungal cells osmotically sensitive and thereby reduce fungal growth (see section 1.2.6). Although this strategy in some respects is similar to the strategy of overexpression of signalling molecules described above, it is much more specific because only one or two genes from the entire defence system are transferred to the new transgenic crop. In contrast to the previous approach, the loss in yield or the interference/antagonism with other defence pathways is most likely to be limited or absent.

The specific roles of these hydrolytic enzymes in resistance to disease have been difficult to prove in non-transgenic plants because *in vivo* rapid accumulation and high levels of these enzymes occur in resistant plants expressing a hypersensitive response, as well as in susceptible plants, and their expression can also be induced by environmental stress and plant senescence (Punja and Zhang, 1993). However, following expression of different types of chitinases and glucanases in a range of transgenic plant species, the important role of these enzymes in resistance to disease has been proven. The rate of lesion development and the overall size and number of lesions in transgenic plants were reduced upon challenges with many fungal pathogens, including those with a broad host range, such as *Botrytis cinerea* and *Rhizoctonia solani*. For example, Lorito *et al.*, (1998) demonstrated that transgenic lines of tobacco and potato plants overexpressing a strongly antifungal endochitinase from a biocontrol fungus, *Trichoderma harzianum*, had no visible effects on the growth and development of the plants, and were highly resistant to the foliar pathogens *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, and the soilborne pathogen *Rhizoctonia solani*. Similarly, expression of the human lysozyme in transgenic tobacco plants resulted in enhanced resistance to *Erysiphe cichoracearum*. Both conidia formation and mycelial

growth were reduced in the transgenic plants (Nakajima *et al.*, 1997). Also, Lusso and Kuc, (1996) reported that constitutive expression of a β-1,3-glucanase in tobacco plants increased resistance of the foliage to the fungi *Peronospora tabacina* and *Phytophthora parasitica.*

There are a number of studies indicating that the combined expression of chitinase and glucanase in transgenic plants was much more effective in preventing disease development by a number of pathogens than either one of them alone confirming the synergistic activity of these two enzymes. For example, Jach *et al.*, 1995 demonstrated that the combined expression of barley chitinase and β-1,3-glucanase genes in tobacco plants confered higher levels of resistance to the fungal pathogen *Rhizoctonia solani* compared with protection levels obtained with transgenic tobacco lines expressing the single transgene to a similar level of expression. Moreover, transgenic tomato plants expressing either the tobacco chitinase gene or the tobacco β-1,3-glucanase gene, showed no protection to infection with *Fusarium oxysporum* f.sp. *lycopersici*, while tomato lines simultaneously expressing both genes, showed a 36% to 58% reduction in disease severity, again indicating a synergistic protective interaction of the co-expressed antifungal proteins *in vivo* (Jongedijk *et al.*, 1995).

Constitutive production of PR proteins not belonging to the PR-2 and PR-3 families can also improve disease resistance. For example, transgenic potato plants overexpressing tobacco osmotin (PR-5) showed a delay in disease progression upon inoculation with spore suspensions of *Phytophthora infestans* (Liu *et al.*, 1994).

The limitation of this approach is that in many cases resistance will be highly specific for only a few pathogens and generally it does not provide a broad-spectrum of disease resistance (Alexander *et al.*, 1993). Nevertheless, these limitations do not weaken the

usefulness of this strategy, as often only a few pathogens are really important per crop.

One of the more significant practical problems encountered with this strategy is that the newly introduced proteins have to fit with the plant's endogenous defence systems. Punja and Raharjo, (1996) demonstrated the difficulty of achieving this result. They transferred different chitinase genes originating from petunia (acidic) and tobacco (basic) into two different crops (carrot and cucumber) and evaluated the transformed lines for response to different fungal pathogens. They found that while the carrot line, transformed with tobacco chitinase, becomes resistance, the carrot line transformed with petunia chitinase and transgenic cucumber lines did not, even when the same pathogens (*Botrytis cinerea* and *Rhizoctonia solani*) were used to challenge the two crops. Moreover, there were no detectable differences in disease development (rate and final levels) with *Alternaria radicini* or *Thielaviopsis basicola* in either group of transgenic carrot plants. These results demonstrate that the efficacy of chitinase gene transformation as a strategy for enhancing disease resistance in plants can be influenced by the nature of the recipient plants, the source and type of chitinase protein expressed, and the characteristics of the fungal pathogen tested.

1.4.4 Phytoalexins

Phytoalexins are low molecular weight secondary metabolites produced in a broad range of plant species, which have been demonstrated to have antimicrobial activity and are induced by pathogen infection, treatment of biotic or abiotic elicitors, or certain stresses (see section 1.2.4) (Grayer and Kokubun, 2001; Hammerschmidt, 1999).

Overexpression of genes encoding certain enzymes that generate phytoalexins resulted in delayed development of disease and symptom production by a number of pathogens

on several plant species. For example, Hain *et al.*, (1993) reported that transgenic tobacco plants overexpressing resveratol synthase genes, an enzyme required for the synthesis of the stilbene-type phytoalexin resveratol, from grapevine (*Vitis vinifera*), reduced colonization by *Botrytis cinerea*. Also Thomzik *et al.*, (1997) demonstrated that transformation and overexpression of the same genes in tomato resulted in a significant increase in the resistance to *Phytophthora infestans*. A similar accumulation of resveratol occurred after inoculation with *Alternaria solani* and *Botrytis cinerea*, but no significant increase in resistance was observed in transgenic tomato plants. Furthermore, transformation of alfalfa (*Medicago sativa*) with a peanut (*Arachis hypogaea*) cDNA encoding resveratrol synthase, resulted in significant inhibition of hyphal growth of the alfalfa fungal pathogen *Phoma medicaginis* as well as reduction of the lesion size (Hipskind and Paiva, 2000).

Although engineering resistance using this strategy has worked in some plants, the number of successes has remained low, and the level of resistance relatively modest. One of the most important reasons for that is that phytoalexins are synthesised through complex biochemical pathways (as mentioned in section 1.2.4), and genetic manipulation of these pathways to enhance phytoalexin production has been difficult to achieve. For example, to synthesise pistatin (the pea phytoalexin) in tobacco, the introduction of genes encoding at least nine new enzymes is required. In addition, the specific activity of these compounds is relatively low, so the amounts needed to confer resistance are extremely high. Accumulation of such high amounts of phytoalexins is difficult to achieve when the appropriate gene/genes required for synthesis is/are transferred to other crops. Moreover, accumulation of such high concentration of phytoalexins is often toxic to the plant cells itself and might also affect the quality of the

crop plants and could present nutritional problems for human and/or animals that use them (Dempsey *et al.*, 1998; Stuiver and Custers, 2001).

1.4.5 Resistance genes and the hypersensitive response

Resistance genes confer race-specific resistance which results from the highly specific recognition between a resistance gene product from a plant and a specific avirulence gene product from a pathogen that matches the resistance gene. This specific gene-for-gene interaction triggers one or more signal transduction pathways that sequentially activate an array of plant defence responses to prevent pathogen growth and restrict the pathogen to the vicinity of the infection sites. The development of a hypersensitive response (HR) is regarded as the most powerful defence response by which plants resist pathogen infection. The strength of the hypersensitive reaction makes it highly suitable for combating a broad spectrum of plant pathogens (see section 1.2.1).

Recently, many plant resistance genes have been isolated. The predicted products encoded by these genes show a high degree of similarity and can be divided into five broad groups depending on the presence of conserved structural domains, such as transmembrane region (TM), a nucleotide-binding site (NBS), a tool/interleukin 1 receptor domain (TIR), a cytoplasmic or extracellular leucine-rich repeat (LRR) and a protein kinase domain (PK) (Bent, 1996). While, efforts to clone an array of resistance genes involved in disease resistance have met with some success, all resistance genes (with a few exceptions) have been shown to lack durability in the field (Pink and Puddephat, 1999). Although the defence response is powerful its limitations, to only one or even sometimes to a limited number of races of the pathogen, are that it is normally triggered only by the highly specific recognition between a resistance gene product and

a specific matching avirulence gene product. Pathogens are usually able to overcome resistance gene-mediated recognition either by shedding the corresponding *Avr*-gene, or by accumulating mutations in the gene, which prevents the gene product from being recognised by the host plant, and consequently a failure to trigger the hypersensitive response. For instance, Joosten *et al.*, (1994) presented evidence that, in nature, a single base-pair change in the biotrophic fungus *Cladosporium fulvum* avirulence gene lead to virulence of races previously avirulent on tomato genotypes carrying the complementary Cf4 resistance gene. Therefore, the use of most known resistance genes is limited in plant biotechnology for conferring disease resistance.

Despite the huge induction of defence responses that results from triggering a hypersensitive response, there are reports indicating that not all pathogens are stopped. In contrast, triggering a hypersensitive response, in some cases, enhanced the infection by a necrotrophic fungal pathogens (Govrin and Levine, 2000) (see section 1.2.1).

1.4.6 *Avr/R* strategy for engineering broad-spectrum disease resistance (non-specific resistance)

Based on the importance of the HR in triggering the activation of the plant defence responses after the specific interaction between the pathogen *Avr*-gene and the matching plant *R*-gene, De Wit proposed an interesting idea, the so called "*Avr/R* strategy" (De Wit, 1992) to engineer plants with a broad-spectrum disease resistance. This involves transfer of a pathogen-derived *Avr*-gene (such as the *Cladosporium fulvum Avr9* gene) into a plant containing the corresponding resistance gene (such as the tomato *Cf9* gene). The expression of this gene is made conditional on pathogen infection by putting it under the control of a tightly regulated pathogen-inducible plant promoter (figure 1-6).

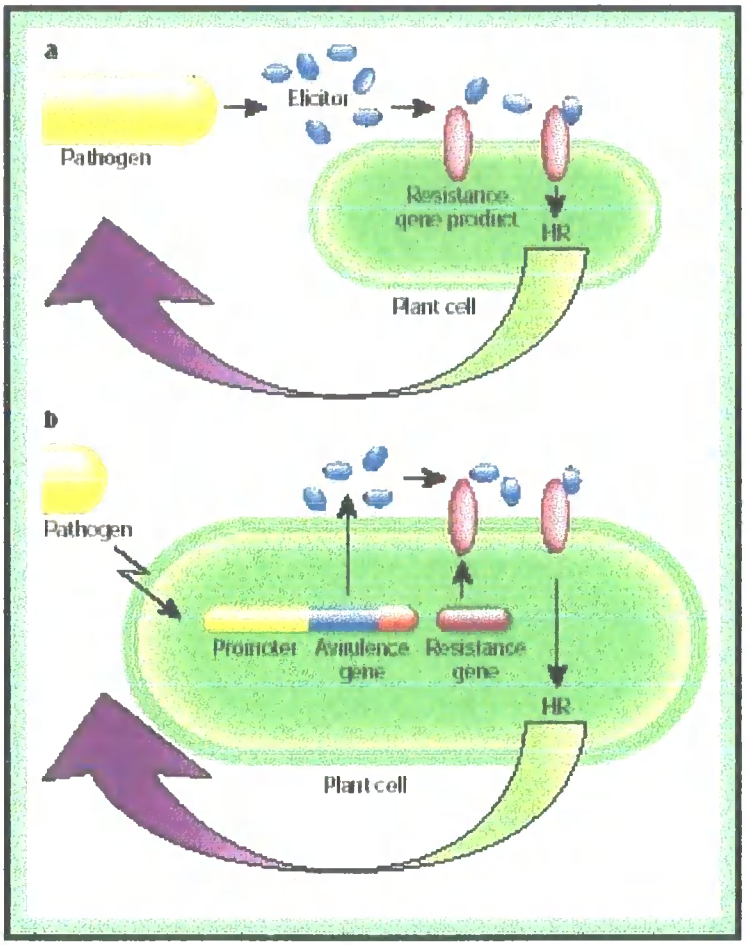


Figure 1-6 The *Avr/R* gene strategy for engineering broad-spectrum disease resistance (Dewit, 1992). (a) The hypersensitive response (HR) is triggered by the highly specific recognition of a pathogen-derived elicitor by a plant resistance gene product. The powerful and concerted defence that constitutes the hypersensitive response stops the pathogen. (b) The transformation of a pathogen-derived *avr* gene (under control of a tightly regulated pathogen-inducible plant promoter) into a plant containing the corresponding resistance gene. A pathogen-inducible plant promoter drives expression of this pathogen elicitor gene. The elicitor formed will trigger a resistance reaction manifested by a hypersensitive response, which will followed by a general defence response that prevents further spread of any invading pathogen (adapted from Stuiver and Custers, 2001).

Pathogen-induced expression of this gene will then stimulate a resistance reaction manifested by a hypersensitive response. A localized HR will then be followed by a general defence response that prevents further spread of any invading pathogen (see section 1.2.1).

Although constitutive expression of an *Avr*-gene in transgenic plants that contain the matching *R* gene results in necrosis and in the end death of the whole plant (Honee *et al.*, 1995), this idea has been exploited using transgenic tobacco and tomato plants which have been successfully engineered with a broad-spectrum disease resistance (Keller *et al.*, 1999; Stuiver and Custers, 2001). The key factor in this strategy is the tight regulation of the pathogen inducible promoter. Any leakiness of the promoter could influence plant vigour and yield. Nevertheless, both the Stuiver and Keller teams have produced transgenic tobacco and tomato plants that show no sign of spontaneous triggering of the hypersensitive response in the absence of pathogen challenge. Therefore, this is one of the most promising approaches to engineer broad-spectrum disease resistance in plants (Stuiver and Custers, 2001).

1.5 The overall conclusions from the literature review

Plants have evolved complex and sophisticated defence systems to survive a variety of pathogens that attack them. Each plant cell is capable of defending itself by a combination of constitutive and induced defences.

Pathogens that attack plants are diverse (necrotrophy, biotrophy, or hemibiotrophy), and each has its own strategy to invade and colonise the plants.

The oomycetes comprise a large number of economically important and highly destructive plant pathogens. Among the oomycetes are the *Phytophthora* species that

cause some of the most destructive plant diseases in the world.

Phytophthora infestans, the causal pathogen of the late blight disease in potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*), is the most damaging microbial pest of potato and tomato crops world-wide.

Due to the complexity of this pathogen, and to limit chemical control, it has been recommended that the development of resistance should be based on breeding potato to incorporate durable forms of genetic resistance (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2000b).

The destructiveness of oomycete diseases, and the difficulty of controlling them, has led to an intensive attempt to develop molecular genetic and genomic tools to investigate these organisms and the genetic bases of pathogenicity.

Various strategies have been developed to engineer plants with enhanced disease resistance to pathogens. Some of these strategies have provided plants with limited disease resistance, while others provide a broad-spectrum disease resistance such as the *Avr/R* strategy (section 1.4.5.1), which is based on engineering plants with an *Avr*-gene whose expression is tightly controlled by a pathogen-inducible plant promoter.

Efforts have been made to engineer durable disease resistance in economically important plants. Unfortunately, many of these attempts have failed, due to the complexity of disease-resistance signalling pathways and the different types of infection mechanisms that different pathogens use.

Further understanding of the molecular mechanisms responsible for the pathogenicity and disease resistance is critical for establishing durable resistance in crops.

Although transgenic plants exhibiting resistance to pathogen diseases are not yet available commercially, the enormous scientific progress made in genetic engineering and in understanding the mechanisms of plant resistance to pathogens is promising to provide commercially broad-spectrum disease resistance crops in the near future.

2 Aims, objectives and project timeline

2.1 Aims and objectives of the present research

The ultimate aim of this project was to analyse genes induced during the interactions between potato plants and the pathogen *Phytophthora infestans*. The majority of studies looking for *Phytophthora*-induced genes in potato have targeted the resistance reactions of the host using an incompatible strain of the pathogen (i.e. race-specific resistance) with the aim of finding factors involved in such resistance (e.g. Avrova *et al.*, 1999; Birch *et al.*, 1999). Unfortunately, race-specific resistance is only effective against certain races of the pathogen, and is easily overcome by rapid evolution of the pathogen resulting in a lack of durability in the field. It has been proposed that for long-term control of late blight disease, potatoes that possess a durable genetic resistance are needed (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2000b). Durable resistance is a phenomenon in which the plant shows some degree of resistance to a compatible pathogen in the field and the resistance is sustainable (section 1.3). Thus, in contrast to the previous studies, the present study aimed to identify the genes induced during the compatible interaction that ultimately leads to the establishment of a long-lasting and durable resistance. In order to achieve this, a compatible strain (strain 9.5.1) of *Phytophthora infestans* (race 1, 2, 3, 4, 6, 7) and a newly developed potato variety, 'Stirling' which exhibits durable resistance were used. This variety was chosen after consultation with Dr Helen Stewart, a plant pathologist at the Scottish Crop Research Institute (SCRI). After years of field observations, the plant pathologists at SCRI have concluded that Stirling is capable of developing durable resistance.

Although the molecular basis of the durable resistance is unknown, resistance is likely

to be controlled by a number of minor genes. Identifying these genes may lead to a better understanding of the molecular processes involved in establishing durable resistance. More importantly, these may also play a major role in the development of biotechnological strategies in the fight against late blight and other potato diseases, such as breeding new cultivars combining good agricultural traits with built-in durable resistance.

The specific objectives for this study were as follows:

1- To establish the experimental conditions for the development of durable resistance in Stirling potato plants by challenging tissue cultured Stirling plants with a compatible strain of *Phytophthora infestans* (races 1, 2, 3, 4, 6, 7)

2- To confirm and characterise the Stirling durable resistance by molecular, cytological and biochemical approaches and by challenging with other potato pathogens such as *Fusarium sulphureum* and *Rhizoctonia solani.*

3- To generate a cDNA library containing sequences induced in the potato variety Stirling during the development of durable resistance.

4- To screen the library for differentially expressed gene sequences, and to identify and characterise some of the selected cDNA clones.

5- To investigate which of these selected clones might be partly associated with establishing durable resistance using appropriate gene expression assays (DNA array technology).

6- To study some of the cytological and biochemical changes in potato leaves in response to *P. infestans* challenged by comparing differences in response between the

control (unchallenged) Stirling plant line and the established durable resistance plant lines.

2.2 Project timeline

Progress of the project is shown in figure 2-1. Preparation of tissue cultured plants and establishing the durable resistant plants to start the project and the subsequent testing of the durable potato variety Stirling took a long period of time. The long term experiment to collect plant materials covering the whole period starting from time point zero until the establishment of durable resistant shoots (after about seven weeks) (section 4.2.9) as well as repeating the experiment to collect enough plant materials for the characterisation experiments and molecular cloning similarly took a very long period.

This project also proved to be problematic. I had to repeat this long term experiment several times due to a failure of the growth room cooling system resulting in the exposure of the tissue cultured plants to very high temperatures (37-39°C). Since this could have influenced the results through the isolation of stress induced (heat shock) gene sequences rather than pathogen-induced sequences, all this material was discarded. Unfortunately this problem happened on three different occasions during the project.

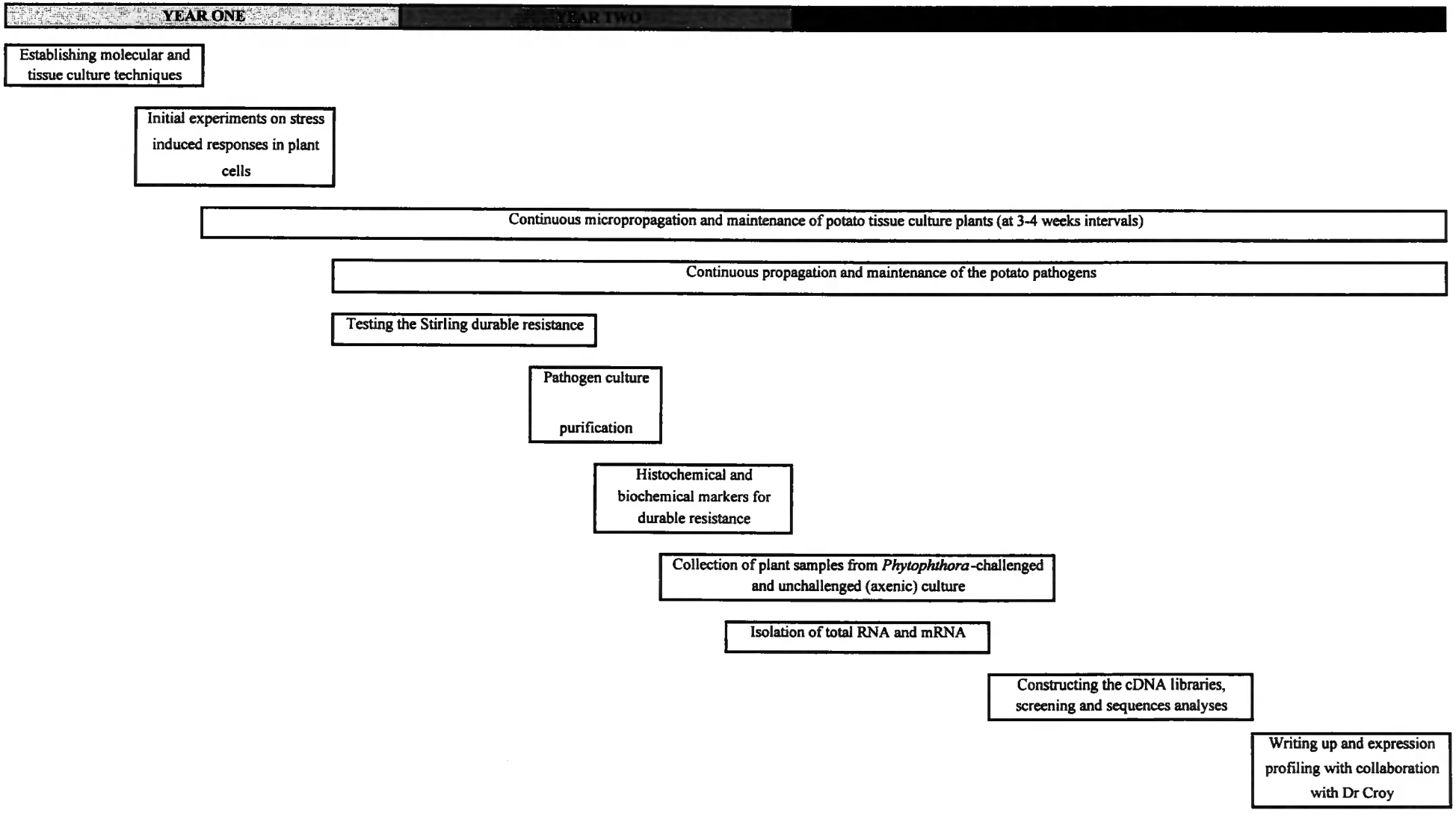
Handling the plant pathogens also proved to be problematical. After the treatment of potato plants with *Phytophthora*, contamination was observed on several occasions. The source of the contamination proved to be the original *Phytophthora* culture, obtained from the SCRI and it was necessary to purify it. This purification process took a long time as the pathogen growth in the selective media was very slow (about 2.5 months) (see section 4.2.3).

Purification of the mRNA from total RNA using the Promega PolyATtract mRNA

isolation kit failed several times, resulting in the loss of much valuable materials (see section 4.3.13).

There was also a significant delay in the installation and setup of the microarraying suite. The instrumentation and software only became available during the last 6 months during which this thesis was being written. Optimisation of media and instruments (by Dr Croy) took up most of the time up to the point of submitting the thesis.

Figure 2-1 Project timeline



3 Materials

3.1 Chemicals and media

All general chemicals, unless otherwise stated, were obtained from Sigma-Aldrich Company Ltd, Poole, Dorset, UK and were of analytical grade or the best grade available. Other materials (product numbers are indicated in brackets) were obtained from the following sources:

Potato dextrose agar (CM139), tryptone (L42), yeast extract (L21) and bacteriological agar grade 1 (L11) were purchased from Oxoid UK Ltd.

Rye seed was kindly provided by Prof. Peter Shewry of the Department of Agricultural Sciences, University of Bristol.

- Silica fines were kindly provided by Dr. David Dixon of the Department of Biological Sciences, University of Durham.

3.2 Kits, enzymes and DNA size markers

PCR-Select™ cDNA Subtraction Kit (K1804-1) and Advantage 2 Polymerase Mix (8430-1) were purchased from Clontech Laboratories UK Ltd., Basingstoke, Hants, UK.

TOPO™ TA Cloning Kit (45-0640) was from Invitrogen BV, CH Groningen, The Netherlands.

GeneRuler 1Kb DNA ladder (SM0311) was purchased from MBI Fermentas, Hanover, USA.

TRI reagent (T-9424) was from Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Dynabeads mRNA purification kit (610.06) was from Dynal A.S, Oslo, Norway.

PCR DIG probe synthesis kit (1636090) was from Roche Diagnostics GmbH, Mannheim, Germany.

Wizard Plus SV minipreps DNA purification System (A1460), Wizard PCR prep DNA purification kit (A7170), restriction endonuclease enzymes (various), PolyATtract mRNA isolation kit (Z5200) and Taq polymerase mini kit (U-1310) were purchased from Promega Corporation, Madison, USA.

Pellet Paint™ co-precipitant was from CN Bioscience Ltd., Beeston, Nottingham, UK.

3.3 Cultured potato plants and pathogens

Tubers of potato varieties Stirling, Bintje and Désirée were kindly provided by Dr. Helen Stewart of Scottish Crop Research Institute (SCRI) in Dundee, Scotland.

Potato pathogens; *Phytophthora infestans* strain 9.5.1 (race 1, 2, 3, 4, 6, 7) (compatible), *Fusarium sulphureum* and *Rhizoctonia solani* were also kindly provided by Dr. Helen Stewart of Scottish Crop Research Institute (SCRI), Dundee, Scotland.

3.4 Potato cDNA clones

Various characterised potato gene sequences and non-plant sequences were collected for use as controls in the gene expression assays.

- A cDNA clone (pSTPR-1) encoding the pathogenesis related protein PR-1 was kindly provided by Dr F. Govers of the Laboratory of Phytopathology, Agricultural University Wageningen. The Netherlands.

Extensin encoding cDNA clones pTEL15 and pTEL16 and three unknown tuber cDNA clones pYP3, 5 and 6 were kindly provided by Dr D. Bown of the Department of Biological Sciences, University of Durham, UK.

Lipoxygenase encoding cDNA clone pLOX28 was kindly provided by Dr S. Rosahl of the Institut fur Pflanzenbiochemie. IPB, Germany.

cDNA clones GluA, GluB2, ChtA2 and ChtB3 encoding acidic and basic glucanases and chitinases were kindly provided by Dr. Erich Kombrink of the Max-Planck Institut fur Zuchtungsforschung, Abteilung Biochemie, Germany.

Polyphenol oxidase (PPO) encoding cDNA clones Pot32 and Pot33 were kindly provided by Dr Simon Robinson of the Division of Horticulture, CSIRO, Australia.

A cDNA clone encoding phenylalanine ammonia-lyase (PAL) was kindly provided by Prof. K. Hahlbrock of the Max-Planck Institute, Germany.

cDNA clones encoding potato chloride-channel protein (pRR-8) a heat shock protein (pRR-12) and alcohol dehydrogenase (pRR-12, pRR20) were clones previously isolated and characterised from a potato subtraction library by Dr. Romaan Raemaekers,

Two non-plant cDNAs, NSE (nerone specific enolase) and GAP43 (growth activating protein) were kindly provided by Dr Stefan Przyborski, Department of Biological Sciences, University of Durham.

3.5 Consumables

Microcentrifuge tubes, pipette tips were obtained from Thistle Scientific, UK.

Cuvettes, and 96 well microtitre plates were obtained from Sarstedt Ltd., Leicester, UK.

Petri dishes were supplied by Bibby Sterilin Ltd (Staffordshire, UK).

Nylon membrane (Hybond-NX) was from Amersham Biosciences Ltd., Little Chalfont, Bucks, UK.

X-Ray film (RX type) was supplied by Fuji, Tokyo, Japan.

3.6 Documentation and computer software/hardware utilised

- Photographs were obtained using an Agfa e-photo 1680 digital camera.

Light and fluorescence micrographs were obtained using a Nikon Coolpix 950 digital camera, MCD lens 0.82-0.29X, fitted to a Nikon Optiphot-2 microscope.

Image manipulation was performed using Paint Shop Pro v6.02 (JASC software) and AGFA PhotoWise image software v1.6 (provided with the digital camera).

Graphical and statistical analyses were performed using GraphPad Prism v3.0 (GraphPad Software) and Excel (Microsoft).

Sequence processing, alignment and primer design were performed using the DNAStar suite of Lasergene programmes (DNASTAR, Inc., Madison, USA).

4 Methods

4.1 Plant tissue culture related techniques

4.1.1 Preparation of tissue culture medium

Throughout the whole project, Murashige and Skoog (MS) medium from Sigma was used for the maintenance of the cultured potato plants. The medium was prepared by dissolving 4.33g (MS), and 30g sucrose in distilled water. The pH was adjusted to 5.7 with 1M NaOH and the volume was made up to 1L then autoclaved at 121°C for 15min after adding 2.0g Phytagel as a solidifying agent (MS-medium).

4.1.2 Micropropagation of potato plantlets from tuber tissues

Micropropagation of potato plants from tubers was carried out using organ culture (bud culture). A potato tuber containing several good buds (eyes) was selected and washed thoroughly but carefully to avoid damaging the bud tissues. To further reduce bacterial and fungal contaminants, the tuber was soaked overnight in a beaker with a continuous flow of water. The tuber was then dried in air before it was sliced at the bud area. The slices taken were about 2 to 3cm in diameter and 1 cm thick at the middle and each contained only one bud. Slices were surface-sterilised by gently shaking in 70% (v/v) ethanol for 10 s, followed by shaking for 10min in 20% (v/v) Clorox with addition of one to two drops of Tween 80. The explants were then washed with sterile distilled water several times to make sure no residual chemicals remained. The sterilised explants were then placed into petri dishes containing a layer of sterile filter paper to absorb the remaining water from the tissue. Following sterilisation, the tuber slices were trimmed into a cube shape of approximately 1 cm width x 1 cm length x 0.75 cm deep

with the bud on the top. The buds were transferred into MS-medium in Kilner jars and sealed with parafilm. The jars were placed in a tissue culture growth room at 23-25°C under a regime of 16h light (90-100μE.m^{-2}.s^{-1}) and 8h dark. Newly developed shoots were excised and transferred onto fresh MS-medium to develop a potato plantlet. Plantlets were propagated and maintained using nodal cuttings every 4-6 weeks as described below.

4.1.3 Maintenance of potato plantlets

Potato plantlets were propagated using nodal cuttings. Prior to subculture, all leaves were removed from the stem of the cultured potato plants and the stem was then cut so that each cutting contained only one node. The cuttings were then transferred onto fresh MS-medium. The jars were then sealed with parafilm and at the sealing area a few holes were made to allow gaseous exchange and to prevent accumulating condensation. All plantlets were kept in the tissue culture growth room under the same conditions as described in section 4.1.2. This maintenance procedure was carried out every 4-6 weeks.

4.2 Microbiological and phytopathological methods

4.2.1 Preparation of pathogen media

4.2.1.1 Rye A broth

Phytophthora infestans was propagated in Rye A broth for elicitor preparation and total RNA extraction. The medium was prepared according to the procedure described by Caten and Jinks, (1968). Briefly, 60g of untreated rye seeds were soaked in distilled water (about 300ml) for 36h at room temperature after which the supernatant was decanted and retained. The swollen rye grains were then covered with distilled water

(about 300ml), blended using a high-speed blender (Waring commercial blender) for 2min. The resulting homogenate was incubated in a water bath for 1h at 68°C. The cooked rye was then filtered through four thicknesses of cheese cloth, squeezed gently to remove residual liquid and the grain sediment discarded. The filtrate was combined with the original supernatant, together with 20g sucrose, made up to one litre and sterilised by autoclaving at 121°C for 15min.

4.2.1.2 Rye A agar

For mycelial growth and long-term maintenance of *Phytophthora infestans* Rye A agar was prepared from Rye A broth as described above but 15g agar was added per litre prior to autoclaving as above.

4.2.1.3 Potato dextrose agar (PDA) medium

For growing *Fusarium sulphureum* and *Rhizoctonia solani,* potato dextrose agar (PDA) medium was prepared according to the manufacturer instructions. 39g PDA was suspended in 1L of distilled water, boiled to dissolve completely and then sterilised by autoclaving as above.

4.2.1.4 Luria-Bertani (LB) media

For growing bacterial clones LB-broth was prepared by suspending 10g tryptone, 5g yeast extract and 10g sodium chloride in 1L of distilled water, boiled to dissolve completely and sterilised by autoclaving as above. Where appropriate antibiotic was added (100μg/ml either ampicillin or kanamycin) after autoclaving and cooling to <50°C.

For LB-agar 15g/L bactoagar was added before autoclaving.

4.2.2 Maintenance of fungal pathogens

All manipulations of fungal pathogens were performed in a negative pressure containment lab using a Microflow class II biological safety cabinet. *Phytophthora infestans* was grown on a Rye A agar at 15°C in the dark and subcultured every 3 - 4 weeks. *Fusarium sulphureum* and *Rhizoctonia solani* were grown on potato dextrose agar also 15°C in the dark but subcultured every 2 weeks.

4.2.3 Purification of *Phytophthora infestans* cultures

Purification of *P. infestans* from contaminated cultures proved to be a difficult task because the pathogen grows very slowly on Rye A agar (see section 4.2.2), thus, it can easily be overgrown *in vitro* by contaminants. *P. infestans* strain 9.5.1 (compatible; race 1, 2, 3, 4, 6, 7) showed evidence of contamination as judged by bacterial colonies growing in the Rye A medium. Purification of the *P. infestans* culture was achieved either by plating the contaminated culture onto *P. infestans* selective media or by direct inoculation of the pathogen onto a potato leaf, or usually by a combination of the two methods. The selective medium was Rye A agar containing rifamycin, ampicillin and nystatin, and was recommended by Dr. Jenny P. Day of the Department of Biological Sciences, University of Bangor, Wales. The medium was prepared by adding 1ml of the antibiotic mixture (250mg rifamycin, 200mg ampicillin and 500mg nystatin dissolved in 10ml dimethyl sulphoxide and filter-sterilised) into 500ml warm autoclaved Rye A agar medium (50°C). Generally, the pathogen grows very slowly on this selective media (about 7 weeks). The second method was by direct inoculation of the contaminated *Phytophthora* culture onto a sterile potato plantlet growing in tissue culture (leaf-bridge bioassay; section 4.2.10). The potato cultivar Bintje was most suitable for this because

Bintje does not posses any *R* gene and the *P. infestans* strains should be able to infect this variety regardless of their compatibility status. Diseased leaf material was then transferred onto the selective medium (above), and checked regularly to monitor growth of *P. infestans*. New mycelial growth of the pathogen was then isolated as soon as it appeared and inoculated onto a fresh Rye A agar plate and maintained as described in section 4.2.2 above.

4.2.4 Maintaining the phytopathogenicity of potato pathogens

Subculturing the pathogens in appropriate medium for a long period usually results in the loss of pathogenicity. In order to maintain the virulence of the potato pathogens throughout the experimental period, pathogen-challenged tissues were used as an inoculum to produce actively virulent pathogen. Infected leaves (those developing disease symptoms) produced as described in section 4.2.3 were excised and placed onto a suitable medium (see section 4.2.2) and incubated at 15°C in the dark. The newly isolated cultures were then used for the experimental purposes and preserved for future use as described in section 4.2.6.

4.2.5 Preparation of *P. infestans* elicitor

P. infestans culture filtrate elicitor was prepared according to the method of Rohwer *et al.*, (1987). Culture filtrate consisted of materials secreted into Rye A Broth medium in which *P. infestans* was cultured for 6 weeks. Four 250ml Erlenmeyer flasks containing 100ml Rye A broth medium were inoculated with two mycelium plugs (0.5cm in diameter each) of *P. infestans* (see section 4.2.6). The flasks were incubated at 15°C with slow shaking (110rpm) in a temperature-controlled orbital shaker (S.H. Scientific, Northumberland). The medium was separated from the mycelia by filtering through

sterile filter paper (Whatman No. 1) under vacuum. The filtrate (about 400ml) was dialysed for 48h against several changes of distilled water (4L) before freeze drying. The dried material was collected and dissolved in about 4ml (1/100 the original volume) phosphate buffer (100mM, pH 7.0), centrifuged at high speed (18,500xg for 30min at 4°C; using a Beckman J2-21 centrifuge and JA-20 rotor) to pellet out undissolved materials and then filter-sterilised using a 0.22-μm sterile acrodisc (Gelman Sciences) and stored at –20°C until needed.

4.2.6 Preparation of mycelium plugs

Mycelium plugs were prepared from actively growing fungal cultures, excised when the size of the colony reached about half of the diameter of the petri dish (~2 weeks old for *P. infestans* and ~1 week old for *F. sulphureum* and *R. solani*). A sterile cork borer of 5mm diameter was used to excise a plug at the advancing edges of the fungal colony. Each plug was assumed to contain mycelium of approximately the same age.

4.2.7 Preserving viable pathogens

Storage on slopes under mineral oil was successfully used to preserve fungal pathogens during this study. After autoclaving, the appropriate medium (see section 4.2.3) was cooled to about 45°C and about 15ml was dispensed into 30 ml sterilised tubes. The tubes were laid at an angle until the medium set. A small plug of actively growing mycelium was placed at the bottom of the slope and incubated at 15°C in the dark until the mycelium covered almost the whole surface of each slant. Double sterile mineral oil was then added to the cultures to make a layer of mineral oil of about 1.5 cm above the agar. The tubes were then stored in appropriate racks at room temperature in the dark

until needed.

4.2.8 Pathogen retrieval from preserved cultures

Retrieval of the fungus from mineral oil preserved cultures was done by removal of a small aliquot of mycelium from under the oil phase. The mycelium was placed on sterile filter paper to remove excess mineral oil, and then inoculated onto plates of appropriate medium (see section 4.2.3) and left to grow in the dark at 15°C. During incubation, the plates were orientated at ~30° to the horizontal to allow excess oil to drain to allow the fungus to grow in the opposite direction. Newly grown mycelium was re-isolated from the edge of the colony and subcultured onto fresh medium.

4.2.9 Exposure of potato plants to *P. infestans*

Actively growing mycelium plugs of *P. infestans* prepared as described in section 4.2.5 were used to challenge potato plants growing in tissue culture. Four-week old plants (after subculturing in killner jars containing MS-medium) were chosen to be approximately the same size and appearance. Plants were challenged by placing a disk of the pathogen mycelium plug (5mm diameter) adjacent to the plant stem. The jars were then sealed with parafilm and the plants (challenged and control) incubated in the phytopathology lab under the same environment conditions described in section 4.1.2 except that the temperature was adjusted to 20°C (suitable for the plants and the pathogen). Two independent groups (eight - ten plants each) were used for each experiment. Treated and untreated samples from each group were collected at different time points (0, 4, 7, 15, 30 and 45 days post challenge), starting from leaf number four going upwards. These plant samples were used for isolating RNA for constructing the subtractive cDNA library (see section 4.3.18). Progress of growth and infection of

plants was recorded photographically using an Agfa e-photo 1680 digital camera.

4.2.10 Treatment of potato leaves with a *P. infestans* concentrated elicitor

The leaf-bridge bioassay method, which was developed in our lab (Abdullah, 1999), was used to study the responses of potato leaves to exposure to the *P. infestans* culture filtrate elicitor (prepared in section 4.2.5). Briefly, three-compartment petri dishes were used in which MS-medium (prepared as described in section 4.1.1) was placed in one compartment of each petri dish. Two detached leaves from the same position on the test plants, and of approximately the same size and appearance, were orientated in order that their petioles were immersed in the medium and the leaf tip laid over the dividing wall into the adjacent empty compartment (See section 6; Fig 6-1). The partition between compartments supported the leaf ("leaf-bridge") keeping it off the surface of the agar thus preventing liquid flow by capillary action. The leaves were elicited by applying 15µl of the concentrated culture filtrate elicitor to the top left half of the leaf and allowing the liquid to infiltrate the leaf tissues. The infiltrated leaves together with appropriate controls (infiltrated with sterile phosphate buffer; 100mM, pH7.0) were collected at different time points and used for the histochemistry and microscopy experiments (see section 4.4).

4.3 Molecular biology techniques

4.3.1 Preparation of RNase-free glassware and plasticware

Glassware was dry-sterilised by baking in an oven at 180°C for at least 8h or overnight, or by autoclaving after washing with DEPC-treated water. Plasticware was either

supplied as DNase and RNase free or was soaked in DEPC-treated water at 37°C for 2h to inactivate nucleases then rinsed several times with sterile water before autoclaving or heating to 100°C for 15min. Electrophoresis tanks used for RNA analyses were cleaned with detergent solution, rinsed in water, dried with ethanol, then filled with a solution of 3% (v/v) H_2O_2 and incubated for 10min at room temperature before rinsing thoroughly with DEPC-treated water.

4.3.2 Preparation of RNase-free water and reagents

RNase-free water was prepared by adding DEPC to deionised water to a final concentration of 0.1% (v/v), left at 37°C with stirring for at least 2h or overnight at room temperature and then autoclaved or heated to 100°C for 15min to decompose any residual DEPC. Whenever possible, solutions for molecular work were similarly treated with 0.1% (v/v) DEPC for at least 2h at 37°C or overnight at room temperature and then autoclaved prior to use. For solutions which could not be treated with DEPC (i.e. those containing chemicals with primary amine groups such as Tris) or autoclaved (i.e. volatile materials such as ammonium acetate), these were prepared in DEPC-treated water from unopened bottles to avoid any nuclease contamination.

4.3.3 Polymerase chain reaction (PCR)

In general, for a single 50µl PCR reaction the following components were added to a 0.5ml sterile microcentrifuge tube: 5µl 10x PCR buffer (100mM Tris-HCl; pH=8.3, 500mM KCl and 0.01% (w/v) gelatine); 5µl dNTP mix solution (2mM, each); 5µl $MgCl_2$ solution (25mM); 1µl of each primer (100pmole/µl); 100ng template DNA; and Zµl sterile distilled water (where Z = 50-sum of all other volumes). The components

were mixed and the tube then centrifuged briefly in a microfuge. The reaction tubes were then placed in the PCR instrument (Omn-E Thermal Cycler), and heated to 94°C for 3min. 2 units of Taq polymerase were then added to each microcentrifuge tube to start the reaction. The thermal cycle was run as follows; template denaturing; 94°C for 30sec, primer annealing; 55°C for 45s, and extension; 72°C for 2min. The cycle was repeated 30 times. On completion of the multiple cycles a further extension period of 7min at 72°C was included before the temperature was finally lowered to 25°C. The amplified products were then analysed on 0.8% (w/v) agarose gel and samples stored at –20°C.

To detect any contamination problems in the PCR reactions, two control reactions were used in parallel with the test samples, a reaction containing no template and another one containing no primers.

4.3.4 DNA Agarose Gel Electrophoressis

Electrophoresis of DNA was carried out according to the method of Sambrook *et al.*, (1989) using a horizontal agarose gel made up and submerged in 1x TAE running buffer (40mM Tris base, 40mM acetic acid, 1mM EDTA). 0.8% (w/v) agarose gel was prepared and ethidium bromide (EtBr) was added to both the gel and the tank buffer, to a final concentration of 1.0 μg/ml. One fifth the DNA sample volume of 6x loading dye (provided with the DNA markers) was added and the samples were loaded onto the gel along with 1Kb DNA markers (MBI Fermentas). Electrophoresis was carried out at 50V initially then raised to 80V until the bromophenol blue dye had migrated two-thirds the length of the gel. DNA bands were detected by the orange fluorescence of the EtBr-DNA complex under UV light (300nm). Gels were photographed through a red-orange

filter (Kodak 23A Wratten) and Polaroid Type 667 (3000 ASA) film or the images cuptured using a Bio-Rad Gel Doc 2000 system.

4.3.5 Purification of DNA from agarose gels

The band of interest was excised from the gel using a sterile scalpel blade and put in a 1.5 ml Eppendorf tube. The DNA was purified from the agarose gel according to the instructions in the Bio-Rad DNA purification kit. Briefly, the standard protocol adopted the following sequence of steps; (1) solubilizing the gels by adding 3x the gel volume of binding buffer (6M Sodium perchlorate; 50mM Tris-HCl, pH 8; 10mM EDTA, pH 8); (2) incubating at 37°C for several minutes; (3) adding 'silica fines' to bind the DNA; (4) pelleting the DNA-containing matrix; (5) washing the matrix two times with washing buffer (800mM sodium chloride, 40mM Tris-HCl pH 7.5, 4mM EDTA pH7.5); (6) washing the matrix with 80% (v/v) ethanol to remove any salts; and finally, (7) eluting the DNA from the matrix by adding sterile distilled water. The DNA was concentrated by precipitation with ethanol (see section 4.3.13).

4.3.6 Cloning PCR products

All cloning (GM) work was subject to appropriate ACGM risk assessment to permit this research to be carried out at the Durham GM centre 40.

The TOPO™ TA cloning kit (Invitrogen) was used to clone PCR products as well as purified DNA bands (amplified by PCR) from agarose gels. The DNA fragments were directly inserted into the linearised plasmid vector (PCR II-TOPO) with a single overhanging 3'thymidine (T) residue by adding the DNA (1-4µl) to 1µl PCR II-TOPO vector and adding sterile water to 5µl if needed, mixed gently and incubated for 5min at

room temperature (TOPO™ Cloning reaction). Competent cells [TOPO One-Shot (50µl) or DH5α (100µl)] were transformed by adding 2-5µl of the TOPO™ cloning reaction mixing gently and incubating on ice for 30min. The cells were then heat shocked for 30s at 42°C without shaking. Immediately the tube was transferred to ice and incubated for 2min. 250µl of SOC medium at room temperature were added to each vial and the vials were shaken horizontally at 37°C for 30-60min. 50–200µl from each transformation reaction were spread onto selective plates (LB-Agar containing 100µg/ml ampicillin, and 60µl X-Gal (40mg/ml) was added to each plate and spread evenly) and incubated overnight at 37°C. Blue/white screening was used to detect the positive clones with inserted DNAs and the presence of the inserted DNAs confirmed by restriction analysis of the isolated plasmids (sections 4.3.8 and 4.3.9).

4.3.7 Bacterial culture preparation and storage

LB-agar medium was used as a general purpose growth medium for bacterial strains and when applicable an appropriate antibiotic was added. Bacterial colonies for regular use were stored on agar plates sealed with parafilm and inverted at 4°C for up to 3-4 weeks. For long-term storage clones were stored as suspensions in glycerol. Bacterial lawns or 'thick streaks' were grown from a single colony on an appropriate selective medium and then transferred into a screw top glass vial containing 1ml aliquots of LB broth, then 80% (v/v) sterile glycerol was added to a final concentration of 40% (v/v).After vortex mixing thoroughly the clones suspended in glycerol were stored at –80°C.

Bacterial suspension cultures were prepared by inoculating 7-10ml of LB broth medium with a single colony (picked from an agar plate) or 5-10µl of a previously frozen clone stored in glycerol. For plasmid-containing strains, the medium was supplemented with

100μg/ml ampicillin. All cultures were incubated overnight at 37°C on an orbital shaker running at 160rpm.

4.3.8 Plasmid isolation

Bacterial cultures (7-10 ml) were grown as described above (section 4.3.7). The cells were harvested by centrifugation at 6000rpm for 5-10min, then the supernatant decanted off and excess media was removed from the pellets by inverting the tube over paper towels for a few minutes.

Two methods were used to isolate plasmid DNA during this study a) the Wizard Plus SV Plasmid purification kit (Promega) following the manufacturer instructions, and b) the alkaline lysis method according to the procedure described by Sambrook *et al.*, (1989) as follows: the bacterial pellets were resuspended in 100μl of ice-cold Solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCl; pH 8.0) by vigorous vortexing and transferred into a 1.5ml Eppendorf tube. 200μl of freshly prepared Solution II (0.2N NaOH, 1% SDS) were added, and the contents were mixed by inverting the tube rapidly five times. The suspension was incubated in ice for 5min. Then, 150μl of ice-cold Solution III (a mixture of 60ml 5M potassium acetate, 11.5ml glacial acetic acid and 28.5ml H_2O) were added and mixed thoroughly by inversion and gentle vortexing for 10s. The tube was incubated in ice for 5min and then centrifuged at 4°C for 10min at 14,000xg. The supernatant was transferred into a fresh 1.5ml Eppendorf tube and an equal volume of phenol:chloroform was added and mixed thoroughly by vortexing. After centrifuging at 14,000xg for 2min at 4°C, the supernatant was transferred to a fresh Eppendorf tube. Two volumes of ethanol were added to precipitate the plasmid DNA at room temperature for 2min. The DNA was then recovered by centrifugation at

14,000xg for 5min at 4^0C. The supernatant was decanted and the DNA pellets were washed by rinsing with 1ml 70% (v/v) ethanol. The supernatant was removed as described above, and the pellet of nucleic acid was allowed to dry in the air for 5-10min. The DNA was redissolved in 100µl of Tris-EDTA buffer (pH 8.0) containing DNAase-free pancreatic RNAase (20µg/ml). 5µl of the plasmid solution were run on 0.8% (w/v) agarose gel to check their purity. Purified plasmids were then restricted with appropriate restriction enzymes to check for the integrity and size of inserts and the plasmids were either sequenced immediately or stored at –20°C until required.

4.3.9 Restriction analysis

Restriction of DNA was carried out by mixing the following components; 5µl plasmid DNA (~1-5µg); 12µl H_2O; 2µl appropriate restriction enzyme buffer (10X); and 1µl of the appropriate restriction enzyme (10U/µl). This mixture was incubated in a water bath at 37°C for 1-2h after which the restricted plasmid DNA was checked for the presence of insert by electrophoresis on a 0.8% (w/v) agarose gel.

In some circumstances (for example, where the intensity of the restricted band was very low because the size of the insert was too small) the volume of plasmid DNA was increased and the H_2O was decreased in order to see a clear band in the gel.

4.3.10 Isolation of total RNA from potato tissues and *P.infestans*

Total RNA was isolated using Trizol reagent (Sigma), a mono-phasic solution of phenol and guanidine isothiocyanate. According to the manufacturer instructions, during sample homogenisation, Trizol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. The method used was based on the

single-step RNA isolation developed by Chomczynski and Sacchi, (1987). 500mg of frozen potato leaf tissues collected at different time points (as described in section 4.2.8) and *P. infestans* mycelium were each ground, separately, to a fine powder with liquid nitrogen in a mortar and pestle. Once the nitrogen had boiled off, the ground samples were then homogenised in 5ml Trizol reagent. The homogenised samples were incubated for 5min at room temperature to permit the complete dissociation of nucleoprotein complexes. Prior to the addition of chloroform (200µl/ml of Trizol reagent), the homogenised samples (about 5ml) were aliquotted into Eppendorf tubes (1ml each). Addition of chloroform was followed by vigorous shaking and centrifugation to separate the solution into an aqueous phase, an interphase and an organic phase. The RNA remained exclusively in the aqueous phase. After recovery of the aqueous phase, the RNA was recovered by precipitation with isopropanol, washed with 75% (v/v) ethanol, briefly dried (5-10min) and finally dissolved in RNase-free water. The isolated RNA samples were then checked for purity and integrity (section 4.3.11). Pure and intact total RNA samples were stored at – 80°C until needed.

4.3.11 Assessment of RNA quality

The integrity of total RNA was assessed by visualisation of intact 18S and 28S ribosomal RNA (rRNA) bands by running the isolated RNA samples on 0.8% (w/v) denaturing agarose gels. The formaldehyde gels were prepared according to the method described by Sambrook *et al.*, (1989). The samples were prepared by mixing 4.5µl RNA sample (up to 20µg) with 2µl of 5x gel-running buffer (50mM sodium acetate, 0.2M MOPS (morpholinopropanesulfonic acid) and 5mM EDTA pH 8.0), 3.5µl formaldehyde and 10µl formamide. The samples were incubated at 55°C for 15min then

loaded onto the denaturing agarose gel after addition of 4µl of 6x loading dye. The size of distinct 18S and 28S rRNAs are approximately 2000 and 4500 bases, respectively. Appearance of sharp 18S and 28S rRNA bands indicated the integrity of the isolated RNA; diffuse and smeared bands were an indication of RNA degradation. The appearance of distinct high molecular weight bands was indicative of possible DNA contamination in the isolated RNA. Low molecular weight 5S rRNA and tRNA sometimes appeared as faster migrating diffuse bands.

The purity of RNA was checked by absorbance at 260nm and 280nm. The desired ratio of absorbances (A260/A280) should be >1.8. In addition, samples were routinely scanned between 240 and 320nm to confirm a characteristic clean spectrum without absorbance due to contaminants such as polyphenols, phenols and proteins.

4.3.12 Quantitation of RNA Preparation

RNA samples were appropriately diluted (1µl RNA in 99µl DEPC-water) with sterile DEPC-treated distilled water and the absorbance measured at 260nm against DEPC-treated distilled water using an acid-washed quartz microcuvette. RNA concentration was calculated using the following formula:

RNA µg/ml = A_{260} x 40 x DF

Where DF = dilution factor.

4.3.13 Purification of Poly (A^+) RNA

At the start of this study a Promega 'PolyATtract' mRNA isolation kit based on Oligo (dT) immobilised on magnetic beads, was used several times, but unfortunately without

success as judged by absence of any amount of poly (A^+) RNA. Subsequently, a Dynabeads mRNA purification kit was used following the manufacturer instructions. The use of the Dynabeads mRNA purification kit also relies on base pairing between the poly (A^+) residues at the 3' end of most messenger RNA and the oligo dT residues covalently coupled to the surface of the Dynabeads oligo $(dT)_{25}$. Other RNA species lacking a poly (A^+) tail do not hybridise to the Dynabeads oligo $(dT)_{25}$ and are readily washed off. The mRNA is captured by the Dynabeads oligo $(dT)_{25}$ and washed thoroughly using a magnetic separator. The mRNA is eluted from the beads by using a low-salt buffer (10mM Tris-HCl; pH 7.5). Briefly, 75µg total RNA (100µl) was added to 100µl binding buffer (20mM Tris-HCl pH 7.5, 1M LiCl and 2 mM EDTA) and heated to 65°C for 2min. The denatured RNA was added to a tube contained the Dynabeads oligo$(dT)_{25}$ in 100µl binding buffer, and annealed by rotating the tube for 5min at room temperature. Using a magnetic separator, the captured mRNA was washed twice with 200µl washing buffer (10mM Tris-HCl pH 7.5, 0.15M LiCl and 1mM EDTA). The mRNA was eluted from the Dynabeads by the addition of 10µl 10mM Tris-HCl and heating to 65°C for 2min. These steps were repeated several times to isolate sufficient mRNA enough for checking the purity and starting the subtractive hybridisation technique (section 4.3.18).

The purity of the isolated poly (A^+) samples was checked by electrophoresis on 0.8% (w/v) denaturing formaldehyde agarose gels to monitor for any traces of the two major ribosomal RNAs (18S and 28S). The RNA concentrations were determined in the same way as described for the total RNA as described in section 4.3.12.

It is unclear why the Promega kit failed to work, though differences in the size of oligo (dT), concentration of oligo(dT) and minor differences in the handling procedure may

have contributed.

4.3.14 Concentrating Poly (A^+) RNA

In order to optimise the initial concentration of poly (A^+) required to synthesise cDNA as required in the Clontech PCR Select procedure (section 4.3.18), the two mRNA preparations (tester and driver) were concentrated to 2µg/µl using ethanol precipitation. In order to follow the RNA precipitate during the washing steps and prevent losses during handling 'Pellet Paint Co-precipitant' (Novagen), a coloured compound which coprecipitates with RNA during ethanol precipitation, was used. To a sample of poly (A^+) RNA, 2µl 'pellet paint' and 0.1 volume of 3M sodium acetate (pH 5.2) was added and thoroughly mixed. This was followed by the addition of two volumes of 100% ethanol. The mixture was vortexed briefly and incubated at room temperature for 2min. The resulting precipitate of RNA was spun at 14,000xg for 5min at 4°C, followed by washing twice with 75% (v/v) ethanol. After each washing, the precipitate was collected by centrifugation at 14,000xg for 5min at 4°C. The pellet was air dried for 5min and then dissolved in an appropriate volume of RNase-free water to a final concentration of 2µg/µl. The concentrated poly (A^+) samples were immediately frozen in liquid nitrogen and stored at –80°C until required.

4.3.15 Synthesis of digoxigenin-labelled probes

For screening the subtracted library with forward, reverse and characterised sequence probes, high specific activity, labelled probes were synthesised by digoxygenin (DIG)-labelling of DNA fragments generated by PCR (see section 7.5). The incorporation of DIG-dUTP into the PCR products was carried out according to the instructions provided with the PCR DIG Probe synthesis kit (Roche, Mannheim, Germany). The PCR reaction

mix comprised 2µl (10x) PCR buffer (100mM Tris-HCl; pH=8.3, 500mM KCl containing 15mM $MgCl_2$), 2µl DIG-labelling mix, 1µl (100 pmole/µl) of each appropriate primer (M13 forward and reverse, SP6 and T7 or nested primers 1&2R), 1µl DNA (0.5-1µg) and sterile distilled water to a total volume of 20µl. After mixing and centrifuging the components, PCR was performed using the same conditions described in section 4.3.3.

4.3.16 Evaluation of probe labelling efficiency

It was important to check the efficiency of each labeling reaction by determining the amount of DIG-labelled product. This facilitated addition of the correct amount of probe to the hybridisation solution (too much probe leads to serious background problems and too little probe leads to little or no hybridisation signal). To estimate the labeling efficiency of the PCR-generated probe, 2µl of each PCR product (both DIG-labelled and unlabelled (i.e., amplified in the absence of DIG-dUTP) versions of the experimental probe) were run on a 0.8% (w/v) agarose gel.

The presence of DIG in the DNA gives it a higher mass than unlabelled DNA as well as slowes the polymerase reaction, so a highly-labelled probe ran slower in the gel and the intensity of the stained DIG-labelled probe should be equal to (or slightly less than) the intensity of the unlabelled probe DNA.

4.3.17 Purification of DIG-labelled Nucleic Acids

DIG-labelled probes were purified from PCR reagents and contaminants using the wizard PCR Preps DNA Purification System (Promega) following the manufacture's instructions. Briefly, the PCR amplified mixture (18µl) was added to 100µl purification

buffer and 1ml binding resin then vortexed briefly three times over a 1min period. The resin plus bound DNA was pushed into a minicolumn (attached to a syringe) using a syringe plunger then the column was washed by passing 80% (v/v) isopropanol solution through it. The remaining isopropanol was removed by centrifugation after transferring the minicolumn to a 1.5ml microcentrifuge tube. The DNA fragment was eluted from the minicolumn with 20µl sterile distilled water and stored in the microcentrifuge tube at –20°C until needed.

4.3.18 Preparation of a subtracted cDNA library from potato plants 'var. Stirling'

4.3.18.1 Strategy for constructing the subtracted cDNA Library

A subtracted cDNA library from *P. infestans*-challenged Stirling plants was constructed using the suppression subtractive hybridisation technique according to the Clontech PCR-Select™ cDNA Subtraction Protocol based on the original method described by Diatchenko *et al.*, (1996) . The principle of the PCR-select technique is complex and the reader is directed to the Clontech web site (http://www.clonetech.com) and the original paper for detailed information about the method by Diatchenko *et al.*, (1996).

In the current study, two subtractions were made (i.e. forward and reverse subtractions). In the forward subtraction two populations of mRNA from two different plant materials being compared i.e. pathogen-treated material is referred to as the 'tester' and the corresponding untreated (control) material is the 'driver' from Stirling plants were used. In the reverse subtraction the driver was used as a tester and the tester as a driver (see below). The two subtraction were carried out in order to isolate gene sequences differentially expressed between the two experimental materials – in the forward

subtraction gene sequences expressed to a higher level in the pathogen-treated material compared with the control (untreated) material are preferentially amplified. In the reverse subtraction gene sequences down regulated in the pathogen-treated material compared with the control (untreated) material are preferentially amplified. The corresponding library clones are isolated using either of these subtracted probes.

The strategy for constructing the subtracted cDNA library is outlined schematically in figure 4-1 below and is discussed in more detail in section 7. Briefly, identical amounts of total RNA isolated from each time point (0, 4, 7, 15, 30 and 45 days post pathogen treatment) were pooled together. Total RNA isolated from *P. infestans* was mixed with the total RNA from the control plant tissue to create the control RNA population (to subtract any pathogen sequences present in the tester). Poly (A^+) RNA (mRNA) was then isolated from each pooled total RNA population as described in section 4.3.13. 2μg of each poly (A^+) RNA was used for the synthesis of the cDNAs, followed by *Rsa*I digestion (a four-base-cutting restriction enzyme that yields blunt ends). The tester cDNA was then subdivided into two portions, and each was ligated with a different cDNA adaptor. The ends of the adaptor do not have phosphate groups, so only one strand of each adaptor attaches to the 5' ends of the cDNA. The two adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in. (See appendix A for sequences of the adaptors and the primers used). Two hybridisations were then performed.

In the first hybridisation, an excess of driver was added to each sample of tester: the samples were heat denatured and allowed to anneal, generating the a, b, c, and d-type molecules in each sample (figure 4-2). The single stranded type a molecules were

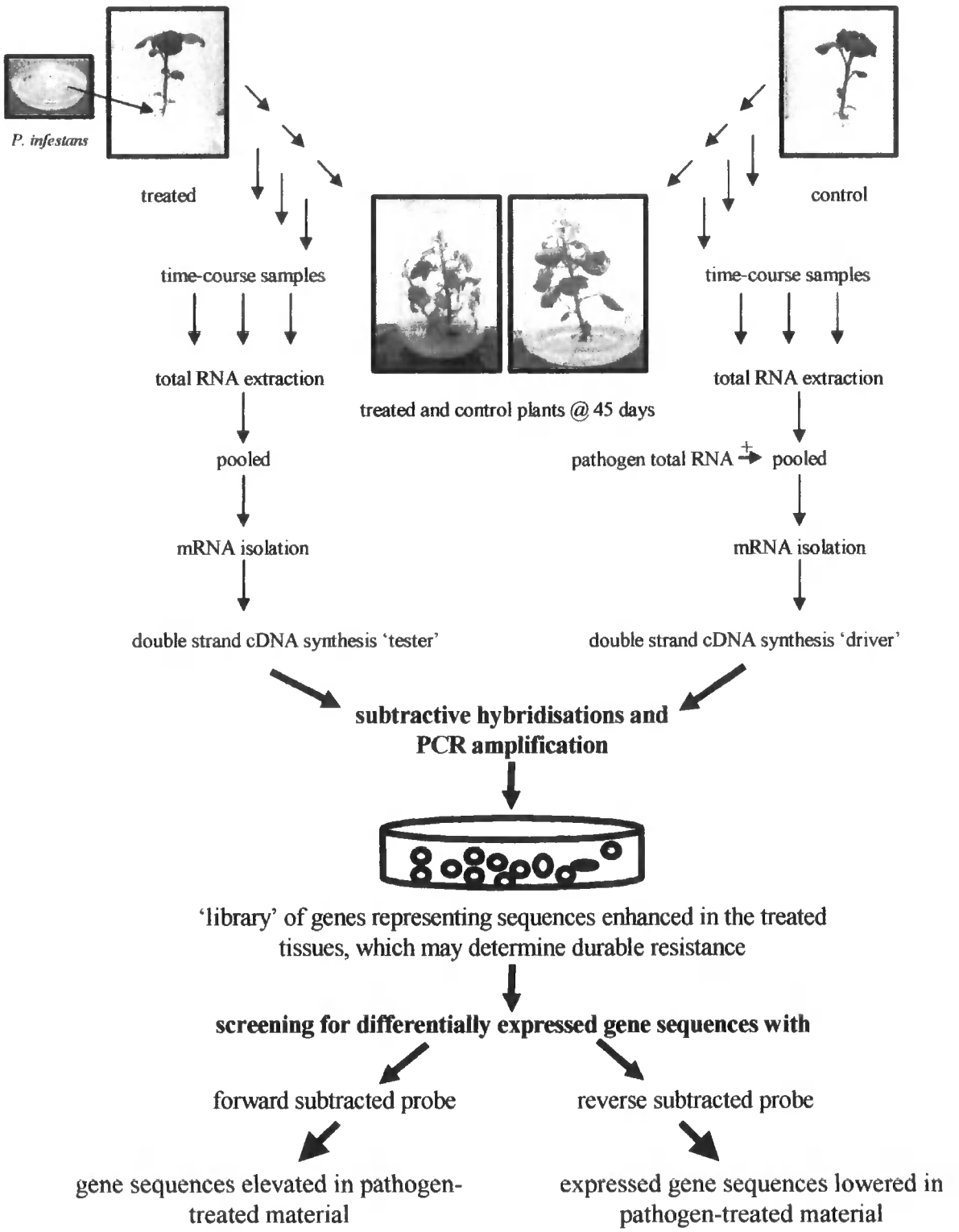


Figure 4-1 Flow chart of the strategy used to construct the subtracted library from *P. infestans* challenged 'Stirling' potato plants

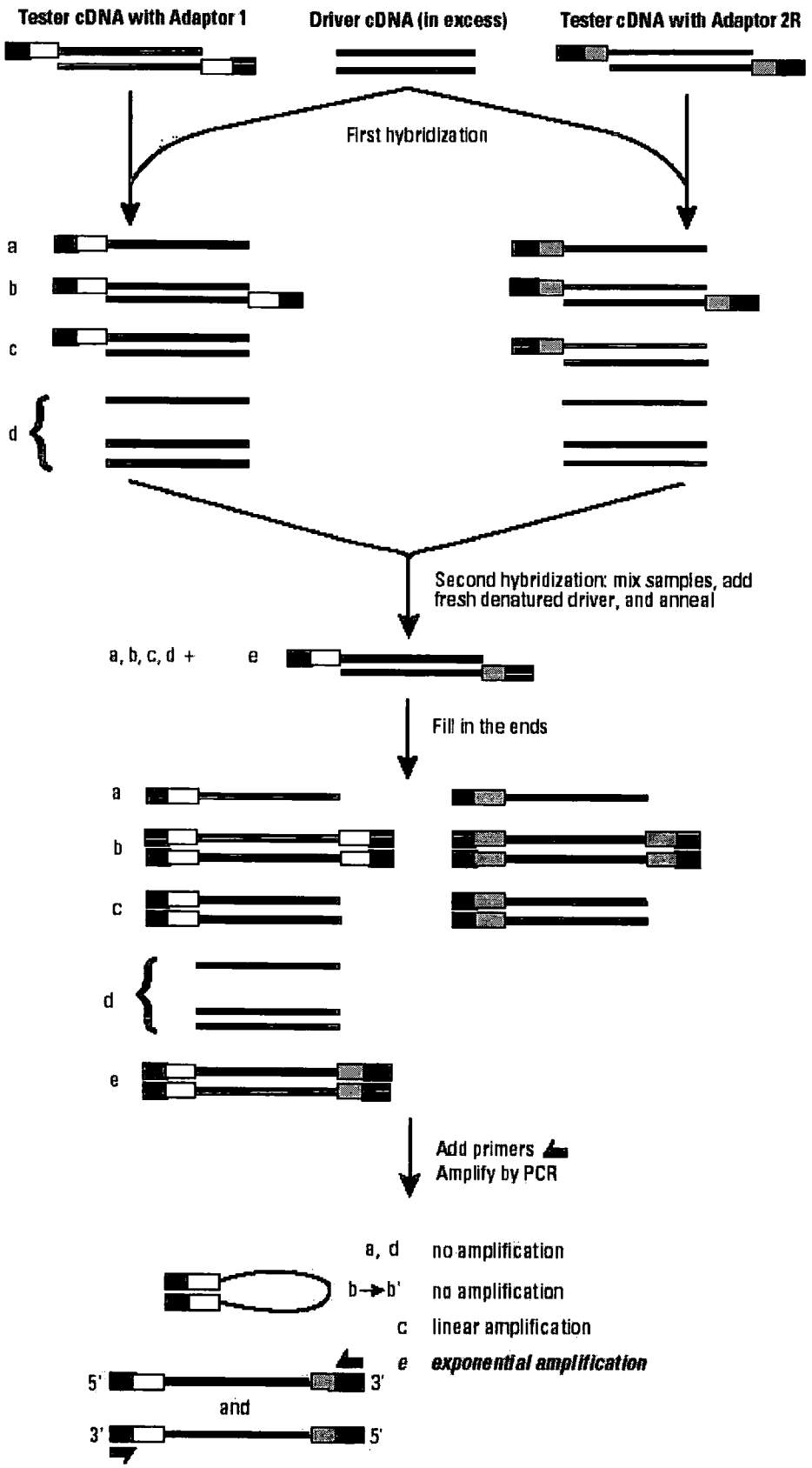


Figure 4-2 The products of the first and second hybridization processes (adapted from Clontech user manual).

significantly enriched for differentially expressed sequences, as cDNAs that are not differentially expressed form type c molecules with the driver.

In the second hybridisation, the two primary hybridisation samples were mixed together without denaturing. Only the remaining equalized and subtracted single stranded tester cDNAs can reassociate and form new hybrids, type e. These new hybrids were double stranded tester molecules with different ends, which correspond to the sequences of adaptors 1 and 2R (appendix A). Fresh denatured driver cDNA was added (again, without denaturing the subtraction mix) to further enrich fraction e for differentially expressed sequences. After filling in the ends by DNA polymerase, the type e molecules (the differentially expressed tester sequences) have different annealing sites for the nested primers on their 5' and 3' ends. The entire population of molecules was then subjected to PCR to amplify the desired differentially expressed sequences. Finally the PCR products were cloned into the PCR II vector following the procedure described in section 4.3.6.

In the reverse subtraction, the 'driver' cDNA was used as a tester without adding the pathogen total RNA and the 'tester' cDNA was used as a driver to produce amplified subtraction products used as probes in the differential screening procedure (4.3.18.4).

4.3.18.2 Growing and transferring the subtracted colonies to nylon membrane (colony arrays)

Randomly selected colonies from the subtraction libraries were picked and grown in 100µl of LB-broth medium containing 100µg/ml ampicillin in a 96-well microplate at 37°C for at least 5h with shaking. Nylon membranes (Hybond-NX; Amersham Biosciences Ltd.) were placed onto LB-agar plates containing 100µg/ml ampicillin. A 48 pin metal inoculator (constructed in University of Durham engineering workshop)

was used to transfer aliquots of each bacterial culture onto the nylon membranes. The membrane plates were then incubated at 37°C overnight. Several replica membranes were prepared for hybridisation with forward and reverse subtracted probes as well as with characterised sequence probes.

For long-term storage, sterile 80% (v/v) glycerol was added to the bacterial cultures in the microplates to a final concentration of 40% (v/v) and mixed by pipetting up and down then the plates were sealed and stored at –80°C until needed.

4.3.18.3 Preparation of the nylon membranes for hybridisation

Colonies on nylon membranes were lysed and the DNA denatured by placing the membranes (colony side up) on Whatman 3MM paper soaked with denaturing solution (0.5M NaOH, 1.5M NaCl) for 15min. Membranes were neutralised by transferring to Whatman 3MM paper soaked with neutralising solution (1.0M Tris-HCl, pH 7.5; 1.5M NaCl) for 15min and then transferred onto filter paper soaked with 2x SSC for 10min, finally the membranes were sandwiched between two sheets of dry Whatman paper and the DNA crosslinked to the membrane with UV-light (150 mJoule) (BioRad cross-linker). The membranes were treated with proteinase K (2mg/ml) for 1h to digest away any interfering proteins before hybridisation with DIG-labelled probes.

4.3.18.4 Hybridisation of DIG-labelled probes to colony arrays

Hybridisations were carried out according to the instruction manual supplied with the DIG luminescent detection kit in a hybridisation incubator (Techne Hybridiser HB-1D). three membranes were placed in a hybridisation bottle and pre-hybridised for 2h at 68°C in 30ml pre-hybridisation solution (5x SSC, 0.1% (w/v) N-lauryl-sarcosine, 0.02% (w/v) SDS, 1% (v/v) blocking reagent and 0.3mg/ml oligonucleotides corresponding to

the nested primers and the adaptor sequences (appendix A). Then 5-25ng/ml denatured probe, prepared by boiling at 95°C for 5min and rapidly cooled on ice, were mixed with fresh 20ml pre-hybridisation solution, pre-warmed to 68°C and added to the membranes. Membranes were incubated in the hybridisation mix overnight at 68°C after which the hybridisation solution was recovered into a falcon tube and stored at –20°C for future use. The membranes were then washed twice with 2x SSC, 0.1% (w/v) SDS for 5min at room temperature and twice in 0.5x SSC, 0.1% (w/v) SDS for 15min at 68°C with gentle agitation. Finally the probe-target hybrids were detected following the procedure described below (section 4.3.18.5).

4.3.18.5 Detection of DIG-labelled probes

The detection of probe-target hybrids was carried out according to the instruction manual supplied with DIG luminescent detection kit following, mainly, a three-step process. In the first step, the membranes were blocked, by gentle agitation for 30-60min in blocking solution (1% (w/v) blocking reagent in maleic acid buffer (100mM maleic acid, 150mM NaCl; pH 7.5)), to prevent non-specific interaction of the antibody with the membrane. In the second step, the membranes were incubated with a diluted alkaline phosphatase-conjugated antibody, specific for digoxigenin, (anti-digoxigenin-AP 1:10,000 (v/v) in blocking solution) to recognise the DIG molecule on the labelled hybrid. Finally, the membranes carrying the hybridised probe and bound antibody conjugate were reacted with a chemiluminescent substrate (1% CSPD® in detection buffer (100mM Tris-HCl, 100mM NaCl; pH 9.5) after washing twice with washing buffer (0.3% (v/v) Tween 20 in maleic acid buffer) for 15min each. The membranes were then exposed to X-ray film (RX type; Fuji, Tokyo, Japan) for 15-25min to record the chemiluminescent signal.

4.3.18.6 Stripping membranes for reprobing

Membranes used for an initial hybridisation experiment were stripped of DIG-labelled probe, with almost no loss of immobilised target sequences, and were then re-hybridised with a different probe. The mild stripping procedures were used to allow multiple, sensitive re-probing experiments. The membranes were rinsed thoroughly in H_2O at room temperature then incubated twice in 0.2M NaOH/0.1% (w/v) SDS for 20min at 37°C, finally rinsed in 2x SSC (0.3M NaCl; 0.03 M sodium citrate; pH 7.0) for 5min. The membrane was then used directly for hybridisation with a different probe or stored in 2x SSC until needed.

4.4 Histochemistry and Microscopy

4.4.1 Histochemical detection of callose deposition

Formation of callose was determined by staining potato leaves with aniline blue according to the method of Dietrich *et al.*, (1994). Leaves for callose deposition examination were collected 7 days post elicitor treatment as described in section 4.2.10 and were cleared by boiling for 2min in alcoholic lactophenol [2:1(v/v) 95% (v/v) ethanol: lactophenol (phenol, glycerol and lactic acid (1:1:1; v/v/v))], rinsed in 50% (v/v) ethanol, and then rinsed in water. Cleared leaves were stained for 1h at room temperature in a 0.01% (w/v) solution of aniline blue in 0.15 M K_2HPO_4. Stained leaves were mounted on slides in 70% (v/v) glycerol in water and examined under ultraviolet epifluorescence using a Nikon Optiphot-2 microscope (excitation filter UV-2A, Ex 330-380nm; Dichroic mirror, DM 400nm; and barrier filter, BA 420nm).

Micrographs were obtained using a Nikon Coolpix 950 digital camera, MCD lens 0.82-0.29X fixed to a Nikon Optiphot-2 microscope. Callose deposition was indicated by a

yellow fluorescence.

4.4.2 Histochemical detection of superoxide anions

Generation of superoxide anions (O_2^-) was detected by infiltrating leaves with nitroblue tetrazolium solution (NBT) following the method described by Schraudner *et al.*, (1998). Elicitor-treated leaves (3, 6 and 12h post treatment) were vacuum-infiltrated with a 0.1% (w/v) solution of nitroblue tetrazolium in 50mM potassium phosphate (pH 6.4) containing 10mM sodium azide. Infiltrated leaves were incubated in the light for 30min and then immersed in a mixture of lactic acid, phenol and water (1:1:1, v/v/v) for 2 days at room temperature in the dark. Cleared leaves were mounted on slides in 70% (v/v) glycerol in water and examined under normal illumination light using a Nikon Optiphot-2 microscope. Generation of superoxide anions was indicated by a blue coloration. Micrographs were obtained using a Nikon Coolpix 950 digital camera fixed to a Nikon Optiphot-2 microscope.

4.4.3 Histochemical detection of hydrogen peroxide

Histochemical detection of hydrogen peroxide (H_2O_2) generation was performed by vacuum infiltrating potato leaves with 3,3'-diaminobenzidine (DAB) according to Schraudner *et al.*, (1998). Elicitor-treated leaves (3, 6, 12, 24 and 48h post treatment) were vacuum-infiltrated with a 0.1% solution of 3,3'-diaminobenzidine-4 HCl (DAB) in 10mM MES (2-(N-morpholino) ethanesulphonic acid), pH 6.5. Infiltrated leaves were incubated in the light for 30min, then cleared by boiling in ethanol (96% (v/v)) for 10min according to the method described by Thordal-Christensen *et al.*, (1997). The DAB reaction was examined under normal illumination light using a Nikon Optiphot-2 microscope. The presence of H_2O_2 was indicated by a reddish-brown coloration.

Micrographs were obtained using a Nikon Coolpix 950 digital camera as described above.

4.4.4 Histochemical detection of pathogen-infected tissues

Leaves for microscopic examination of *P. infestans*-infected tissue were first cleared and then stained according to the method described by Dietrich *et al.*, (1994). Selected leaves were harvested and vacuum infiltrated with lactophenol for 10min and then incubated at room temperature with at least three changes of lactophenol overnight or until the leaves were clear. To stain the fungal tissue, cleared leaves were incubated for 1h at room temperature in 0.06% (w/v) aniline blue in lactophenol. Stained tissues were examined using Nikon Optiphot-2 microscope and micrographs were obtained using a Nikon Coolpix 950 digital camera as described above.

4.5 Microarraying

This part of the project was carried out in collaboration with Dr Ron Croy.

4.5.1 Large-scale preparation of cDNA probes for arraying

Plasmids from the subtraction library clones were prepared as described previously (section 4.3.8). A number of characterised potato clones were obtained from other sources, as detailed in section 3.4, including acidic and basic glucanases and chitinases, the pathogenesis related protein PR-1, lipoxygenase, polyphenol oxidase, phenylalanine ammonia lyase, alcohol dehydrogenase and extensins. Clones preserved as glycerols or in arrays in 96-well microplates, were inoculated directly into deep well microplates containing 1ml LB-amp broth (100µg/ml ampicillin) using a sterile 48 pin replicating fork. The plates were sealed with a sterile silicon mat and the plate incubated at 37 °C

on an orbital shaker at 600rpm overnight. The cells were harvested by centrifuging the culture plates at 4,100rpm (2,800g) for 10min at 4°C in a Jovan BR4 centrifuge. The supernatants were aspirated using a vacuum line and the pelleted cells resuspended in 500µl of 1mM EDTA, pH 8.0 using a vortex mixer. The cells were lysed by heating at 95 °C for 5min and the cell debris removed by centrifugation at 4,100rpm (2,800g) for 30min at 4°C. 200µl of the supernatants containing the released plasmids were transferred to a fresh microplate and stored frozen at -20°C.

10µl aliquots of each of the purified mini-prepped plasmids (section 4.3.8) or as released from lysed clone cultures were diluted into 200µl of imM EDTA, pH 8.0. 20µl aliquots of the deluted mini-prepped plasmids containing about 1-5ng plasmid were used for PCR amplifications.

4.5.2 Amplification of cloned cDNA fragments

PCR reactions were carried out on aliquots of the plasmids prepared from the selected clones. 100µl reaction mixtures were prepared in 96-well thin wall PCR plates (Greiner thermoquick plates, # 651570) as follows: 50µl Taq polymerase enzyme mix (Biomix Red 2x), 0.5µl of each primer (0.5µM each), 29µl water. Biomix Red (Bioline) was a 2x concentrate providing the following components: 125mM Tris-HCl pH=8.8, 32mM ammonium sulphate, 0.02% Tween 20, 2mM dNTP's, 0.05 units Taq polymerase/µl, 3.0mM $MgCl_2$. Primers were purchased from Helena Biosciences (Genset Oligos) with the following sequences (Table 4.1).

Taq mix, primers and water were combined in each well of a 96 well PCR microplate using a 8-channel multipipette. The reactions were held at 0°C until after the final

Table 4.1 Primer sequences used for amplifying the cDNA inserts from purified plasmids.

primer designation	sequence	Tm
30-mer forward primer	5'-CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA-3'	73
30-mer reverse primer	5'-AAC AAT TTC ACA CAG GAA ACA GCT ATG ACC-3'	62

Table 4.2 PCR amplification programmes

step	programme 1		programme 2	
initial denaturation	T = 94°C	4 min	T = 94°C	2 min
	x 1 cycle		x 1 cycle	
cycle denaturation	T = 94°C	30 secs	T = 94°C	30 secs
primer annealing	T = 55°C	45 secs	T = 55°C	45 secs
extension	T = 72°C	1min	T = 72°C	1min
	x 35 cycles		x 5 cycles	
final extension	-	-	T = 72°C	7min
interrupt	after 35 cycles		-	
hold	-	-	T = 10°C	until required

addition of 20µl of the templates and thorough mixing by multichannel pipette. The plate was then transferred to an Eppendorf Mastercycler Gradient 5331 thermocycler programmed with the settings shown in Table 4.2. Two programmes (Table 4.2) were used sequentially to allow extra nucleotides to be added at the end of the first set of cycles to increase the yield of product. After cycling was completed the PCR plates were sealed and stored at -20 °C until required. 1-5µl of each sample was diluted to 10µl with 1 x TBE containing marker dyes and 3% glycerol and analysed on a FAST gel stretch system (Abgene) (see section 8, figure 8.1).

4.5.3 Purification of PCR products

PCR products in 96-well microplates were purified using either a PCR cleanup kit (Whatman, # 7905-0002) based on DNA binding to glass fibre filters or Wizard MagneSil magnetic silica beads (Promega, #A1930) using a Magnabot 96 well magnetic separation device (#V8151) used according to the manufacturers instructions except in both cases elution of the DNA from the filters or beads was carried out with only half the recommended elution volume of sterile water (50µl). The purified DNA's in water were stored at -20°C. The concentration of DNA's recovered was estimated either by fluorescence assay using Hoescht 33258 benzimidazole fluorescent dye as decribed by Sambrook and Russell, (2001) or by ethidium bromide fluorescence following electrophoresis. For arraying 10µl aliquots of the DNA samples were diluted 1:1 with DMSO.

4.5.4 Microarraying

Microarrays were printed on coated glass slides using a Genomic Solutions GeneTAC

G3 workstation equipped with a 48-pin printing tool. Samples for arraying were made 50% in dimethyl sulphoxide and transferred to 384-well microplate for printing. Purified, PCR amplified probes were printed as 8x8 matrices with quadruplicated samples as illustrated in figures 8.2 & 8.3 (section 8). Overall the array dimensions were (~20mm x ~40mm). To establish microarray formats and to check the performance of the microarrayer, test arrays were printed with food dyes diluted 1:1 with DMSO on agarose coated slides as follows. Standard microscope slides were thoroughly cleaned, acetone washed and coated with 0.5% (w/v) agarose in 50% (v/v) methanol. Once the agarose had set the slides were transferred to a Bio-Rad gel drier and the agarose dried down to an invisible film. DNA microarrays were printed on slides with aminopropylsilane coating (APS) from Sigma (Sigmascreen APS slides, # S9936) or Corning (UltraGAPS, #40016).

After printing the slides were allowed to dry for 30min and then prior to hybridisation were processed as follows. Arrays were rehydrated in steam by inverting the slide over a container of hot water (95-100°C) for 5s. Immediately the slide was transferred, DNA side up, to a heating block at 100°C to snap-dry it for 5-10s. The DNA was then immobilised by heating the slides at 80°C for 2-4h in a clean oven (Techne, HB-1D). Printed slides were then stored desiccated at room temperature until used for hybridisation. The DNA on test arrays were visualised by staining in a 1:10,000 dilution of SYBR green I dye (Sigma #9430) in TBE buffer for 3-5min, washed briefly in TBE buffer and then in water before drying the slide by centrifugation at ~1000g for 5min. Slides at various stages in the processing and storage kept singly or in pairs (back to back) in Falcon 50ml centrifuge tubes.

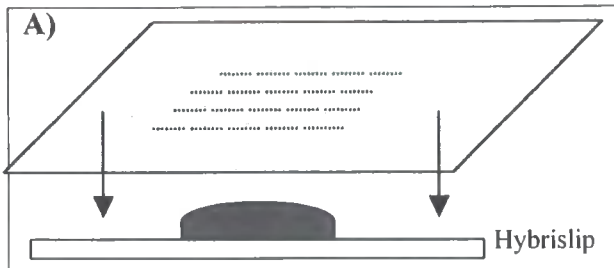
4.5.5 Labelling target RNA's

Pooled-control and pooled-treated plant tissues from all stages (50mg each) during the development of durable resistant (section 4.2.9) were used for total RNA preparations (section 4.3.10). The two populations of total RNA's (from control and treated Stirling plants) were individually labelled with one of the fluorescent dyes Cyanine 3 (Cy3) and Cyanine 5 (Cy5). Labeling was carried out on total RNA preparations using a Micromax direct labelling kit essentially as described in the instruction manual (PerkinElmer Life Sciences, #MPS502). Briefly, total RNA's from the specified materials were isolated and the quality and quantity checked as described previously by electrophoresis and spectrophotometry (sections 4.3.11 and 4.3.12). The control RNA's were labelled with Cy5 and the pathogen challenged RNA's with Cy3. The labeling reactions contained: 100µg RNA (either control or pathogen challenged), 1µl primer mix (FF concentrate) and RNase-free H_2O to 16µl total volume. The tubes were then incubated at 65°C for 10min to denature the RNA's, cooled to 25°C for 5min to anneal the primers to the mRNA's. 1µl of the cyanine nucleotide triphosphate (Cy3 to the pathogen challenged RNA's; Cy5 to the control, untreated RNA's) was added to each sample and the tubes prewarmed to 42°C for 2-3min prior to addition of 2.5µl 10xRT reaction buffer and 2µl AMV Reverse transcriptase with RNase inhibitor mix. After mixing thoroughly the samples were incubated at 42°C for 60min to synthesise the labelled target cDNA. After the incubation the samples were cooled to 4 °C for 5min and then 2.5µl of 0.5M EDTA, pH=8 was added to stop the reaction and 2.5µl of 1M NaOH to hydrolyse the RNA template. The mixtures were incubated at 65 °C for no more than 30min and then cooled to 4 °C for 5min and 6.5µl of 1M Tris-HCl buffer, pH7.5 added. The labelled cDNA's were purified prior to hybridisation by isopropanol precipitation. The two

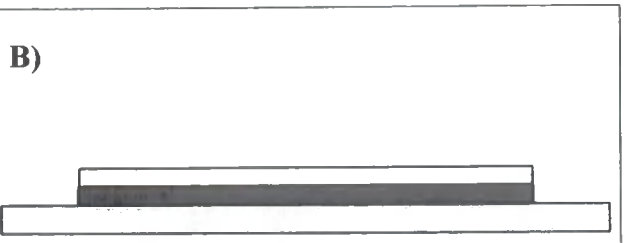
cDNA samples (Cy3 + Cy5) were combined and mixed with 3µl of 5M ammonium acetate and then 62µl of 100% isopropanol was added and the mixture vortexed and incubated at 4°C for 30min. The cDNA's were recovered by centrifugation (10,000g x 15min) at 4°C. The supernatant was removed and the pellet washed twice with 100µl of ice-cold 70% (v/v) ethanol with centrifuging at 10,000g for 10min each time. The final supernatant was removed and the pellet dried briefly and then dissolved in 20-40µl of the hybridisation buffer. Hybridisation buffers from Micromax (Perkin Elmer Life Sciences, #MPS502) or EasyHyb (U-Vision Biotech, #UVH002-1000) were used according to manufacturer instructions. All operations involving the cyanine dyes or the labelled cDNA's were performed in foil wrapped containers or in secluded fluorescent lighting to avoid any photodegradation of the dyes.

4.5.6 Hybridisations

Microarrays were hybridised with the labelled target cDNA's as follows: 20-40µl of labelled target cDNA's were pipetted as a single droplet directly onto the surface of a sterile HybriSlip (20 x 40mm) (Grace Biolabs GS40, Sigma #Z36,591-2) on a flat clean surface (figure 4.3). HybriSlips were used to eliminate loss of any probe which can occur by binding to conventional glass coverslips. A printed slide with the microarray on the lower surface was gently touched onto the surface of the droplet ensuring the microarrayed spots were centred on the droplet and carefully lowered avoiding trapping of any air bubbles until the HybriSlip was drawn up onto the slide. The slide assembly was then placed right way up in a hybridisation chamber (Corning, # 2551), with 10µl of 2xSSC in each reservoir and then the chamber carefully assembled and fully immersed in a water bath. Hybridisations were carried out at 52°C for appropriate times

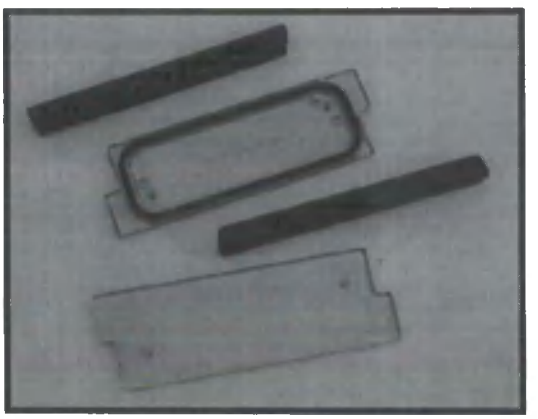


Assembly of microarray, labelled probe and HybriSlip



Assembled microarray slide ready for insertion into hybridisation chamber

C) Hybridisation chamber (Corning)



The microarray slide is inserted in the base of a Corning hybridisation chamber (#2551) and 10µl of 2xSSC placed in each of the two reservoirs, before the chamber is reassembled and immersed in a water bath at 52°C for the duration of the hybridisation period.

Figure 4.3 Hybridisation of microarrays

according to the buffer manufacturer instructions (using EsyHyb buffer this was 2h). After the hybridisation period the chamber was removed, disassembled and the microarray slide removed and immersed intact into the first wash solution in a 50ml falcon tube. The HybriSlip detached itself and sank to the bottom of the tube. Arrays were processed through wash solutions and conditions as recommended in the suppliers instruction manuals (Micromax - Perkin Elmer Life Sciences, #MPS502; EasyHyb hybridisation solution kit -U-Vision Biotech, #UVH002-1000). All washing steps were carried out in 50ml Falcon tubes. After the final wash slide arrays were dried by centrifuging at ~1000g for 5min and stored in foil wrapped 50ml Falcon tubes. Slides were scanned using a Genomic Solutions LSIV array scanner using lasers/filters optimised for detection of Cy3 (excitation 552nm; emission 565nm) and Cy5 (excitation 650 nm; emission 667nm).

5 Establishment and testing of durable resistant potato plants

5.1 Introduction

The oomycete *Phytophthora infestans*, the causal pathogen of late blight disease of potato and tomato, is assessed as to be the most damaging pathogen of potato and tomato crops worldwide. Also, it is one of the major problems in the potato industry worldwide since this devastating pathogen attacks both tuber and foliage during all stages of crop development and causes severe losses in potato fields every year. Many efforts have been made to try to control this pathogen including chemical controls and genetic engineering. Unfortunately, it remains a severe problem and the control measures are seldom very successful. One of the main reasons for this is that *P. infestans* reproduces both sexually and asexually, thereby increasing genetic flexibility that enables it to adapt rapidly to, and overcome chemical control measures and genetic resistance bred into the host plants. In recent years, new isolates of *P. infestans* have shown more virulence and higher resistance to previously effective chemicals such as metalaxyl and the severity of this disease has increased dramatically. Annual losses due to late blight and control measures are estimated to exceed $5 billion world wide (see section 1.3).

After the failure of vertical resistance programmes, based on the gene-for-gene hypothesis, and in order to limit needs for chemical controls and to tackle this disease in an environmentally friendly way, potato breeding programmes to incorporate durable forms of genetic resistance are underway. Although, this type of resistance is believed to be effective against all known races of *P. infestans* and provides in additional some level of general resistance, until now the genetic bases of this type of resistance is still

unknown and the molecular mechanisms poorly understood. A more profound understanding of the mechanisms of resistance to *P. infestans*, particularly leading to the establishment of durable resistance, is needed in order to develop novel control strategies. Therefore, it was of interest in this study to look at a molecular level in order to elucidate some biochemical and molecular events induced during the interaction between *P. infestans* and the establishment of durable resistance in the potato cultivar Stirling. It was anticipated that these initial studies would form the basis for further studies in the future.

The potato variety Stirling was chosen as an experimental variety after consultation with Dr Helen Stewart, a plant pathologist at the SCRI. This selection was based on the unique character of this variety i.e. 'durable resistance' to late blight disease.

It was essential to monitor the establishment of durable resistance to late blight in this variety before starting the ultimate objective of the current study, namely the isolation and identification of genes induced during the interactions between potato plants and *P. infestans*.

5.2 The response of Stirling and Désirée plants to *P. infestans* challenge

In order to establish and test the resistance to *P. infestans* of the potato variety Stirling, axenic plants in culture were challenged with a compatible strain of the pathogen (race 1, 2, 3, 4, 6, 7) (as described in section 4.2.9) and these responses compared with those of the variety Désirée, which exhibits moderate resistance to *P. infestans*. Cultures of the potato varieties Stirling and Désirée were initiated from tubers into plantlets by direct bud culture under sterile conditions (see section 4.1.1). Four-week old plants

(after subculturing into new MS-medium) of approximately the same size and appearance were chosen and infected by placing a disk of a virulent *P. infestans* mycelium plug (5mm diameter) adjacent to the plant stem (see section 4.2.9). Progress of the infection during 8 weeks was recorded photographically. The two potato varieties showed different responses to *P. infestans*. As illustrated in figure 5-1 the pathogen rapidly established infection in Désirée plants after 2 weeks post challenge (figure 5-1B), while at the same time stage no obvious disease symptoms appeared in Stirling plants (figure 5-1A). After 4 weeks post challenge, Désirée plants had collapsed completely (figure 5-1D), while the pathogen had only started to establish the infection of Stirling and the plant had started to produce new shoots (figure 5-1C). Although, the pathogen had infected the Stirling plants completely after 8 weeks the newly developed shoots continued to grow under this strong infection but without developing disease symptoms. The plants also produced microtubers (figure 5-2A).

Surprisingly, when the microtubers were transferred into new MS-medium, new shoots started to grow still under the presence of the pathogen without developing any disease symptoms (figure 5-2B). Moreover, when the newly developed shoots, from the infected plants, were excised and subcultured in new MS-medium and then re-challenged again with the fungus the pathogen also failed to infect them (figure 5-2 C). The newly developed shoots showing this strong resistance were subsequently designated 'durable shoots' and the plants produced from them (after subculturing in new MS-medium) as 'durable plants' (figure 5-2).

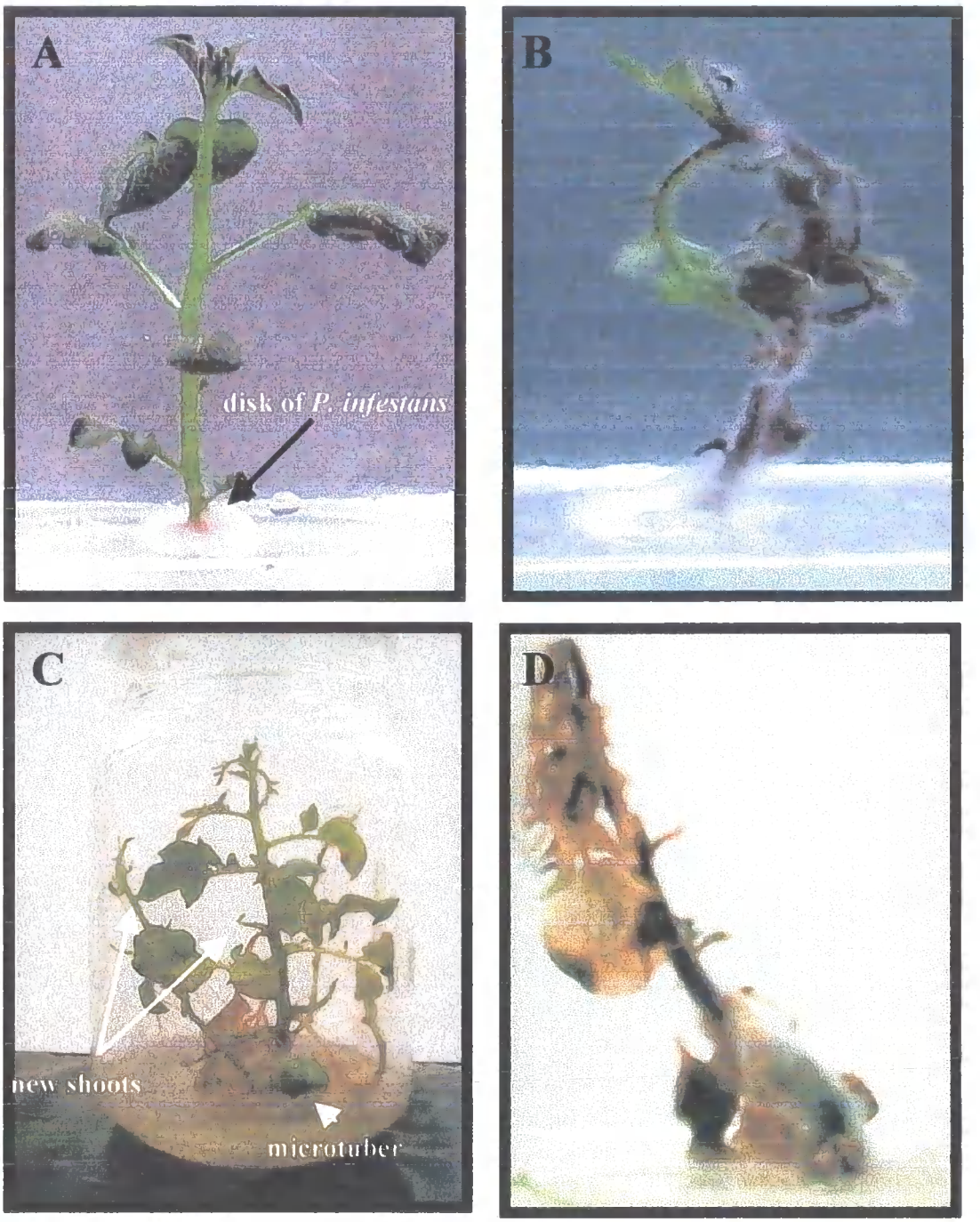


Figure 5-1 The response of Stirling and Désirée plants to *P. infestans* challenge. **(A&B)** Four week-old tissue culture Stirling and Désirée plants were challenged by placing a disk (5mm diameter) of infective *P. infestans* mycelium adjacent to the plant stem. 2 weeks post challenge: **(A)** no obvious disease symptoms in Stirling plants and **(B)** the established infection in Désirée plants.
(C&D) 4 weeks post challenge: **(C)** the pathogen has started to establish the infection of Stirling and the plants have started to produce new shoots and microtubers, while **(D)** Désirée plants have collapsed completely and show surface mycelial infection.

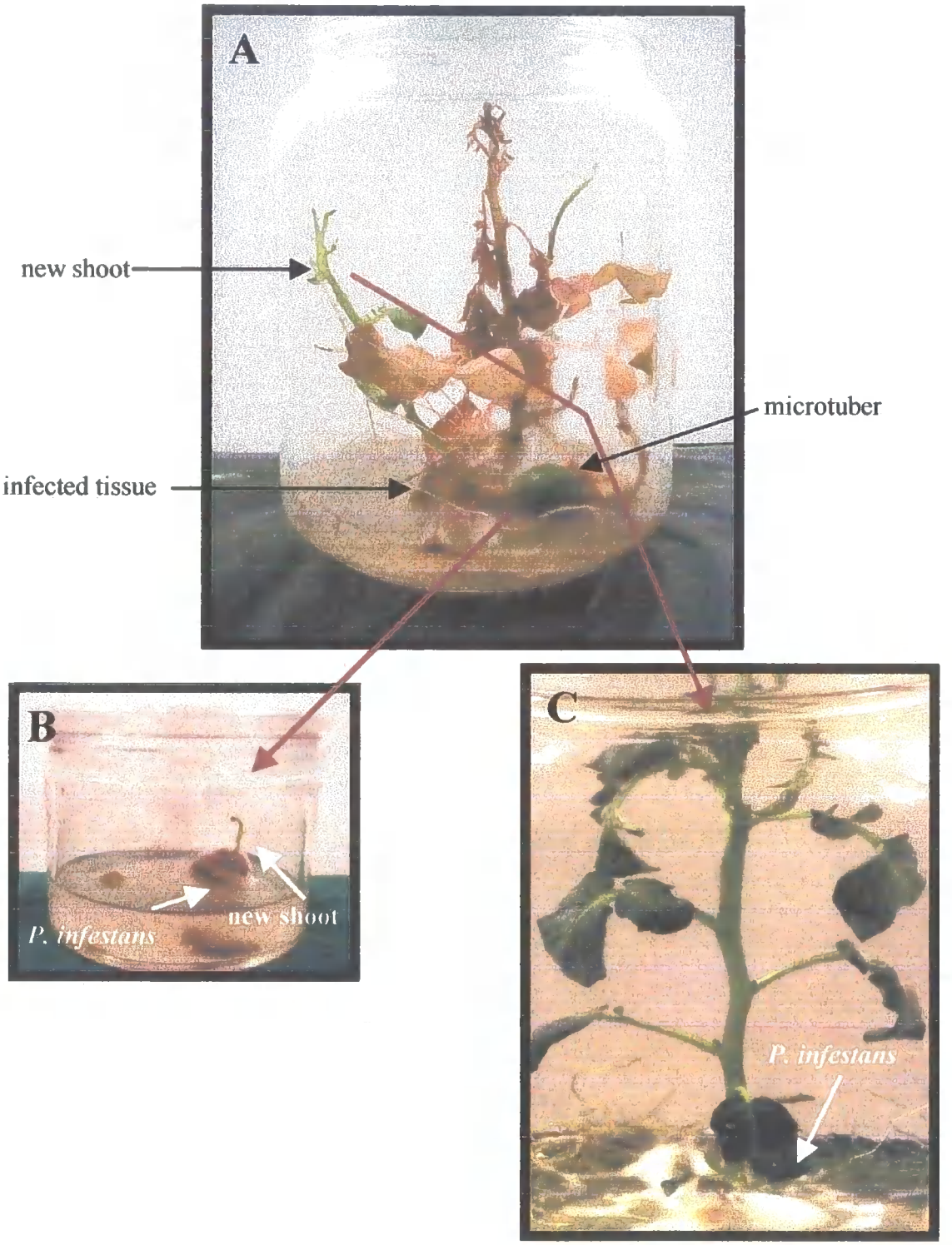


Figure 5-2 Production of durable resistant Stirling plants. (A) Stirling plants, 8 weeks post challenge with *P. infestans* showing the developed resistant shoots and microtubers; **(B)** a microtuber when transferred to new MS-medium produced new resistant shoots 2 weeks after subculturing in the presence of the pathogen; **(C)** Durable resistant plants developed from the excised newly developed shoots from the infected plants, growing without any observed disease symptoms 4 weeks after rechallenging with *P. infestans*.

5.3 Is the durable resistance in Stirling a general pathogen resistance?

In order to test whether the observed Stirling resistance was only specific to *P. infestans* or if it was a general resistance to other pathogens, the Stirling control plants and the durable Stirling plants established from the durable shoots excised from infected Stirling plants (section 5.2), were independently challenged with two other potato pathogens-*Rhizoctonia solani* and *Fusarium sulphureum*. After three-weeks post challenge the durable plants showed strong resistance to both pathogens (no disease symptoms) compared with the control Stirling plants treated with the same pathogens, in which the young leaves started 'rolling' and the plants started to die back after three weeks post challenge (figure 5-3).

5.4 Do the durable shoots inherit the resistance?

In order to test whether the durable shoots inherit the resistance response property or that the newly developed plant showed this resistance simply because it is growing under the presence of the pathogen, the durable shoots were subcultured for two generations in MS-medium in the absence of any pathogen. The second generation of disease-free plants were then re-challenged with *P. infestans*. These plants continued to exhibit more resistance than the control Stirling plants when treated with the pathogen. This experiment confirmed that the durable plants maintained the resistance compared with the original Stirling plants (data not shown).

5.5 Monitoring the progress of infection

The ultimate objective of this study was to identify potato genes induced during the

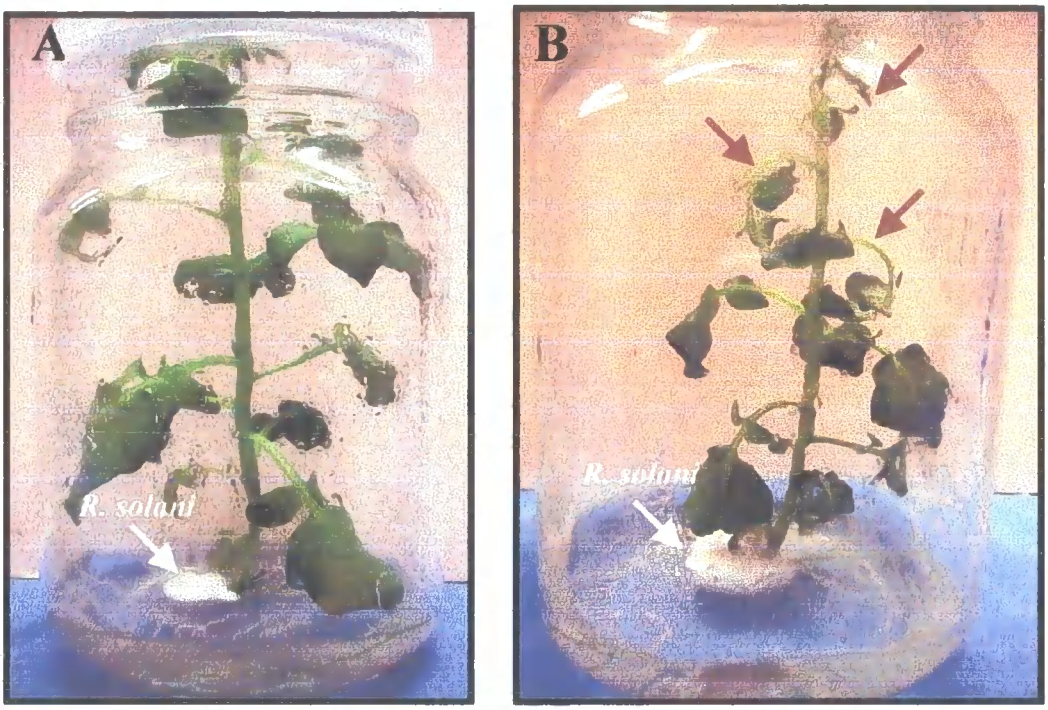


Figure 5-3 The response of durable and control Stirling plants to the challenge with *R. solani*. Axenic potato plants were challenged after 4 weeks subculturing in new MS-medium. Plants were challenged as before by placing a disk of *R. solani* culture adjacent to the plant stem. **(A)** A durable plant three weeks post challenge growing normally without any evidence of disease symptoms; **(B)** control plant after the same period of challenge. The pathogen infected the lower part of the plants and the young leaves started rolling indicating stress responses and the plants started to die. The examples shown were representative of the replicates of similarly treated plants. The response was essentially the same with the pathogen *F. sulphureum* challenge.

establishment of resistance during the interaction with *P. infestans* using the suppression subtractive hybridisation technique (see section 4.4.18). In order to accurately select appropriate times for sampling and to avoid contamination with genes of pathogen origin, monitoring the progress of infection was crucial.

Plant tissues were collected at different time points post pathogen challenge (4, 7, 15, 21, 30, 45 and 60 days) and two methods were used to check for the presence of the viable pathogen in the plant tissues. 1) sampled plant tissues were placed on Rye A agar medium and the growth of the pathogen mycelium from them observed (figure 5-4A), or by 2) microscopic examination following clearing the plant tissues and staining them with aniline blue to detect the pathogen infection structures as described in section 4.5.5 (figure 5-4B). Figure 5-5 shows the progress of infection over this time scale measured as the mean number of pathogen infected leaves on challenged test plants. In the first three weeks the progress of infection was very slow, the pathogen only infected the lower two leaves. Subsequently, the rate of infection increased exponentially until the pathogen infected the whole plant except for the newly established durable shoots.

5.6 Conclusion

The experiments in this study established the conditions necessary to establish durable resistant Stirling plants in culture. Additionally the observations indicated that potato plants of the variety Stirling develop a durable resistance response to *P. infestans* as judged by the delay in the infection process, compared with the moderately resistant variety Désirée, and by the production of newly green viable shoots in the presence of the pathogen. The derived durable shoots and plants showed a strong general resistance response as judged by their resistance to the compatible strain of *P. infestans* as well as

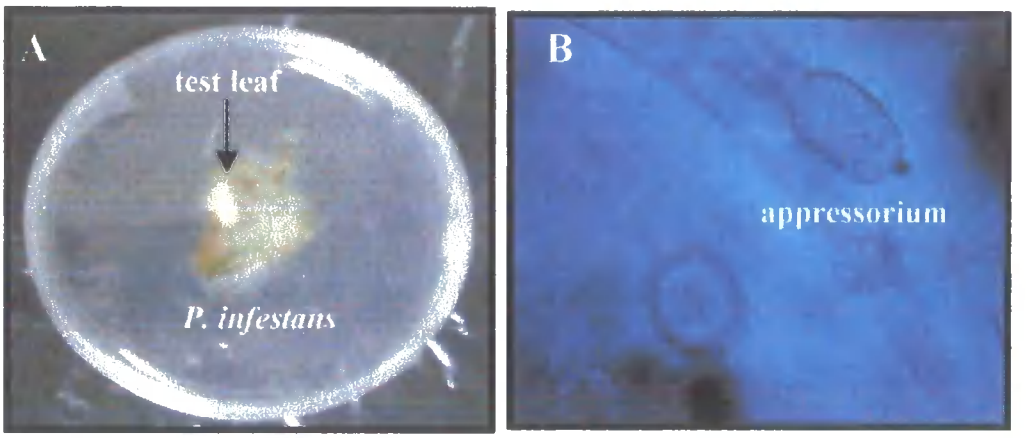


Figure 5-4 Checking for the presence of *P. infestans* infection in Stirling leaves. (A) The presence of the pathogen in the challenged plant tissues was checked by placing the leaves on Rye A agar medium and the growth of the mycelium in the medium observed and **(B)** by clearing the plant leaves with lactophenol and staining for the pathogen infection structures by aniline blue

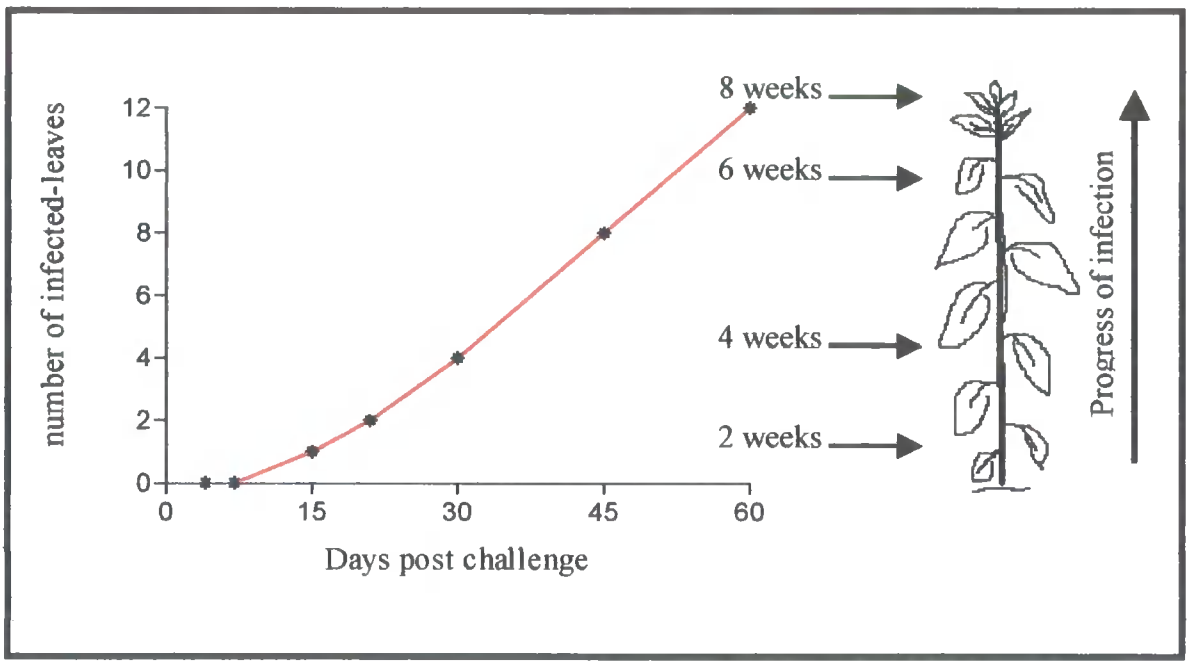


Figure 5-5 Quantifying the progress of infection of Stirling plants by *P. infestans*. Test Stirling plants, 4-week old (after subculturing into MS-medium) were treated with *P. infestans* by placing a disk of the actively growing mycelium plug (5mm diameter) adjacent to the plant stem. Plant tissues were harvested at different time points to check for the presence of the pathogen in it as described in section 5.5.

to the other potato pathogens *R. solani* and *F. sulphureum* even after two generations culture of the plants in the absence of the pathogen.

The progress of infection of Stirling plants by *P. infestans* was very slow during the first three weeks and then the rate of infection increased exponentially. Thus the Stirling plants can recognise the pathogen and activate mechanisms that play an important role in halting or delaying the pathogen infection process. Eventually the pathogen overcomes these mechanisms and infects the plant. However during this process the plants established new shoots that the pathogen could not infect.

For the purposes of clarification throughout the remaining sections of the thesis the types of experimental material used for testing were designated as indicated in table 5-1.

Table 5-1 The designations of the experimental materials used throughout this study

Potato variety	Plants produced by	Designation
Désirée	Axenic culture of nodal cutting	Désirée control plants
Désirée	Axenic culture of nodal cutting and challenged with Pathogen	Désirée treated plants
Stirling	Axenic culture of nodal cutting	Stirling control plants
Stirling	Axenic culture of nodal cutting from control plants and challenged with pathogen	Stirling treated plants
Stirling	Generated from excised durable shoots from infected plants	Stirling durable plants

6 Histochemical characterisation of Stirling durable resistance

6.1 Introduction

Plants react to pathogen invasion by inducing an array of defence mechanisms (see section 1.2). Pathogen recognition by the plant triggers intra- and intercellular signalling mechanisms to generate both local and systemic responses to pathogen infection. Following the signal transduction, biosynthesis and/or release of molecules acting to stop the progress of pathogen growth occurs.

The production of active oxygen species (AOS) such as superoxide ions, hydrogen peroxide and hydroxyl radicals is one of the earliest responses to pathogen recognition. AOS have been associated with several aspects of plant defence responses, including direct toxicity to invading pathogens, strengthening of plant cell walls, a mobile signal inducing local and systemic acquired resistance by itself or its derivatives, triggering the transcription of defence-related genes, and induction of cell death by either oxidative damage of cell components or by triggering the programmed death of challenged cells (Guo and Li, 2000; Vranova *et al.*, 2002) (see section 1.2.2).

Recognition of the pathogen, also, leads to a rapid tissue necrosis at the site of infection, which is called the hypersensitive response (HR). The HR deprives the pathogen of nutrients and/or releases toxic compounds, thereby limiting pathogen growth to a small region of the plant. This response provides resistance to the great majority of potential pathogens. Deposition of structural compounds such as callose at and around sites of hypersensitive cell death may be part of a complex cell wall-strengthening process meant to halt pathogen invasion. Also lignification, which is considered as a general

response to pathogen attack in several plant species, appears to play a role in resistance (see sections 1.2.1 and 1.2.3).

One of the molecules responsible for the activation of the plant defence system is the elicitor. The term elicitor is used for molecules that stimulate any plant defence mechanism (Dixon and Lamb, 1990). There is a wide range of elicitors involved in plant-pathogen interactions ranging from glycopeptides, polypeptides, oligosaccharides and fatty acids (reviewed in Hahn, 1996). These elicitors are responsible for the induction of many defence mechanisms including the induction of a hypersensitive response (Kamoun *et al.*, 1993; Kamoun *et al.*, 1998), oxidative burst (Fauth *et al.*, 1998; Nurnberger *et al.*, 1994) and the production of lignin and other structural materials (Oelofse and Dubery, 1996).

After the impressive observations of the establishment of Stirling durable resistance (section 5), it was of great interest to use some histological and biochemical markers to get an idea or explanation why the durable shoots showed strong resistance to the pathogen. Markers which correlate with a plant's response to pathogen invasion such as callose deposition, hypersensitive response, and active oxygen species were used to check the difference in the response between Stirling control and the durable plants. In some cases the Désirée plants were included in the comparison as an example of a moderate cultivar.

In this study, crude elicitor was prepared from a *P. infestans* culture filtrate (as described in section 4.2.4) and was used to infiltrate potato leaves excised from the test plants and placed in MS-medium as described in section 6.2 below.

6.2 Leaf-bridge bioassays

The leaf-bridge bioassay method, which was developed in our lab for investigating the progress of pathogen infection through leaves (Abdullah, 1999), was used for this study. In this method, sterile, detached leaves from potato plants grown in tissue culture were used. The excised leaves were placed in a three-compartment petri dish containing Murashige and Skoog (MS) medium as shown in figure 6-1. The MS-medium was used to support the base of the leaves and also to supply water and nutrients needed to prolong the life of the leaf *in vitro*. A quarter strength MS medium supplemented with 20 $g.L^{-1}$ sucrose and 2 $g.L^{-1}$ phytagel was used. The sterile medium was poured into one compartment of the three-compartment petri dish (9cm) used for the bioassay. Leaflets of identical size and position on the plants were taken from 4 week-old potato plantlets growing in tissue culture. The leaves were cleanly cut with a sterile scalpel blade at the base of the petiole to avoid damaging the tissue. The excised leaflets were examined to ensure they were free from any physical damage caused by cutting. Any such damaged materials were discarded. Two leaflets were orientated in the bioassay plate so that the end of the petiole was completely embedded in the agar medium and the rest of the leaf was raised over the partition (bridge) to make contact with the base of the plate at the leaf tip as shown in figure 6-1.

6.3 The response of Stirling, durable and Désirée plants to elicitor treatment

Disease symptoms in the detached potato leaves infiltrated with *P. infestans* culture filtrate elicitor were monitored using the leaf-bridge bioassay method described above. Stirling, durable and Désirée plants were grown in tissue culture as described in section 4.1.3. Detached leaves, from 4-week old plants (from the same position in the test

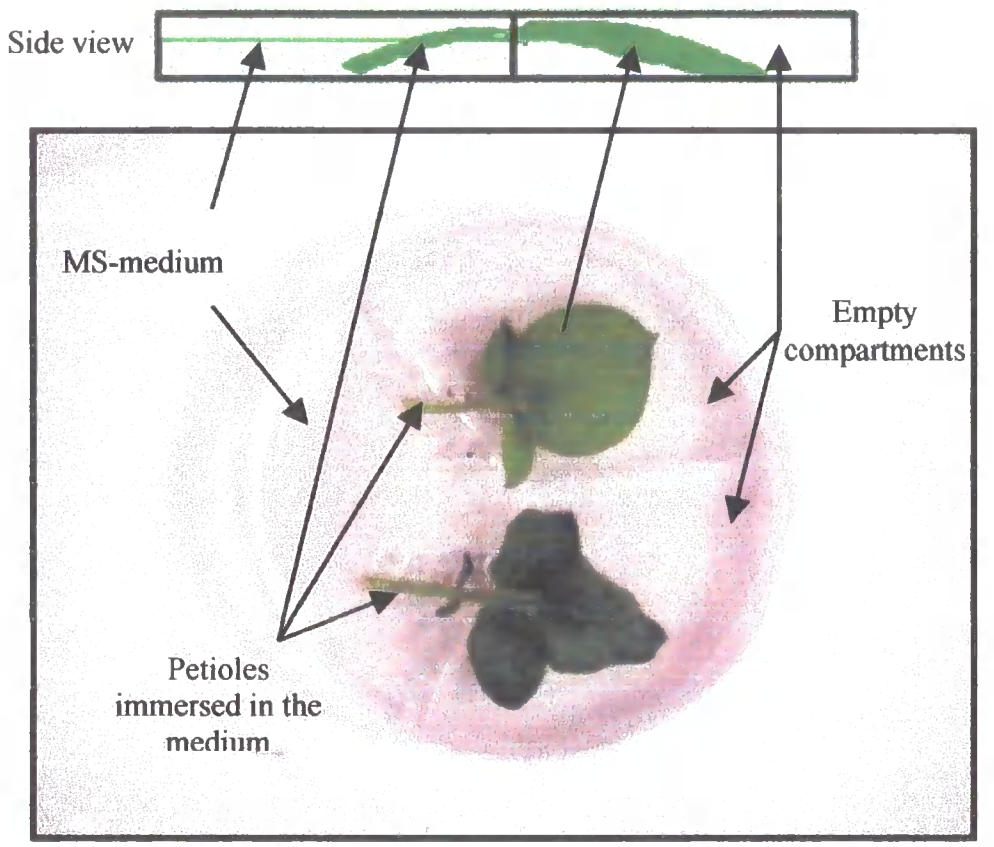


Figure 6-1 Leaf-bridge bioassay set-up. Three-compartment petri dishes were used in which MS-sucrose medium was placed in one compartment and the other two left empty. Two detached leaves, approximately the same size and appearance, from the tested plants were orientated so that the petioles were immersed in the medium and the leaf tip in the empty compartment. The plastic wall of the compartments supported the body of the leaf above the medium and preventing direct contact with it.

plants, approximately the same size) were orientated in a three-compartment petri dish as described above. The leaves were elicited by applying 15µl of the concentrated solution of culture filtrate elicitor (prepared as described in section 4.2.4) to the top left half of the leaf and the liquid allowed to infiltrate to the leaf tissues. The response of the different potato lines to the elicitor treatment (seven days post treatment) is illustrated in figure 6-2. The treatment resulted in a large necrotic area in Désirée leaves (figure 6-2C) and small necrotic areas in Stirling leaves (figure 6-2A). It was difficult to observe any necrosis occuring in the durable line (figure 6-2B). The microscopic examination revealed that the durable line reacted hypersensitively to the elicitor treatment but in only a few cells and these cells were surrounded by a border of callose deposition to restrict the pathogen spread as described in section 6.4 (figure 6-3A) below.

These results confirmed the suggestion of Vleeshouwers *et al.*, (2000b), which correlated the effectiveness of the HR with the level of resistance to *P. infestans* and indicated that the durable line was particularly primed for the HR.

Vleeshouwers *et al.*, (2000b) studied the relation between the HR and the cessation of *P. infestans* growth using *Solanum* clones displaying different types and levels of resistance to *Phytophthora*. The authors found that although all *Solanum* clones reacted with a similar type of response to *P. infestans*, major differences were observed in severity and timing of the HR between the *Solanum* clones.

In the nonhost *Solanum* clones such as *S. nigrum*-SN18, the HR was induced extremely quickly. One to three cells displayed the HR at 22 hai (hours after inoculation). In most cases the response remained limited to these cells and *P. infestans* was not detected at 46 hai. In *S. berthaultii*-9, the HR was established at a slower rate but was finally

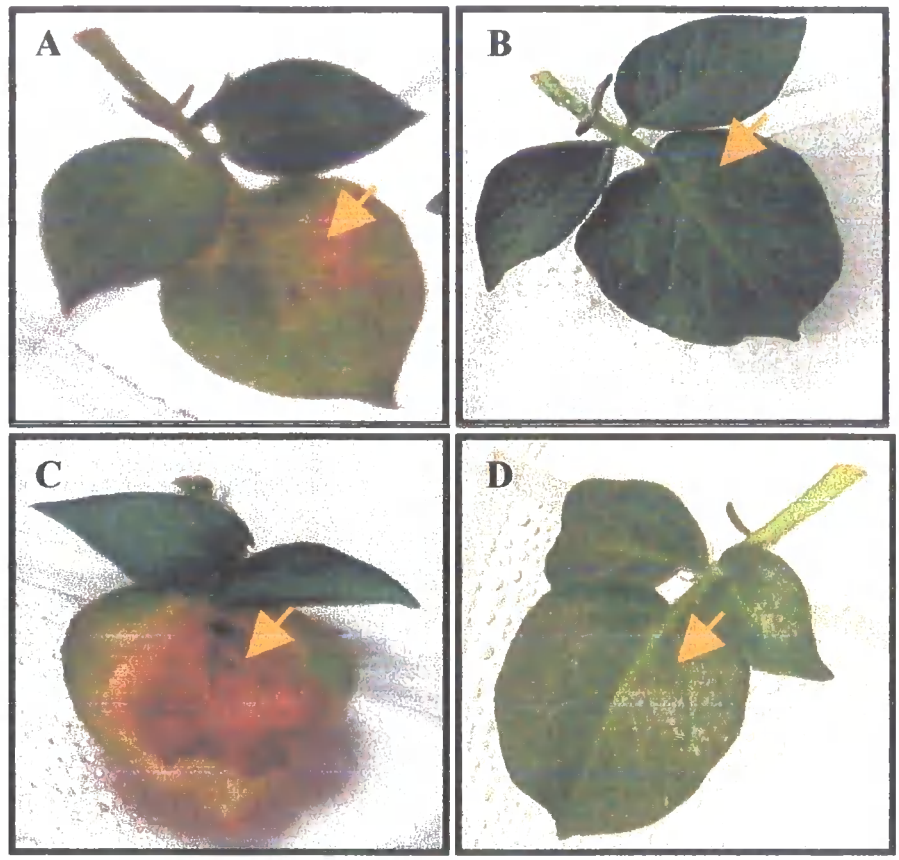


Figure 6-2 The necrotic lesion as a response of elicitor treatment. The leaf-bridge bioassay (described in section 6.2) was used to check the response of **(A)** Stirling, **(B)** durable and **(C)** Désirée plants to the treatment with *P. infestans* culture filtrate elicitor. Detached potato leaves from 4-week old plants were orientated in a three-compartment petri dish as described before. The leaves were elicited by applying 15µl of the concentrated culture filtrate *P. infestans* elicitor the top left half of the leaf (arrowed) and the liquid allowed to infiltrate to the leaf tissues. A Stirling control leaf infiltrated with phosphate buffer (100mM; pH7.0) is shown as non-elicitor control **(D)**. Photographs were taken 7 days post treatment.

completed at 46 hai.

Partially resistant *Solanum* clones such as *S. berthaultii*-11 exhibited a less effective HR as more cells displayed the HR before the pathogen was restricted. At 46 hai, it appeared that hyphae had grown out of the initially responding epidermal cells into mesophyll cells. These cells subsequently responded with the HR, resulting in increased sizes of HR lesions. In more susceptible clones, such as *S. arnezii* x *hondelmannii*-72, the HR occurred later, hyphae escaped and growing disease lesions were formed.

In the susceptible clones such as *Bintje*, no early plant response was visible. At 46 hai, the entire leaf disc was overgrown by hyphae, and extensive necrosis near the inoculation spot observed.

These results suggest a correlation between the efficiency of the HR and the level of resistance to *P. infestans*.

Kamoun *et al.*, (1999) also reported that HR is associated with all known forms of genetic resistance to *Phytophthora* and downy mildew oomycetes.

6.4 Cytological examination of callose deposition

Detached potato leaves were treated as described above in section 6.3. Seven days post treatment the leaves were cleared by boiling in alcoholic lactophenol, and then stained for 1h in a 0.01% (w/v) solution of aniline blue as described in section 4.4.1. The stained leaves were examined with ultraviolet illumination (UV) for callose deposition, normal light for HR response or both UV using an Optiphot-2 microscope. A comparison between durable and Stirling plant responses is shown in figure 6-3. Cells adjacent to HR cells usually showed callose deposited on cell walls, whereas HR cells

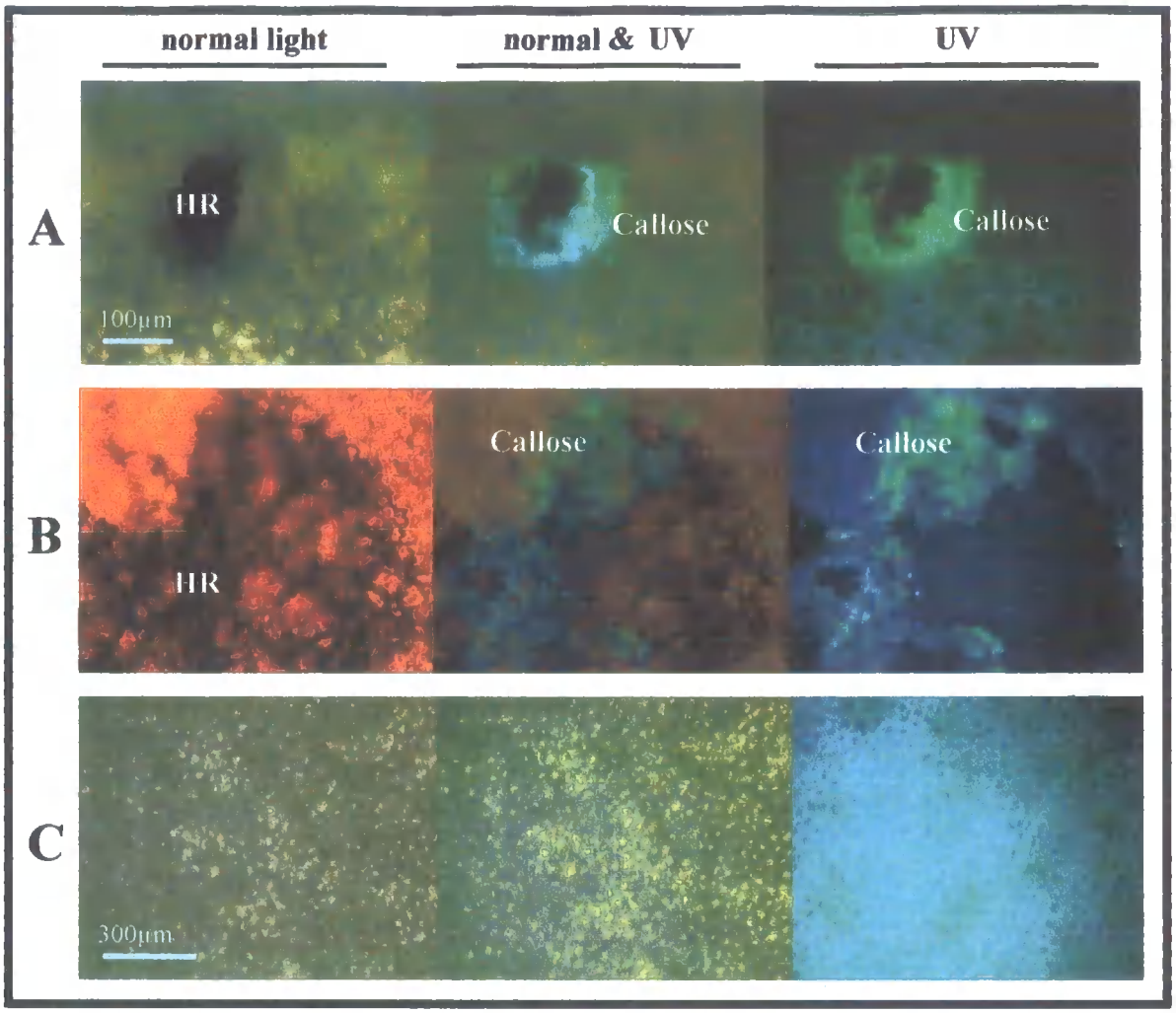


Figure 6-3 Histochemical detection of callose deposition. Detached potato leaves were treated with *P. infestans* elicitor or phosphate buffer (100mM; pH7.0) as described before. Seven days post treatment the leaves were cleared by boiling in alcoholic lactophenol, and then stained in aniline blue as described in section 4.4.1. The leaves were examined under normal, UV and combination of both UV and normal light using Optiphot-2 microscope. Callose is seen deposited on cell walls of the cells adjacent to HR cells.

Horizontal rows display leaf morphology from **(A)** durable plant infiltrated with *P. infestans* elicitor, **(B)** Stirling plant infiltrated with *P. infestans* elicitor and **(C)** control Stirling plant infiltrated with phosphate buffer (100mM; pH 7.0).

The magnification used was 20X in (A) and (B); 10X in (C). Scale bars: (A) and (B) 100µm; (C) 300µm. HR = hypersensitive

usually did not show any callose staining (figure 6-3). In the durable line a very small HR area was observed and was found totally surrounded by a border of callose deposition (figure 6-3A), while the Stirling line showed a larger HR area also surrounded by callose deposition but not as tightly defined as found in the durable line (figure 6-3B). In the latter case, the HR appears ineffective in blocking the pathogen completely resulting in escaping hyphae and a typical phenotype of trailing HR, in which the pathogen hyphae remain ahead of the plant response.

These results may explain why the durable plants were able to grow in the presence of *P. infestans* without any observed disease symptoms as the plants respond to the pathogen infection by a rapid HR response and callose deposition, thereby restricting the pathogen to the infection site and activating the plant defence responses.

Although the deposition of callose observed in the Stirling plants, the large HR area, compared with durable plants and the more diffuse callose deposition may indicate that the response was slightly delayed compared with the durable plant response. It may also explain why the *Phytophthora* managed to infect the Stirling plants, but the induction of the HR response might activate the plant defence responses and the plants responded to the infection by the production of the resistant shoots (see section 5).

In compatible and incompatible *P. infestans*-potato interactions, cell wall appositions with accumulated callose have been found (Cuypers and Hahlbrock, 1988; Gees and Hohl, 1988). Recently, Vleeshouwers *et al.*, (2000b) demonstrated that lesions following HR were often found completely surrounded by callose depositions. In regions of penetration and hyphal growth, callose deposition was also found in papillae. Susceptible *Solanum* clones displayed a higher number of papillae as a result of *Phytophthora* growth throughout the tissue, whereas resistant clones mainly showed

callose deposition around HR cells. Partially resistant clones showed an intermediate phenotype.

6.5 Histochemical detection of hydrogen peroxide

The production of hydrogen peroxide (H_2O_2) in response to elicitor treatment was examined by vacuum infiltration of potato leaves with 3,3'-diaminobenzidine (DAB) as described in section 4.4.3. DAB polymerizes and turns deep brown in the presence of H_2O_2, and the intensity of the coloration can be qualitatively assessed and photographed. DAB can detect H_2O_2 in concentrations at levels as low as 0.1 μM, but a strong colour develops only at higher concentrations of about 1–10 μM (Thordal-Christensen *et al.*, 1997). DAB has been used for the detection of H_2O_2 *in vivo* in different plant species such as tobacco (Schraudner *et al.*, 1998), barley (Thordal-Christensen *et al.*, 1997) and in 18 plant species including potato and tomato (Orozco-Cardenas and Ryan, 1999).

The development of the DAB-H_2O_2 reaction product in durable and Stirling potato leaves in response to elicitor treatment at different time points (3, 6, 12, 24 and 48h post treatment) is shown in figure 6-4. In durable leaves, H_2O_2 was detectable as early as 3h after elicitor treatment, with the colour deepening at 6h, where it remained high for about 12h, then declined (figure 6-4A). In comparison the production of H_2O_2 in the Stirling leaves was detected only after 6h post treatment with the colour deepening at 12h, before declining. The colour initially was visible only at the elicited site, and then deepened in the tissues surrounding the elicited site.

These results indicated that the generation of H_2O_2 production was earlier and for a longer period in the durable plants. This timing difference may also explain why the

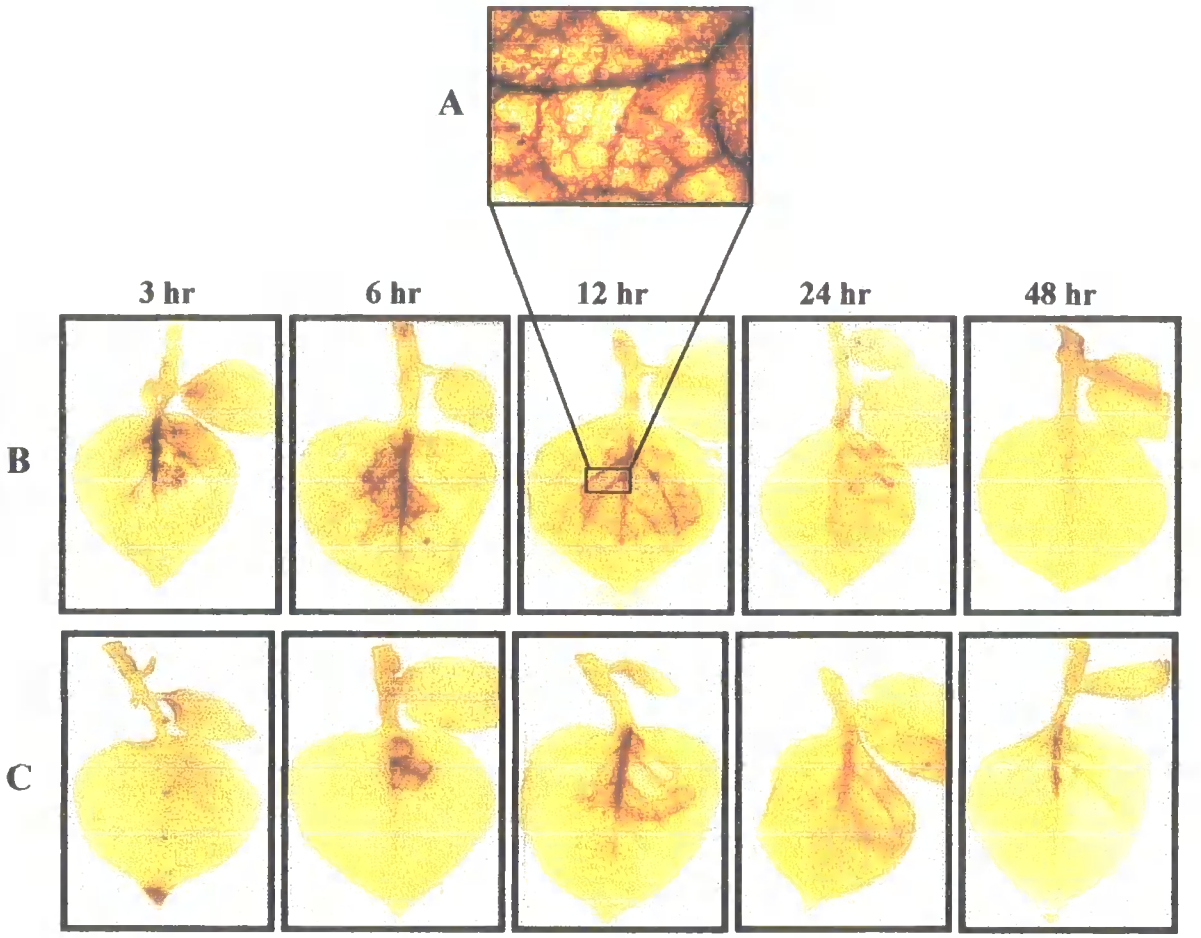


Figure 6-4 The generation of H_2O_2 in potato leaves in response to elicitor treatment. Detached potato leaves from 4-week old plants were orientated in a three-compartment petri dish and elicited as described before (section 6.2). The generation of hydrogen peroxide in response to elicitor treatment at the different time points (as indicated above) was examined by vacuum infiltrating the leaves with 3,3'-diaminobenzidine (DAB) as described in section 4.4.3.

(A) The microscopic examination of the leaf under normal light using Optiphot-2 microscope (10x magnification).

(B) The time course of H_2O_2 production in leaves from durable plants.

(C) The time course of H_2O_2 production in leaves from Stirling control plants.

durable plants showed strong resistance to infections with *P. infestans*, *R. solani* and *F. sulphureum* as timing of the induction of defence responses in which ROS play an important role, can be a significant factor in the success or failure of the plant to defend itself against pathogen attacks (Dangl *et al.*, 1996).

It has been demonstrated that potatoes transformed with a constitutive glucose oxidase that generated a low level of H_2O_2 in cells throughout the plants, exhibited elevated levels of plant defence proteins (Wu *et al.*, 1997), and the tubers were strongly resistant to a bacterial soft rot and late blight diseases (Wu *et al.*, 1995).

6.6 Histochemical detection of superoxide anion

The generation of superoxide anion (O_2^-) in potato leaves in response to elicitor treatment was examined by nitroblue tetrazolium (NBT). NBT has been used for *in vivo* detection of superoxide anion in different plants species such as tobacco (Schraudner *et al.*, 1998) and *Arabidopsis* (Jabs *et al.*, 1996). In this study, elicitor-treated leaves (3, 6, 12 and 24h post treatment) were vacuum-infiltrated with a 0.1% (w/v) solution of NBT in 50mM potassium phosphate (pH 6.4) as described in section 4.4.2. The development of the scattered dark-blue formazan spots in leaves of durable and Stirling potato leaves in response to elicitor treatment at the different time points is shown in figure 6-5.

In durable leaves, superoxide anions were detectable as early as 3h after elicitor treatment, with the colour deepening at 6h and reaching a maximum level at 12h before declining (figure 6-5A). The production of superoxide anions in Stirling leaves was only detected after 6h post elicitor treatment with the colour deepening up to 12h before declining. The colour initially was visible at the elicited site, and then deepened

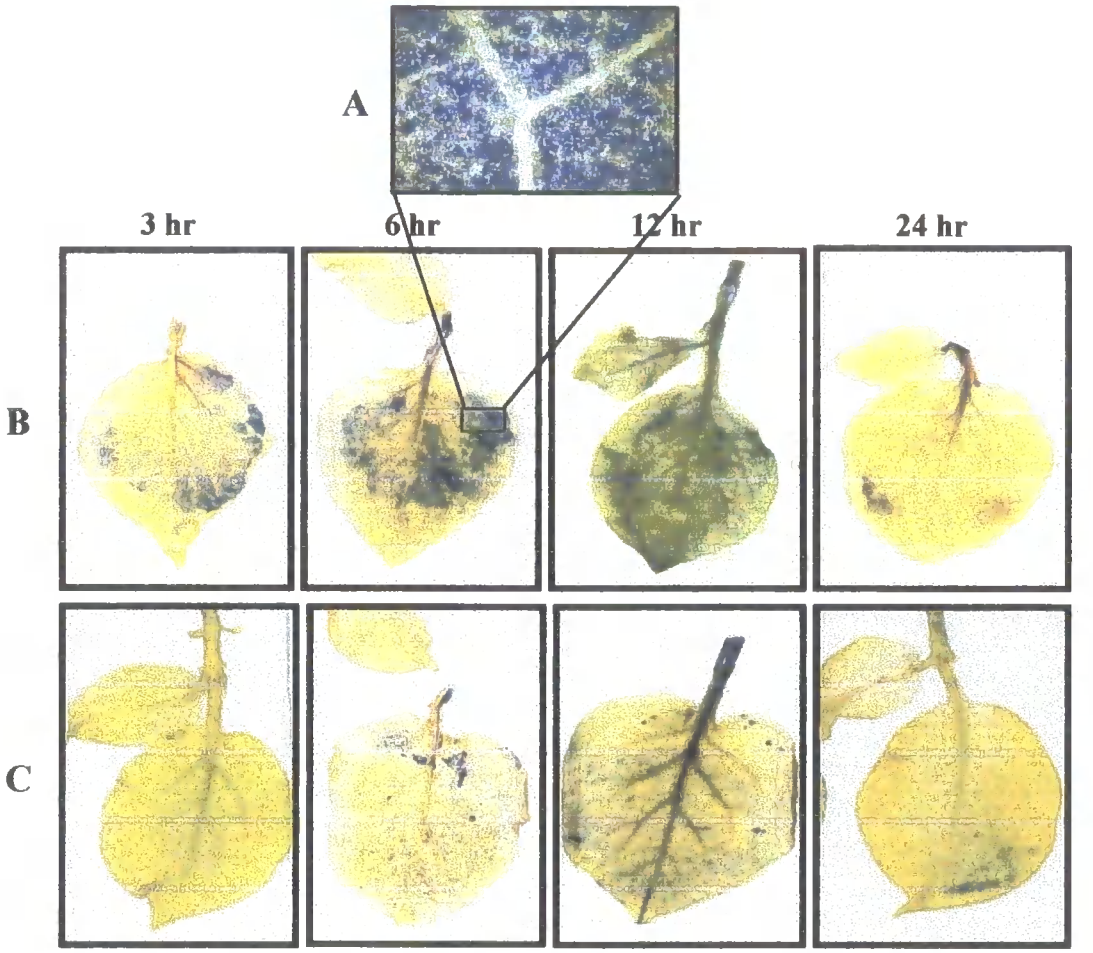


Figure 6-5 The generation of superoxide anion in potato leaves in response to elicitor treatment. Detached potato leaves from 4-week old plants were orientated in a three-compartment petri dish and elicited as described before. The generation of superoxide anion in response to elicitor treatment at different time points (as indicated above) was examined by vacuum infiltrated the leaves with nitroblue tetrazolium (NBT) as described in section 4.4.2.

(A) The microscopic examination of the leaf under normal light using Optiphot-2 microscope (10x magnification).

(B) The time course of superoxide anion production in leaves from durable plants.

(C) The time course of superoxide anion production in leaves from Stirling control plants.

in tissue surrounding the elicited site as well as in the other half of the leaf.

These results together with the production of H_2O_2 support the suggestion that durable plants resist pathogen infection as a result of early induction of ROS, which play an important role in the induction of plant defence responses.

6.7 Conclusion

The resistance of durable plants to pathogen infection could be explained by the effectiveness of the HR and the tight deposition of callose surrounding the HR cells, which deprive the pathogen of nutrients and limit pathogen growth to a small region of the plant. Moreover, the fast production of ROS, which play important roles in the activation of plant defence responses and in the direct toxicity to invading pathogens, can also be an important factor in the success of durable plants to defend themselves against the pathogens attack.

These fast durable plant responses and timing differences between the Stirling plants and the durable plants propagated from them after the pathogen challenge, indicate that genetic changes have occurred. In section 7 the supression subtractive hybridisation (SSH) cloning was used in an attempt to identify gene sequences upregulated during the *P. infestans*-potato interaction leading to the establishment of durable resistance.

7 Isolation of genes that are induced during the establishment of durable resistance in response to *P. infestans* challenge

7.1 Introduction

Late blight disease of potato and tomato is the most damaging disease of potato and tomato crops worldwide. How to tackle this disease in an environmental friendly way and how to reduce the expenses for the control measures, which are estimated to exceed $5 billion, of this disease are a major problem in the potato industry. A more profound understanding of the molecular and biochemical mechanisms underlying the resistance to *P. infestans* is needed to develop novel control strategies.

As mentioned earlier (section 1.3), in spite of concerted research on the genetics and physiology of *P. infestans* and its interaction with potato, progress in understanding the molecular processes involved in infection and resistance is still limited. Several studies have demonstrated that the potato attack by *P. infestans* leads to transcriptional activation of various genes in potato (Avrova *et al.*, 1999; Beyer K. *et al.*, 2001; Birch *et al.*, 1999; Zhu *et al.*, 1995a). Unfortunately, the most commonly studied type of resistance, in the *P. infestans*-potato interaction, is race-specific resistance, which is governed by single dominant resistant genes (R genes). Unfortunately, race-specific resistance is only effective against certain races of the pathogen, and is easily overcome by rapid evolution of the pathogen resulting in a lack of durability in the field (see section 1.3). In contrast, race-non-specific resistance is effective against all known races of the pathogen. This type of resistance is thought to be based on multiple genes, and may be durable (Vleeshouwers *et al.*, 2000a). The identification and characterisation of these genes might lead to a better understanding of the molecular processes involved in

resistance, as well as potentially contributing to the development of biotechnological strategies for the fight against this disease.

The experimental system of choice for this study was a compatible strain (strain 9.5.1) of *Phytophthora infestans* (race 1, 2, 3, 4, 6, 7) and tissue cultured potato plants of the variety Stirling, which exhibits durable resistance (see section 5). Tissue culture plants, growing under sterile and defined conditions were used to study the genes induced in the response to *P. infestans* infection without interference from external factors.

7.2 Strategies for constructing subtracted cDNA libraries

In this work, gene sequences induced during the compatible interaction between potato plants and *P. infestans* were isolated using the polymerase chain reaction-based suppression subtractive hybridisation (SSH) method (Diatchenko *et al.*, 1996), following the instructions provided with the PCR-Select cDNA subtraction kit (Clontech) (section 4.3.18). Suppression subtractive hybridisation (SSH) is a PCR-based method that has been developed to enrich rare transcripts and low-abundance genes in animal systems (Diatchenko *et al.*, 1996). Recently, several applications, using this method, have been reported in plants (e.g. Beyer K. *et al.*, 2001; Birch *et al.*, 1999; Kim *et al.*, 1999; Caturla *et al.*, 2002). SSH is a powerful technique that produces a library of cDNA clones that are differentially expressed between one mRNA-population (tester) compared with a second, control, mRNA-population (driver). Here, the method was used to enrich gene sequences differentially expressed in potato upon challenge with *P. infestans*.

Subtractive cDNA cloning is both technically challenging and very expensive so it was

essential early in the experimental programme to establish which stages during the development of resistant plants to focus on. In other words, which materials to sample and which pair or pairs to use for subtraction. After detailed considerations made in the light of the findings reported in sections 5 and 6 it was decided to use control Stirling tissues and treated Stirling tissues sampled at various time stages starting from the pre-challenged plants "time 0" through to the establishment of the durable resistant shoots "45 days" post challenge (section 4.2.9) thus covering genes up/downregulated during early to late responses. This strategy was deemed the most likely to yield a set of differentially responding gene sequences which could be investigated in more detail in the later stages of this project or in subsequent research programes using techniques such as transcript profiling.

Strenuous efforts were made to avoid cloning, selecting and sequencing genes of fungal (i.e. *P. infestans*) origin. A detailed explanation of this is given in section 7.4.

To generate cDNA subtracted libraries enriched for gene sequences induced during the compatible interaction, two mRNA populations were prepared: a target sample from a pool of total RNA from potato plants challenged with *P. infestans* (tester) and a control from a pool of total RNA from untreated potato plants (driver). Starting from these samples, forward and reverse subtracted cDNA pools were made (section 4.3.18).

All RNA samples were routinely assessed for quality (intactness) and quantity by electrophoresis and spectrophotometry (section 4.3.11). The isolated total RNAs appeared to be intact as judged from routine electrophoretic analyses in which the appearance of the band of 28S rRNA and the 18S rRNA band were discrete and showed no evidence of smearing which might indicate degradation (figure 7-1, A). In addition,

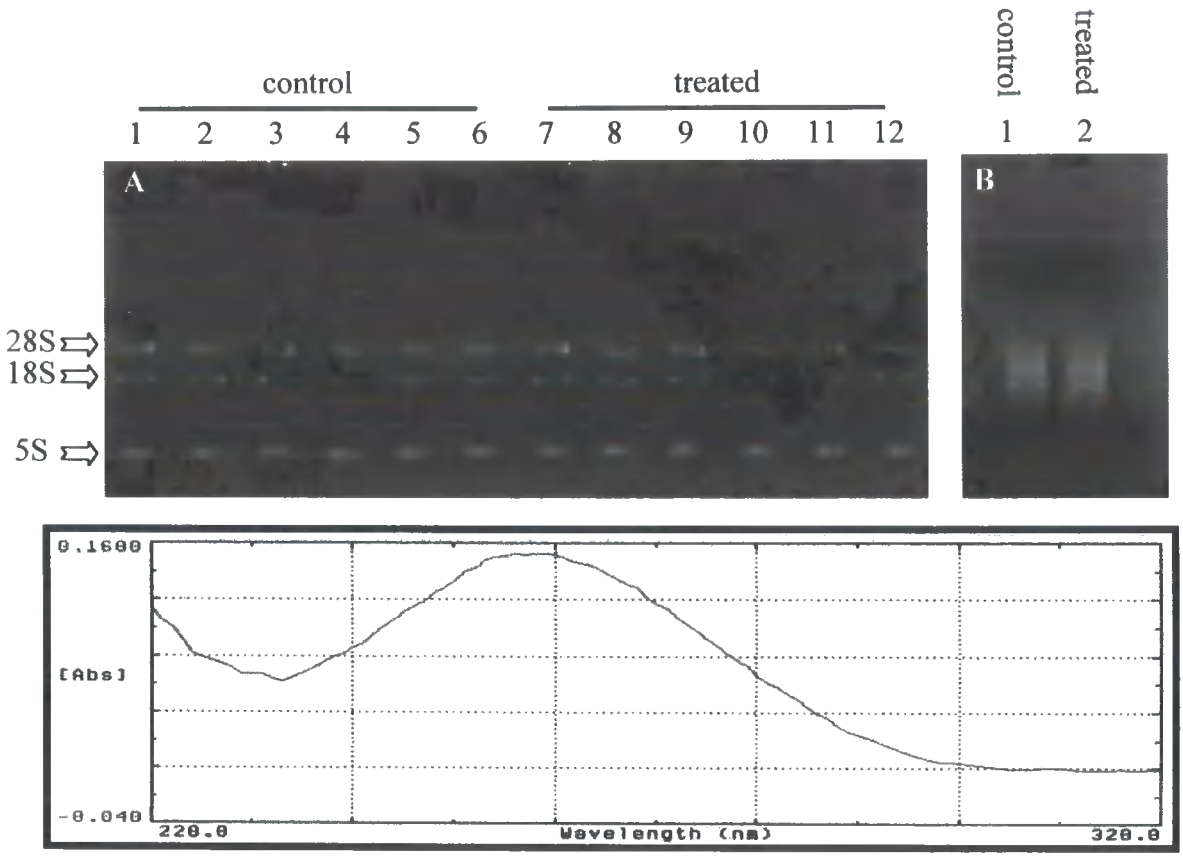


Figure 7-1 Representative electrophoretic and spectrophotometric analyses of RNA preparations. Preparation of total RNA and poly A^+ RNA for subtraction hybridization was carried out using Trizol reagent and Dynabeads mRNA purification kit, respectively. **(A)** electrophoretic analysis of total RNA isolated from control (lanes 1-6) and treated Stirling potato plants with *P. infestans* (lanes 7-12) at different time points (0, 4, 7, 15, 30 and 45 days) using Trizol reagent. Each lane contains about 10µg total RNA. **(B)** typical electrophoretic analysis of poly (A^+) RNA preparations . Total RNA from each time point following challenge, were pooled (400µg total RNA) and poly (A^+) RNA was purified using Dynabeads mRNA purification kit. Each lane contains about 2µg Poly A^+. **(C)** example of UV spectrum (scanned between 220nm and 320nm) of a typical RNA preparation to check its purity.

the absorbance 260nm/280nm ratios of the total RNA samples were routinely measured and typically were more than 1.8 indicating that the RNA preparations were pure and free from protein, polyphenols and phenol. Identical amounts of total RNA from each time point were pooled and used for isolating poly (A^+) mRNA. The poly (A^+) mRNA preparations were also pure as judged by the appearance of discrete mRNA bands with minimal smearing and without visible traces of rRNAs (28S and 18S rRNAs) (Figure 7-1, B). A characteristic UV absorbance spectrum was obtained with RNA preparations scanned between 220 and 320nm (example shown in figure 7-1, C). Preparations failing to meet these quality criteria were either repurified or if degradation was evident, were discarded.

7.3 Constructing cDNA libraries

For cDNA synthesis, adaptor ligation, hybridisations, and PCR amplification, the recommended PCR-select cDNA subtraction procedure was followed (Clontech instruction). For forward subtraction, mRNA from potato plants challenged with *P. infestans* was used as the 'tester' and mRNA from axenic plants as the 'driver'. A reverse subtracted cDNA pool was made with mRNA from challenged plants as a driver and control mRNA as a tester.

To make these subtracted cDNA pools, tester and driver double stranded-cDNAs (ds-cDNAs) were prepared from the corresponding mRNA populations. The ds-cDNAs produced form both control and challenged plants were in the size range of about 0.2 – 2.0Kb as estimated by agarose gel electrophoresis and these ds-cDNAs were subjected to *Rsa*I digestion, separately, to generate short, blunt-ended fragments (figure 7-2, A). Two tester populations with different adaptors were made, but no adaptors were ligated

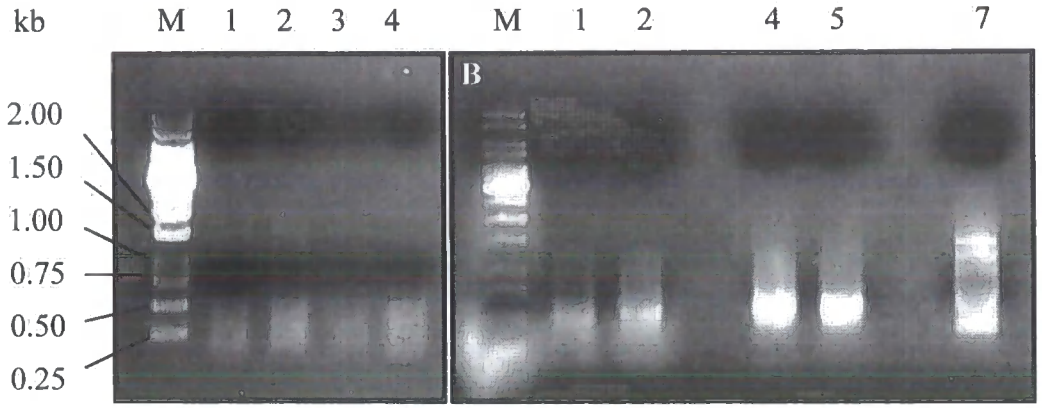


Figure 7-2 Electrophoretic analyses of ds cDNA preparations, the corresponding *Rsa*I digests and the secondary PCR products. (A) ds cDNA from the challenged (lane 1) and unchallenged Stirling plants (lane 2), the corresponding *RsaI* digest from challenged (lane 3) and unchallenged Stirling plants (lane 4). **(B)** the secondary PCR products from the forward subtraction (lane 1), reverse subtraction (lane 2), forward unsubtracted tester (lane 4), reverse unsubtracted tester (lane 5) and the subtracted skeletal muscle, control subtraction provided with the kit, (lane 7). M = size marker-GeneRuler 1kb DNA ladder.

to the driver cDNA. Following the PCR-select subtraction procedure, the tester was subtracted twice by the addition of the driver and the differentially expressed sequences were subjected to PCR amplification (figure 7-2, B).

The forward subtracted cDNA pool, enriched in upregulated cDNAs obtained from the RNA population of Stirling plants challenged with *P. infestans*, was cloned in a TOPO TA PCRII cloning vector (section 4.3.6). These forward subtraction and cloning procedures were repeated twice during this study. In the first attempt 144 clones were isolated, so the procedure was repeated again after gaining more experience and getting familiar with the technique. In the second attempt another 384 clones were isolated. Thus, a total of 528 randomly clones were picked and transferred to 96-well microplates, grown, glycerol added and stored at –80°C until needed (section 4.3.7).

The secondary PCR products from the forward and reverse subtraction were labelled with digoxigenine (section 4.3.15) and were used for the differential screening (section 7.5).

7.4 Avoiding isolation of clones carrying fungal gene sequences

In order to avoid isolating clones in the subtraction library carrying gene sequences of fungal origin, several checks were made at all stages of the cloning operation starting from harvesting the plant tissues, through the cloning procedures, to searching for sequence similarities in the databases. These checks were:

I) Sampling pathogen-free plant tissues. A preliminary experiment was designed to monitor the progress of the infection and the spread of the pathogen in the plant tissues (see section 5.5). Plant leaves were collected at different time points and the presence of

P. infestans in the plant leaves was checked by (i) microscopic examination (section 4.4.5); (ii) placing the leaves in Rye A agar medium and observing the growth of the fungal mycelium (see section 5.5).

II) Subtracting the *P. infestans* sequences from the tester "challenged plants" sequences by mixing the total RNA from a *P. infestans* mycelium culture with the pooled total RNA from the control plant tissues to create the control RNA population for the forward suppression subtractive hybridisation (SSH) method (section 4.3.18.1). Recently, Beyer *et al.*, (2001) used the same idea to subtract constitutively expressed *P. infestans* sequences from potato plants challenged with zoospores of *P. infestans*, while constructing a library to screen for genes induced in potato during the interaction between potato and *P. infestans* using the SSH technique.

III) Screening the subtracted library with *P. infestans* DIG-labelled cDNA probe. The *P. infestans* dsDNA ligated to adaptors 1 and 2R, prepared according to the subtraction protocol, was used as a template. The PCR primer 1 was used for the PCR amplification to incorporation of DIG-dUTP into the PCR products (section 4.3.15). None of the selected clones showed a significant hybridisation with this probe.

IV) During the bioinformatic processing of the sequenced clones, BLAST searching did not reveal any significant similarity to sequences in the *P. infestans* ESTs database at the *Phytophthora* Genome Initiative (PGI) database (http://www.ncgr.org/pgc). In contrast most of the cloned sequences showed very high homology to characterised plant sequences.

7.5 Screening for differentially expressed sequences

The subtracted cDNA library was intended to greatly enrich for differentially expressed

sequences; even so, the subtracted sample will still contain some cDNAs that are common to both the tester and driver samples through 'leakage' at the hybridisation stages. To determine which clones in the subtracted library truly represented transcripts that accumulated during the resistance response, and in order to determine the clones corresponding to the low-abundance mRNA, the PCR-select differential screening procedure was used instead of using probes synthesised as first-strand cDNA from tester and driver as recommended by Clontech. Two sets of probes were prepared using the PCR-select subtraction protocol performed in both directions. The forward-subtracted probe was made from the same subtracted cDNA used to construct the subtracted library, while the reverse-subtracted probe was prepared by performing the reverse-subtractive hybridisation (i.e. the subtractive hybridisation was performed with the original tester cDNA as a driver and the driver cDNA as a tester) as described in the user manual for the PCR-select cDNA subtraction kit (Clontech).

The probes were synthesised by direct digoxigenin (DIG)-labelling of DNA fragments generated by PCR using the nested primers for amplification (see appendix A for primer sequences). The arrayed libraries were spotted onto Hybond-NX membrane (Amersham Biosciences) in duplicate, grown and processed to lyse the cells and crosslink the DNA (sections 4.3.18.2 and 4.3.18.3). The membranes were hybridised, separately, with the forward- and reverse-subtracted cDNA probes. One example of the resulting blots is shown in figure 7-3).

Two hundred and sixty eight clones hybridised only with the forward subtraction probe such as clone IA5 in figure 7-3 (clone designation; I = microplate I, A = row letter and 5 = column number). These clones were considered as representing sequences that are truly upregulated compared with the control plants (i.e. genes induced during the

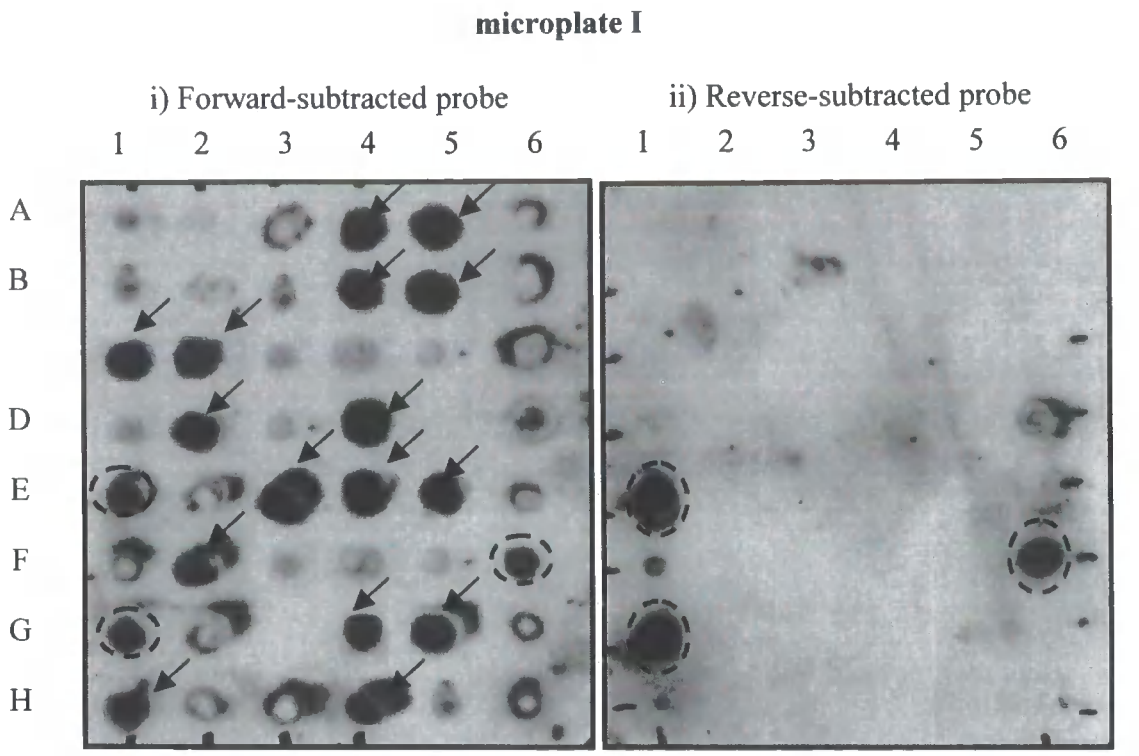


Figure 7-3 Screening the subtracted library for differentially expressed cDNA sequences. PCR-Select subtraction was performed using Stirling plants challenged with *P. infestans* (tester) and unchallenged Stirling plants (driver). The subtracted cDNA was cloned using the TOPO TA Cloning Kit. 528 clones were randomly picked, transferred to microplates for storage and also arrayed in duplicate onto nylon membranes. The membranes were screened by hybridization with DIG-labelled cDNA probes prepared from i) forward subtracted cDNA (cDNA used to construct the library) and from ii) reverse subtracted cDNA (cDNA derived from subtractive hybridisation of the original tester as a driver and the driver as a tester). Arrows indicate differentially expressed clones; circles indicate clones that considered as representing sequences that are never differentially expressed. The other library plates were tested in a similar manner. This shows a typical result in which most clones hybridised to the forward probe and comparatively few hybridised to the reverse probe. Only clones showing positive hybridisation with forward probe were selected for future study.

pathogen challenge or establishment of durable resistance). Forty-three clones hybridised with both the forward- and reverse-subtracted probes with almost the same intensity such as clone IF6 in figure 7-3. These clones were considered as representing sequences that are not differentially expressed in this system (i.e. genes expressed more or less at the same level as in the control plants, throughout the pathogen challenged and establishment of durable resistance). Thirty-two clones hybridised with both probes, but with different intensities. These clones were regarded as probably corresponding to differentially expressed genes. The rest (one hundred eighty five clones) did not hybridise with neither of the subtracted probes such as clones IA1 in figure 7-3. These clones were regarded as representing nondifferentially expressed cDNAs (Clontech; PCR-select differential screening kit).

Defined probes such as phenylalanine-ammonia lyase (PAL), polyphenol oxidase (PPO), pathogenesis-related protein-1 (PR-1), superoxide dismutase (SOD), which was amplified during this project and published in the NCBI database (AC; AF354748), and acidic chitinase (AC) were also synthesised by direct digoxigenin (DIG)-labelling of DNA fragments generated by PCR using the M13 forward and reverse primers for amplification (figure 7-4). These probes were also used for screening the library. Table 7-1 summarises the screening results.

7.6 cDNA sequencing and sequence handling

Sixty of the clones that hybridised with the forward subtracted probe as well as with defined probes but not with the reverse probe were selected for sequencing. The plasmids of these selected clones were isolated (section 4.3.8) and the cDNA inserts were checked by electrophoresis after restriction with *EcoR* I (section 4.3.9) (examples

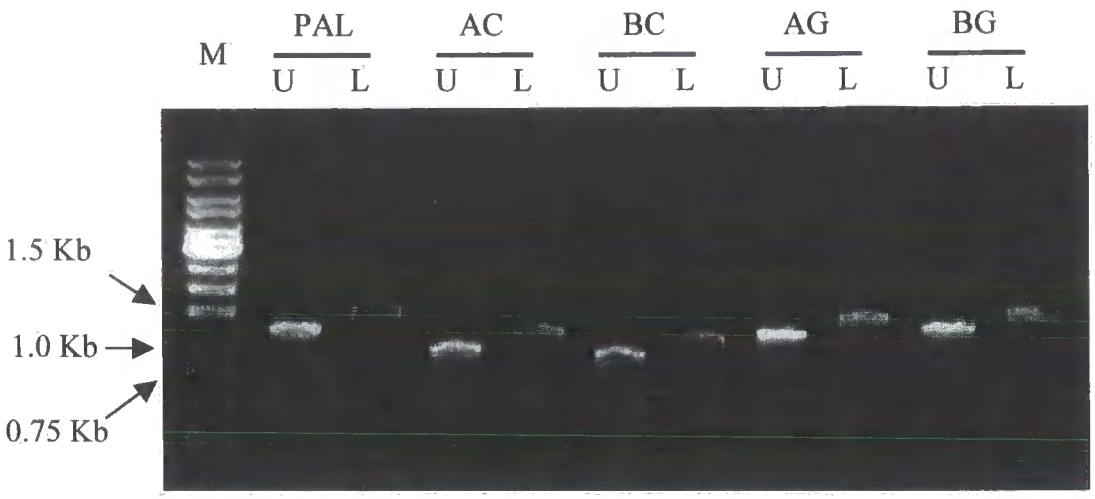


Figure 7-4 Evaluation of DIG-probe labelling efficiency. Digoxigenin-labelled probes were synthesised as described in section 4.3.15. 2µl of each PCR product (both DIG-labeled and unlabeled (i.e., amplified in the absence of DIG-dUTP) versions of the experimental probe) were run on a 0.8% (w/v) agarose gel. The presence of DIG in the labelled probe DNA gives it a higher mass than unlabelled DNA and the intensity of the stained DIG-labeled probe is slightly less than the intensity of the unlabeled probe DNA.

M = size marker- GeneRuler 1kb DNA ladder; U = unlabelled PCR product; L = labelled PCR product. Probes were: PAL = phenylalanine ammonia-lyase; AC = acidic chitinase; BC = basic chitinase; AG = acidic glucanase and BG = basic glucanase.

Table 7-1: Summary of the screening results using defined probes

Probe used	Number of hybridised clones	Clone designation
PAL	7	IIH1, IIH4, IIF3, AB9, AD12, CF10 and DD1
SOD	2	AF12 and DF11
PR-1	7	IIB1, IIC2, IIC6, IID2, IIE5, CC8, and DB4
AC	4	IIG5, AH2, CD5 and CE2
PPO	3	IIA6, AG5 and DC9

are shown in figure 7-5). The cDNA inserts were sequenced using either the SP6 or T7 promotor primers, flanking the vector's multiple cloning site (see appendix B). Sequence determination was carried out using the dideoxynucleotide method, using fluorescent bases and analysed on an automated Applied Biosystems Model 373 sequencer (Stretch Version) by the DNA Sequencing Service, Biological Sciences, University of Durham.

DNA sequences were edited to remove any primer or vector sequences associated with the sequence using the EditSeq module from the Lasergene suite of programmes (DNAStar). DNA sequence comparisons were carried out with those entries in the primary databases, Genbank (at National Centre for Biotechnology Information (NCBI), Maryland, USA) and the TIGR consortium (The Institute for Genomic Research) potato, tomato and *Arabidopsis* databases using their corresponding BLAST (Basic Local Alignment Search Tool) search engines at (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and (http://tigrblast.tigr.org/tgi/), respectively.

The TIGR *Solanum tuberosum* gene index integrates research data from international *Solanum tuberosum* EST sequencing and gene research projects. The ultimate goal of the TIGR gene index projects is to represent a non-redundant view of all *Solanum tuberosum* genes and data on their expression patterns, cellular roles, functions, and evolutionary relationships.

Just after this project started it was announced by the TIGR consortium, which is funded by the NSF (National Science Foundation), that a potato EST and genomic programme was starting to produce and sequence large EST libraries (comprising 60,000 sequences

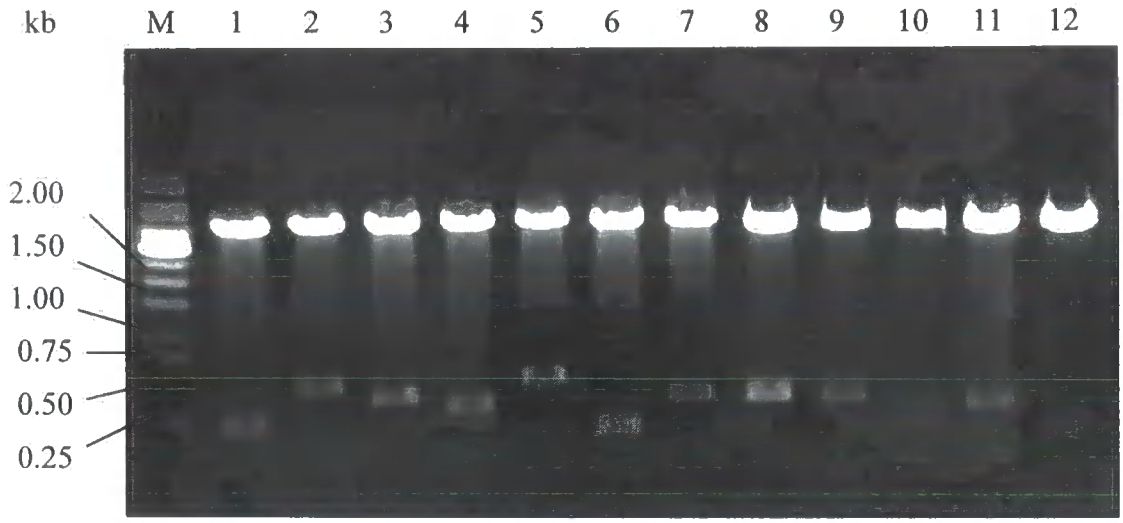


Figure 7-5 Electrophoretic analyses of *EcoR* I restricted plasmid preparations. plasmids of the selected clones were isolated using Wizard Plus SV minipreps DNA purification system as described in section 4.3.8 and the cDNA inserts were checked by electrophoresis on a 0.8% (w/v) agarose gel after restriction with *EcoR* I (section 4.3.9). The restricted plasmid preparations presented here were from clones: AG2 (lane 1), AG1 (lane 2), AE7 (lane 3), AE6 (lane 4), AE4 (lane 5), AC5 (lane 6), AA3 (lane 7), IG9 (lane 8), IG7 (lane 9), ID2 (lane 10), IIG1 (lane 11) and IIB2 (lane 12); M = size marker- GeneRuler 1kb DNA ladder. The other plasmid preparations were tested in a similar manner.

from: stolons, *P. infestans*- challenged (incompatible and compatible), tubers, leaves, sprouting eyes and roots). These sequences formed the basis of a TIGR primary database but not all of these have been included in Genbank. Thus, during the sequence characterisations in the present project sequence searching had to be carried out not only just in Genbank but also duplicated in the TIGR databases. Many of the new sequences appearing in the TIGR databases were only published after those in the present project so routine searches had to be performed several times with sequences, which had not yet been identified.

During this study the blastn programme, which compares a nucleotide query sequence against a nucleotide sequence database, was mainly used. A high similarity in the nucleotide sequence indicates a higher (or at least the same level of similarity) at the protein level.

Multiple alignment of DNA or protein sequences was carried out using the Clustal W method in the Megalign module from DNAStar suite (Lasergene) program. Generally, this software was used to align the nucleotide sequence of the selected clone with the best hits retrieved from the databases, but when the length of the sequence was too large to fit the clustal alignment onto one page such as clones AB9 and AD12 (775 bp and 768 bp; figure 7-8) and clone IC9 (599 bp; figure 7-9), or there were many sequences to be aligned such as proteinase inhibitors (figure 7-14), the protein sequence was used instead.

Due to incompatibilities between Microsoft Word and the DNAstar software, the aligned DNAStar sequence images had to be imported into Word following image cupture and editing in Paint Shop Pro V6.2 (an imaging program).

7.7 Sequence analysis

A summary of the homology search results against Genbank and TIGR (potato, tomato and *Arabidopsis* gene indices) databases is given in table 7-2. The detailed description of the sequences revealed follows in sections 7.7.1 to 7.7.8. Most of the cDNA inserts of the selected clones showed very high similarity (95% or more) with identified nucleotide sequence entries in the databases. Among the sixty clones, which were sequenced, one was found to be a mixed clone, so this clone was discarded from the table 7-2 and the following analyses. As expected the sequence search of the selected clones revealed homology to diverse classes of genes, and thus were organized into categories according to their putative function as summarised in figure 7-6. Inevitably in a classification scheme of this design many sequences may contribute to more than one type (i.e. may fit in more than one category). One such example was PAL, which could be categorised as a metabolism related sequence or as a stress response related sequence because it plays an important role in the phenylpropanoid metabolic pathways leading to the production of phytoalexins that are shown to be induced under various stress responses. In such cases, the most likely biological function for this sequence was chosen. The categories included: (A) defence and stress related sequences, which was the largest category of sequences – as expected – 37 sequences (comprising 63%), including sequences such as proteinase inhibitor (PR-6), peroxidase (PR-9), chalcone synthase, and heat shock protein; (B) signalling-related sequences: 5 sequences (comprising 8.5%), such as ADP-ribosylation factor 1 and patatin; (C) transcription related sequences: 3 sequences (5%), such as Myb-like DNA-binding domain and CCR4-associated factor 1; (D) metabolism related sequences: 5 sequences (8.5%), such as malate dehydrogenase and myo-inositol-1-phosphate synthase; and (E) those with

Table 7.2 : Summary of the BLAST similarity search results of selected differentially expressed cDNA transcripts

Clone	Length (bp)	Sequence homologue	Species	Identities	E value*	AC**	Database	Putative function	Notes
Stress and defence response related sequences									
IC9	599	Chalcone synthase	Potato	258/259 (99%)	4.9E-131	TC25786	TIGR	catalyse the first step in flavonoid biosynthesis pathway	
AC5	293	hydroxymethylglutaryl coenzyme A reductase	Potato	293/293 (100%)	2.5E-61	TC16595	TIGR	Catalyze the rate-limiting step in terpinoid phytoalexin production	
AB9	775	phenylalanine ammonia-lyase	Potato	711/722 (98%)	1.6E-154	TC21425	TIGR	Catalyzes the first step in the biosynthesis of phenylpropanoids	
AD12	768	phenylalanine ammonia-lyase	Potato	714/720 (99%)	2.2E-156	TC21425	TIGR		
AG2	176	flavonol synthase	Potato	173/176 (98%)	1.5E-34	TC15264	TIGR	Phytoalexin production	
		EST538349 *P. infestans*-challenged leaf	Potato	173/176 (98%)	2.0E-87	BI435588	NCBI		
AF12	296	Superoxide dismutase	Potato	264/264 (100%)	e-146	AF354748	NCBI	Oxidative stress	
IID1	277	GSH-dependent dehydroascorbate reductase	Potato	276/277 (99%)	3.8E-58	TC25547	TIGR	Oxidative stress	
IB4	443	metallothionein-like protein type 2 b	Potato	432/444 (97%)	2.4E-90	TC25557	TIGR	Induced by heat shock, oxidative stress, wounding, plant pathogens	

* E value = Expectation value. The lower the E value, the more significant the score

** AC = Accession number

Table 7.2 continued

Clone	Length (bp)	Sequence homologue	Species	Identities	E value	AC	Database	Putative function	Notes
AA1	412	Heat shock protein 90	Tomato	399/413(96%)	0.0	AF123259	NCBI	Stress response	Novel
IG12	382	70-Kd heat shock protein	Potato	330/382 (86%)	1.8E-60	TC21404	TIGR		
AF5	358	low molecular weight heat-shock protein (hsp18p protein) tobacco	Potato	335/341 (98%)	1.6E-70	TC23617	TIGR		
AC2	240	EST276997 tomato callus similar to heat shock protein 80	Tomato	209/249 (83%)	6.6E-32	AW033426	TIGR		Novel
IIG1	261	aspartic proteinase inhibitor	Potato	258/261 (98%)	9.5E-54	TC27525	TIGR	Biotic and abiotic stresses	PR-6
		proteinase inhibitor, complete cds		257/261 (98%)	E-136	D17330	NCBI		
IB12 & ID9	261	aspartic proteinase inhibitor	Potato	258/261 (98%)	9.5E-54	TC27525	TIGR		
		Kunitz-type proteinase inhibitor	Potato	261/261 (100%)	E-145	STU30814	NCBI		
IIE1	261	Kunitz-type proteinase inhibitor	Potato	257/261(98%)	E-135	STU30814	NCBI		
IH1	255	aspartic (Kunitz-type) proteinase inhibitor	Potato	254/255 (99%)	4.0E-53	TC27525	TIGR		
IE9	315	P23 protein	Potato	303/316 (97%)	1.1E-61	TC21391	TIGR	pathogenesis-related protein	PR-5
		EST537212 *P.infestans*-challenged	Potato	295/301 (98%)	E-149	BI434451	NCBI		
IG9	339	secretory peroxidase (Tobacco)	Potato	338/340 (99%)	6.9E-72	TC16647	TIGR	Stress response	PR-9
		EST536542 *P. infestans*-challenged leaf	Potato	339/339 (100%)	0.0	BI433781	NCBI		
IIC2	256	Ubiquitin conjugating protein	Potato	255/256 (99%)	2.5E-53	TC27621	TIGR	Protein turnover and stress response	
AE3	310	Ubiquitin conjugating protein	Potato	308/313 (99%)	2.1E-64	TC27621	TIGR		
IID2	317	Ubiquitin conjugating protein (*Avicennia marina*)	Potato	305/307 (99%)	6.7E-64	TC27621	TIGR		

Table 7.2 continued

Clone	Length (bp)	Sequence homologue	Species	Identities	E value	AC	Database	Putative function	Notes
IA4	391	IAA 4.1 deduced protein mRNA	Tobacco	128/137 (93%)	2.0E-49	AF123509	NCBI	water & osmotic stresses	Novel
IIB2	85	formate dehydrogenase	Potato	84/85 (98%)	5.7E-14	TC19814	TIGR	Stress response (one-carbon metabolism)	
IIB1	134	Ci21A gene (cold inducible)	Potato	133/135 (98%)	2.9E-25	TC23711	TIGR	Cold inducible	
AH2	240	40S RIBOSOMAL PROTEIN SA	Potato	240/240 (100%)	2.9E-50	TC21387	TIGR	Osmotic stress response	
IG4	322	putative protein (*A. thaliana*)	Potato	322/322 (100%)	1.6E-62	TC22102	TIGR		
		EST536811 *P. infestans*-challenged	potato	322/322 (100%)	0.0	BI434050	NCBI		
AE6	246	water-stress induced tonoplast intrinsic protein	Potato	246/246 (100%)	1.5E-51	TC18260	TIGR	Channel protein in tonoplast	
		EST537479 *P. infestans*-challenged leaf	Potato	246/246 (100%)	E-136	BI434718	NCBI		
AH4	310	Chlorophyll a/b-binding protein type I precursor (Tomato)	Potato	307/310 (99%)	7.7E-65	TC23477	TIGR	activated under Iron-stress condition	
		EST534164 *P. infestans*-challenged leaf	Potato	307/310 (99%)	E-167	BI431403	NCBI		
AD2	289	unknown protein (*A. thaliana*)	Tomato	280/290 (96%)	2.0E-57	TC21450	TIGR		Novel
		EST497292 *P. infestans*-challenged leaf	Tomato	261/263 (99%)	E-114	BG589450	NCBI	Stress response	
AE1	296	EST536915 *P. infestans*-challenged leaf	Tomato	145/150 (96%)	3.0E-65	BI434154	NCBI	Stress response	Novel

Table 7.2 continued

Clone	Length (bp)	Sequence homologue	Species	Identities	E value	AC	Database	Putative function	Notes
IC1	503	EST286026 mixed elicitor	Tomato	299/312 (95%)	E-144	AW092846	NCBI		Novel
IE7	268	EST284406 mixed elicitor	Tomato	263/267 (98%)	E-139	AW041542	NCBI		Novel
ID2	165	EST307746 tomato mixed elicitor	Tomato	138/140 (98%)	2.0E-68	AW442816	NCBI		Novel
IIH5	474	Unknown function EST538609 potato leaf *P. infestans*-challenged	Potato Potato	97.90% 93.50%	8.5E-55 3.0E-33	TC24109 BI435848	TIGR NCBI	Stress response	
IF11	370	Dehydration-responsive protein RD22 precursor	Potato	356/362 (98%)	1.7E-75	TC21411	TIGR	Dehydration-stress response	
IE12	217	Dehydration-responsive protein RD22 precursor {Arabidopsis} EST494327 cSTS	Potato Potato	185/209 (88%) 159/159 (100%)	9.7E-32 2.0E-84	TC21411 BG595649	TIGR NCBI	Dehydration-stress response	
Signaling related sequences									
IG7	327	patatin-like protein	Potato	293/300 (97%)	2.5E-60	TC18191	TIGR	Identical to cytosolic phospholipase A2, which involves in signal transduction	
IH12	310	patatin-like protein	Potato	292/301 (97%)	9.7E-60	TC18191	TIGR		
IG10	238	patatin	Potato	224/238 (94%)	3.5E-42	TC13720	TIGR		
IH4	191	patatin	Potato	191/191 (100%)	3.2E-39	TC13720	TIGR		
IA5	368	ADP-ribosylation factor 1	Potato	366/368 (99%)	3.3E-78	TC25479	TIGR	Cellular regulator for phospholipase D (PLD)	

Table 7.2 continued

Clone	Length (bp)	Sequence homologue	Species	Identities	E value	AC	Database	Putative function	Notes
Transcription related sequences									
AH3	263	Similar to CCR4-associated factor 1 (CAF1) from Mus musculus. EST287244tomato mixed elicitor	Tomato Tomato	256/263 (97%) 256/263 (97%)	1.6E-52 E-129	TC94090 AW094064	TIGR NCBI	A general transcriptional factor	Novel
IG5	284	Similar to Myb-like DNA-binding domain (*A. thalaina*)	Potato	174/180 (96%)	1.0E-33	TC27655	TIGR	transcription factor	
IE5	108	Squamosa promoter binding protein	Tomato	93/105 (98%)	5.0E-12	TC77067	TIGR		Novel
Metabolism related sequences									
AE5	383	myo-inositol-1-phosphate synthase	Potato	381/383 (99%)	9.0E-82	TC25589	TIGR		
IH9	209	myo-inositol-1-phosphate synthase (Topacco)	Potato	207/214 (96%)	6.4E-40	TC21755	TIGR		
AD1	76	cytosolic NADP-malic enzyme (malate dehydrogenase)	Tomato	73/76 (96%)	9.2E-11	TC89528	TIGR		Novel
IH8	149	Serine O-acetyltransferase EST523216 cTOS	Tomato Tomato	142/149 (95%) 142/149 (95%)	4.1E-26 6.0E-62	TC94268 BI205176	TIGR NCBI		novel
AE7	325	arogenate dehydrogenase mRNA	*A. thaliana*	126/158 (79%)	3.0E-07	AF434681	NCBI		Novel
Unknown function related sequences									
AD3	276	Unknown	Potato	276/276 (100%)	2.2E-58	TC25538	TIGR		
AG7	364	HYPOTHETICAL PROTEIN AT1G67360 (A. thaliana)	Potato	266/271 (98%)	7.4E-66	TC27841	TIGR		
IG11	99	Similar to JD1(tobacco) EST503942 cSTS	Potato Potato	92/93 (98%) 92/92 (100%)	4.3E-16 5.0E-28	TC26322 BG599042	TIGR NCBI		

Table 7.2 continued

Clone	Length (bp)	Sequence homologue	Species	Identities	E value	AC	Database	Putative function	Notes
AA3	325	cTOS cDNA clone	Tomato	221/235 (94%)	1.0E-86	BI209137	NCBI		Novel
AG5	254	Unknown	Tomato	236/256 (92%)	5.8E-44	TC85863	TIGR		novel
		EST527177 cTOS	Tomato	221/235 (94%)	3.0E-87	BI209137	NCBI		
AG1	425	PGPD14 (*Petunia x hybrida*)	Tomato	406/425 (95%)	2.8E-84	TC94044	TIGR	Expressed in early stages of flavonol-induced pollen germination in Petunia	Novel
		EST559223 potato roots	Potato	379/379 (100%)	0.0	BM111687	NCBI		
IH11	294	Unknown	Tomato	274/298 (91%)	2.9E-52	TC88008	TIGR		Novel
		EST257834 from *Pseudomonas* resistant tomato	Tomato	260/284 (91%)	E-100	AI776734	NCBI		
AD4	204	putative splicing factor (*Arabidopsis thaliana*)	Tomato	101/166 (60%)	1.1E-02	TC21810	TIGR		Novel
		putative glucosyl transferase (*Arabidopsis thaliana*)	Tomato	111/185 (60%)		AW944800	NCBI		
AF1	322	putative protein kinase	Tomato	99/162 (61%)	2.2	TC92844	TIGR		Novel

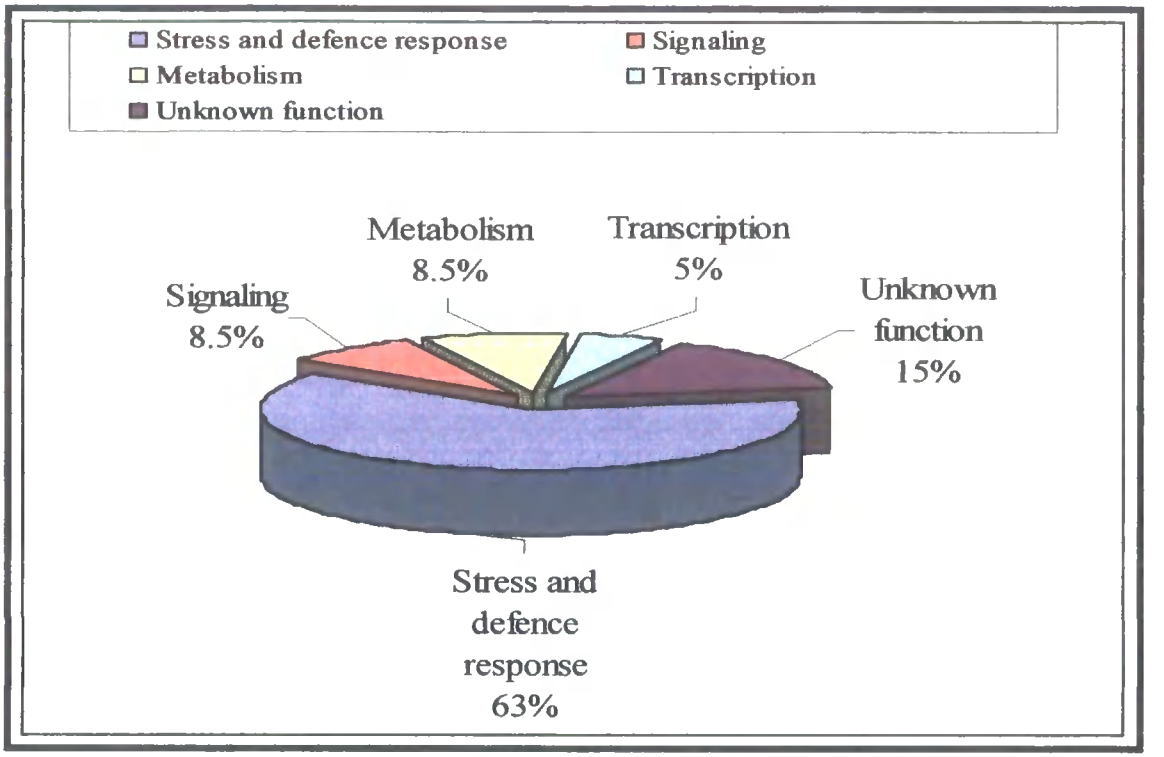


Figure 7-6: Functional grouping of the 59 selected sequences

Table 7-3: Representation and sequence identities of the abundant cDNAs from the selected clones.

Sequence representation	Identity
5	Proteinase inhibitor (PR-6)
4	Patatin and patatin-like protein
4	Heat shock proteins (HSPs)
3	Ubiquitin conjugating protein
2	Phenylalanine ammonia-lyase (PAL)
2	Myo-inositol-1-phosphate synthase
2	Dehydration-responsive protein

unidentified functions: 9 sequences (15%), such as JD1 and hypothetical protein AT1G67360.

Only two sequences (3%), clones AD4 and AF1, showed low similarity 60% and 61%, respectively, when compared with nucleotide and protein sequence databases, and probably represent new undescribed potato sequences. The rest had significant matches to known genes or EST sequences present in either Genbank or the TIGR databases at the time of writing the thesis.

The level of the redundancy in the selected clones was calculated. Among the sixty sequences, seven (12%) were found at least twice. The most abundant sequence, of which 5 copies were found, was proteinase inhibitor, which is one of the pathogenesis–related proteins (PR-6), (table 7-3). Four clones were homologous to heat shock proteins, three clones were homologous to ubiquitin conjugating protein and two clones of each of patatin, patatin like protein, phenylalanine ammonia-lyase, and myo-inositol-1-phosphate synthase were found in the selected clones. The abundance of these sequences may reflect their importance in establishing the potato resistance response.

Due to limitations on space only certain of these sequences will be discussed in detail regarding their significance in the present experimental context.

7.7.1 Sequences related to phytoalexin production

Phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plants as a result of infection or stress. Phytoalexins represent a chemically diverse group of compounds from a number of different metabolic pathways (see section 1.2.4). Since they are complex secondary metabolites, many genes are involved in the synthesis and regulation of phytoalexins. For example, phenylalanine ammonia-lyase (PAL) and

chalcone synthase (CHS) both function early in the biosynthetic pathway for phenylpropanoid phytoalexins, while flavonol synthase (FLS) functions on intermediates in the flavonoids biosynthetic pathway to produce a class of flavonols. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), functions in the early mevalonate pathway for sesquiterpenoid phytoalexins biosynthesis.

In the present study five clones, of the selected sixty clones, showed very high homology to sequences related to enzymes involved in phytoalexins production. These clones were: clone AC5 showed homology to HMGR, clones AB9 and AD12 showed homology to PAL, clone IC9 showed homology to CHS and clone AG2 showed homology to FLS (table 7-2).

7.7.1.1 Clone AC5, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)

cDNA clone AC5 is 293 bp long. Database searching revealed that it has a 100% homology, in the 293 bp overlapped region, to the nucleotide sequence of potato hydroxymethylglutaryl coenzyme A reductase (HMGR) (TC16595) in the TIGR database (see table 7-2 and figure 7-7 for the sequence homology and clustal alignment).

Hydroxymethylglutaryl-coenzyme A reductase (HMGR) (EC 1.1.1.3), an enzyme functioning early in the mevalonate pathway, catalyses the synthesis of mevalonate from 3-hydroxy-3-methylglutaryl-CoA. In plants, mevalonate is the precursor of all isoprenoid compounds. HMGR is essential for the biosynthesis of the sesquiterpenoid phytoalexins and steroid derivatives following stress imposed by wounding and pathogen infection. For example potato (*Solanum tuberosum* L.) tubers synthesize

(A)

(B)

```
1     ACGAATACGAACCGTGAAAGCGTGGCCTAACGATCCTTTAGACCT  AC5.seq
3079  ACGAATACGAACCGTGAAAGCGTGGCCTAACGATCCTTTAGACCT  TC16595.seq

46    TCGGAATTCGAAGCTAGAGGTGTCAGAAAAGTTACCACAGGGATA  AC5.seq
3124  TCGGAATTCGAAGCTAGAGGTGTCAGAAAAGTTACCACAGGGATA  TC16595.seq

91    ACTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGA  AC5.seq
3169  ACTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGA  TC16595.seq

136   TCCTTCGATGTCGGCTCTTCCTATCATTGTGAAGCAGAATTCACC  AC5.seq
3214  TCCTTCGATGTCGGCTCTTCCTATCATTGTGAAGCAGAATTCACC  TC16595.seq

181   AAGTGTTGGATTGTTCACCCACCAATAGGGAACGTGAGCTGGGTT  AC5.seq
3259  AAGTGTTGGATTGTTCACCCACCAATAGGGAACGTGAGCTGGGTT  TC16595.seq

226   TAGACCGTCGTGAGACAGGTTAGTTTTACCCTACTGATGACAGTG  AC5.seq
3304  TAGACCGTCGTGAGACAGGTTAGTTTTACCCTACTGATGACAGTG  TC16595.seq

271   TCGCAATAGTAATTCAACCTAGT                         AC5.seq
3349  TCGCAATAGTAATTCAACCTAGT                         TC16595.seq
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Figure 7-7 Clustal alignment of the nucleotide sequence of clone AC5 with the nucleotide sequence of potato hydroxymethylglutaryl coenzyme A reductase (HMGR) (TC16595) retrieved from the TIGR database. The block diagram (**A**) at top represents the overlapped region. The alignment (**B**) shows the actual nucleotide sequence aligned by the Clustal W method using Megalign. The shaded boxes represent identical residues.

antifungal sesquiterpenoid phytoalexins in response to fungal infection or arachidonic acid, an elicitor present in *Phytophthora infestans*, elicitation and toxic steroid glycoalkaloids in response to wounding via the mevalonate pathway. The activity of HMGR has been shown to increase rapidly in response to these stimuli. For example, Bianchini *et al.*, (1996) demonstrated that in potato tubers the HMGR levels increased 30-fold following arachidonic acid treatment and 15-fold following wounding.

RNA gel blot analyses using probes of three classes of cDNAs encoding potato HMGR (hmg1, hmg2, and hmg3) showed that hmgl was strongly induced in tuber tissue by wounding, but the wound induction was strongly suppressed by treatment of the tissue with the fungal elicitor arachidonic acid or by inoculation with an incompatible or compatible race of the fungal pathogen *Phytophthora infestans*. The hmg2 and hmg3 mRNAs also accumulated in response to wounding, but in contrast to hmgl, these mRNAs were strongly enhanced by arachidonic acid or inoculation with a compatible race of *P. infestans* (Choi *et al.*, 1992).

Laxalt *et al.*, (1996) found that when tuber discs were treated with eicosapentaenoic acid (EPA), an elicitor found in *P. infestans*, the expression of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a stress-related gene, is induced. This induction was parallel to that of the HMGR. Glucans obtained from the *P. infestans* cell wall acts synergistically with EPA on GAPDH and HMGR gene induction.

Chappell *et al.*, (1991) found that addition of cell wall fragments from *Phytophthora* species or cellulase from *Trichoderma viride*, to tobacco (*Nicotiana tabacum*) cell suspension cultures induced the accumulation of the extracellular sesquiterpenoid capsidiol due to the induced activities of the enzymes of the sesquiterpene biosynthetic

pathway, HMGR and sesquiterpene cyclase.

Kang *et al.*, (1998) have shown that HMGR and phenylalanine ammonia-lyase (PAL), involved in isoprenoid and phenylpropanoid biosynthesis, respectively, were mildly induced in tobacco leaves upon TMV infection at the late stage of the normal hypersensitive response (HR) or after salicylic acid treatment when compared with the PR-gene expression such as PR-1, beta-1, 3-glucanase and chitinase. However, in acute HR, they were strongly expressed at an early stage in the infection.

Using transgenic tobacco plants expressing a construct containing 2.3 kb of the tomato hmg2 gene promoter fused to the beta-glucuronidase (GUS) reporter gene, Westwood *et al.*, (1998) demonstrated that parasitisation by *O. aegyptiaca*, one of the *Orobanche* species that live parasitically on the roots of other plants and are capable of significantly reducing the yield and quality of their crop hosts, induces expression of the hmg2 gene. The expression of hmg2 was detected within 1 day following penetration of the host root by the *O. aegyptiaca* radicle and was localised to the region immediately around the site of parasite invasion. This expression continued and intensified over the course of *O. aegyptiaca* development.

Nelson *et al.*, (1994) suggested that HMGR may also play a key role as a component of the inducible defence mechanism in monocot plants as they found that it is strongly and rapidly induced in rice suspension cells by a fungal cell wall elicitor from the pathogen *Magnaporthe grisea*, the causal agent of rice blast disease.

All the above examples and many others support the important role that HMGR plays in the production of sesquiterpenoid phytoalexins as a plant defence mechanism to the pathogen attack and wounding. The isolation of an HMGR clone (AC5) from the

subtracted library in this study provides further support for the involvement and importance of this enzyme in potato defence to *P. infestans*.

7.7.1.2 Clones AD12 and AB9, Phenylalanine ammonia-lyase (PAL)

cDNA clones AD12 and AB9 are 768 and 775 bp long, respectively. A search for homologous sequences in the nucleotide sequence databases revealed identity with phenylalanine ammonia-lyase (PAL) as seen in the TIGR database (TC21425). The two inserts are slightly different in length but appear to be from the same gene. They showed 99% and 98% homology with TC21425 in the 720 bp overlapped region, respectively. The alignment of the predicted amino acid sequences of the inserts of these clones showed some mismatched residues at the end of the sequence that could be due to sequencing error especially because these were long sequences and were only sequenced in one direction (see figure 7-8).

Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) is a key enzyme of plant phenylpropanoid metabolism, which is involved in the biosynthesis of a wide variety of secondary metabolites such as isoflavanoid phytoalexins and lignin (a cell wall component). These compounds have many important roles in plants during normal growth and in responses to environmental stress. PAL catalyses the first committed step in the biosynthesis of phenylpropanoids by removing an ammonia group from L-phenylalanine to form *trans*-cinnamate. This reaction is considered to be a key step in the phenylpropanoid pathway (Hahlbrock and Scheel, 1989).

The wide range of PAL inducers ranging from abiotic to biotic stresses may be explained by the many intermediates that are associated with the phenylpropanoid pathway. Some of these intermediates are involved in disease resistance while others are

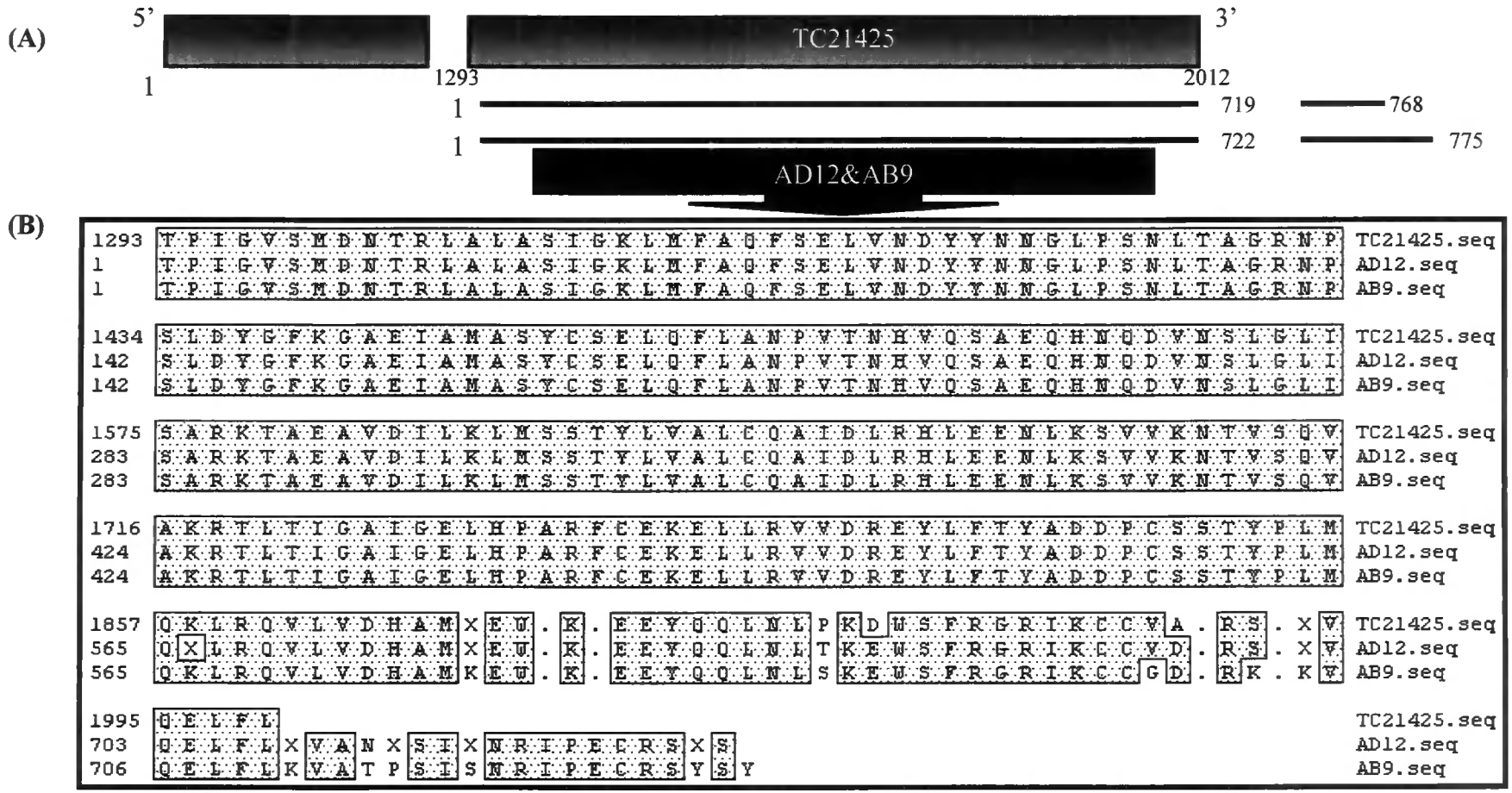


Figure 7-8 Clustal alignment of the predicted amino acid sequences of clones AD12 and AB9 with the amino acid sequence of potato phenylalanine ammonia-lyase (PAL) (TC21425) retrieved from the TIGR database. The block diagram (**A**) at the top represents the overlapped regions. The alignment (**B**) shows the predicted amino acid sequence aligned by the Clustal W method using Megalign. The Shaded boxes represent identical residues.

involved in wound healing and development.

Since change in PAL activity is a key event in controlling the synthesis of phenylpropanoids, PAL has become one of the most extensively studied enzymes in plants. There are many published articles, some of which are recent, which prove that PAL activity is induced upon pathogen attack as well as during other stress responses, using cell suspension cultures or plants. For instance, Schmidt *et al.*, 1998 demonstrated that rapid and transient increases in PAL activity was concurrent with induced incorporation of 4-hydroxybenzaldehyde, 4-hydroxybenzoate, and N-4-coumaroyl- and N-feruloyltyramine into the cell wall and secretion of N-4-coumaroyl- and N-feruloyltyramine into the culture medium of potato cell suspension cultures treated with an elicitor from *P. infestans*. These phenolic compounds are thought to be involved in cell wall reinforcement and may also affect fungal growth in the apoplastic space (Schmidt *et al.*, 1998).

In potato tuber discs treated with compatible and incompatible races of *P. infestans*, the induction of PAL activity was observed. The levels of PAL mRNA in both the total RNA and the polysomal RNA fractions, as well as the enzymatic activity, were higher in the incompatible than in the compatible interaction (Yoshioka *et al.*, 1996).

Ramamurthy *et al.*, (2000) studied the effect of gamma irradiation on lignin biosynthesis during wound healing in potato tubers. They found that the level of PAL, the first enzyme involved in lignin biosynthesis was five-fold higher in irradiated potatoes than in control tubers during wound healing.

El Modafar *et al.*, (2001) showed that the inoculation of the roots of resistant and susceptible cultivars of date palm seedlings by *Fusarium oxysporum* f, sp. *albedinis* or the elicitation with a hyphal wall preparation (HWP) induces PAL activity. The

PAL activity post-inoculation in the resistant cultivar was faster and to a higher level than in the susceptible cultivar. However, the elicitation of the seedlings by the HWP induced an identical PAL response in both resistant and susceptible cultivars, which indicated that the HWP elicitor was non-specific.

Li *et al.*, (2001a) demonstrated that the expression of PAL in wheat is organ-specific. Although, no detectable PAL expression was found in leaves, infection of leaves with *Puccinia graminis* f. sp. *tritici* (Pgt) induced, within 4 to 8 dayes, a high level of expression PAL transcripts in the Pgt-resistant line, while a delayed induction was observed in the Pgt-susceptible line. In cell suspension cultures, treatment with chitin oligomers or an elicitor derived from *P. graminis* germ tube walls also activated PAL gene expression. Kervinen *et al.*, (1998) demonstrated that PAL activity was induced in infected or elicitor-treated leaves and in cell suspension- cultures of barley as well as by the treatment with mercuric chloride.

Meena *et al.*, (2000) demonstrated that application of a plant growth-promoting rhizobacterium, *Pseudomonas fluorescens* strain Pf1 significantly controlled late leaf spot and rust diseases of groundnut (*Arachis hypogaea* L.) due to the induction of pathogenesis-related proteins, phenolics and PAL. Spraying the plants with *P. fluorescens*, showed increase in PAL activity 1 day after application and the maximum enzyme activity was detected 3 days after treatment.

Sharan *et al.*, (1998) studied the effects of methyl jasmonate and an elicitor on phenylpropanoid metabolism in tobacco. They found that the treatment of tobacco cell suspension culture with methyl jasmonate or with an elicitor from the plant pathogenic fungus *Fusarium solani* induced PAL activity. PAL was induced transcriptionally and enzymatically faster in elicitor-treated cells as compared to MJ-treated cells.

As mentioned earlier, PAL activity has been used as an early response indicator to biotic and abiotic stress, so as expected two clones (AB9 and AD12) from the 60 sequenced clones from the subtracted library showed strong homology to the PAL sequence (TIGR; TC21425) from the TIGR potato database.

7.7.1.3 Clone IC9, chalcone synthase (CHS)

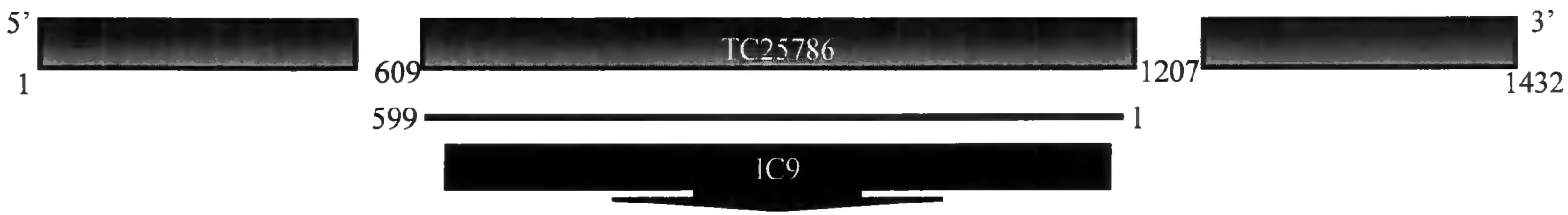
The subtracted cDNA clone IC9 was 599 bp long and showed 99% homology, at the nucleotide level, to potato chalcone synthase (CHS) (TC25786) from the TIGR database. The alignment of the translated nucleotide sequence is shown in figure 7-9.

Like PAL, chalcone synthase (CHS) (EC 2.3.1.74) is a classic stress responsive enzyme. CHS is the key enzyme of flavonoid biosynthesis. This enzyme catalyses the addition of three molecules of malonyl-CoA to 4-coumaroyl-CoA, producing a chalcone, the first compound in the flavonoid pathway.

Biosynthesis of flavonoids, which are well characterised as defence substances including UV protectants and antimicrobial compounds, is one of the most significant defence responses that plants have adapted to protect themselves against environmental stresses such as UV, mechanical wounding and pathogen attack. CHS is induced by all of these stimuli (Sakuta, 2000). For example, chalcone synthase accumulated rapidly in barley leaves in response to inoculation with the fungus *Blumeria graminis* f.sp. *hordei*, and the accumulation was also elicited by UV light (Christensen *et al.*, 1998).

Cui *et al.*, (1996) found that the inoculation of sorghum seedlings with the sorghum fungi *Peronosclerospora sorghi* or the maize pathogen, *Bipolaris maydis*, which also elicits a hypersensitive response in sorghum, caused rapid accumulation of both CHS and PAL transcripts. Although, seedlings of both resistant and susceptible cultivars

(A)



(B)

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74   S L G E K S S A K R M E D L I F L V C A S S M N G Y D S L V   TC25786 REV.PRO
1    S L G E K S S A K R M E D L I F L V C A S S M N G Y D S L V   Ic9.pro

104  Q R T A Q L K E P K L G L K L E V Q D L I A P G G P H A I W   TC25786 REV.PRO
31   Q R T A Q L K E P K L G L K L E V Q D L I A P G G P H A I W   Ic9.pro

134  F I S N W D S I G L P Q F A E I L S K E I N K S I L G F V D   TC25786 REV.PRO
61   F I S N W D S I G L P Q F A E I L S K E I N K S I L G P V D   Ic9.pro

164  K L L H F T L G V E R L H G D I A G E S D P L L T Q A A S V   TC25786 REV.PRO
91   K L L H F T L G V E R L H G D I A G E S D P L L T Q A A S V   Ic9.pro

194  L E F L P R E V E P L P D S G I I M A A A G D G F L A Q G V   TC25786 REV.PRO
121  L E F L P R E V E P L P D S G I I M A A A G D G F L A Q G V   Ic9.pro

224  M S D L H T D S P G S F T V A T I E S C V V L V R A G K M N   TC25786 REV.PRO
151  M S D L H T D S P G R F T V A T I E S C V V L V R A G K M N   Ic9.pro

254  E A L D K A L R I V T G G A F C G Q Q                       TC25786 REV.PRO
181  E A L D K A L R I V T G G A F C G Q Q                       Ic9.pro
```

Figure 7-9 Clustal alignment of the predicted protein sequence of clone IC9 and the protein sequence of potato chalcone synthase (CHS) (TC25786) retrieved from the TIGR database. The block diagram (**A**) at the top represents the overlapped region. The alignment (**B**) shows the actual protein sequence aligned by the Clustal W method using Megalign. The shaded boxes represent identical residues.

accumulated higher levels of CHS and PAL mRNAs than uninoculated controls, the accumulation of mRNA in resistant cultivars was higher and longer lasting than that in susceptible cultivars indicating the important of these enzymes in the resistance response.

Ebel *et al.*, (1984) demonstrated that a glucan elicitor from cell walls of the fungus *Phytophthora megasperma* f. sp. *glycinea*, a pathogen of soybean (*Glycine max*), induced rapid increases in the activities and the transcription of CHS and PAL in suspension-cultured soybean cells. The induction of transcription and activities of these enzymes was correlated with phytoalexins accumulation. Similar degrees of induction were also observed when the soybean cells were treated with diverse microbial compounds such as xanthan, an extracellular polysaccharide from *Xanthomonas campestris*, and endopolygalacturonase from *Aspergillus niger* (Ebel *et al.*, 1984).

Like PAL, CHS was expected to be represented in the subtracted library as one of the most important elements in the production of phytoalexins and defence responces.

7.7.2 Signalling related sequences

7.7.2.1 Patatin and patatin-like sequences

Four cDNA sequences, of the sixty clones that were sequenced, showed homology to patatin and patatin-like protein. These clones were IG10, IH4, IG7 and IH12 (table 7-2). Clones IG10 and IH4 had different insert sizes and represented different regions of the patatin EST sequence (TC13720) when compared with the nucleotide sequences in the TIGR database (table 7-2). IG10 clone was 238 bp long and was shown to have 94% homology at the nucleic acid level, while the IH4 clone is 191 bp long and was shown to have 100% homology in the 191 bp overlapped region to the 3' end of TC13720 (see

figure 7-10 for the clustal alignment).

Clones IG7 and IH12 also had different insert sizes but they represent the same sequence region of the gene. They showed homology with the patatin-like protein sequence (TC18191) in the TIGR nucleotide sequence database (table 7-2). IG7 clone was 327 bp long, while IH12 clone was 310 bp long and both were shown to have 97% homology to patatin-like protein sequence (TC18191) in 299 bp overlapped region. These are truncated cDNAs containing the 3' coding and non-coding regions of the mRNA. A putative polyadenylation signal is located at the 3' end of the nucleotide sequences. The alignment of these sequences with the retrieved sequence from the database showed three gaps and five mismatched residues (figure 7-11). These mismatched sequences were the same in the isolated clones and there were no gaps which meant that there are genuine differences between the isolated clones and the sequence in the database, which indicate that they may represent different genes.

Patatin is a member of a multigene family of vacuolar glycoproteins with a molecular mass of about 40 kDa. It represents 40% (w/w) of the total soluble potato tuber protein and is considered to be a storage protein- providing a source of nitrogen, sulphur and carbon for use when the tuber germinates and produce a new potato plant. However, unlike other storage proteins patatin also displays enzyme activity- lipid acylhydrolase and acyltransferase activities (see below) (Andrews *et al.*, 1988).

Two patatin multigene families with different expression patterns have been identified: class I patatin genes are mainly expressed in tubers whereas class II genes are found both in tubers and roots although at a much lower level as compared to class I genes (Pikaard *et al.*, 1987).

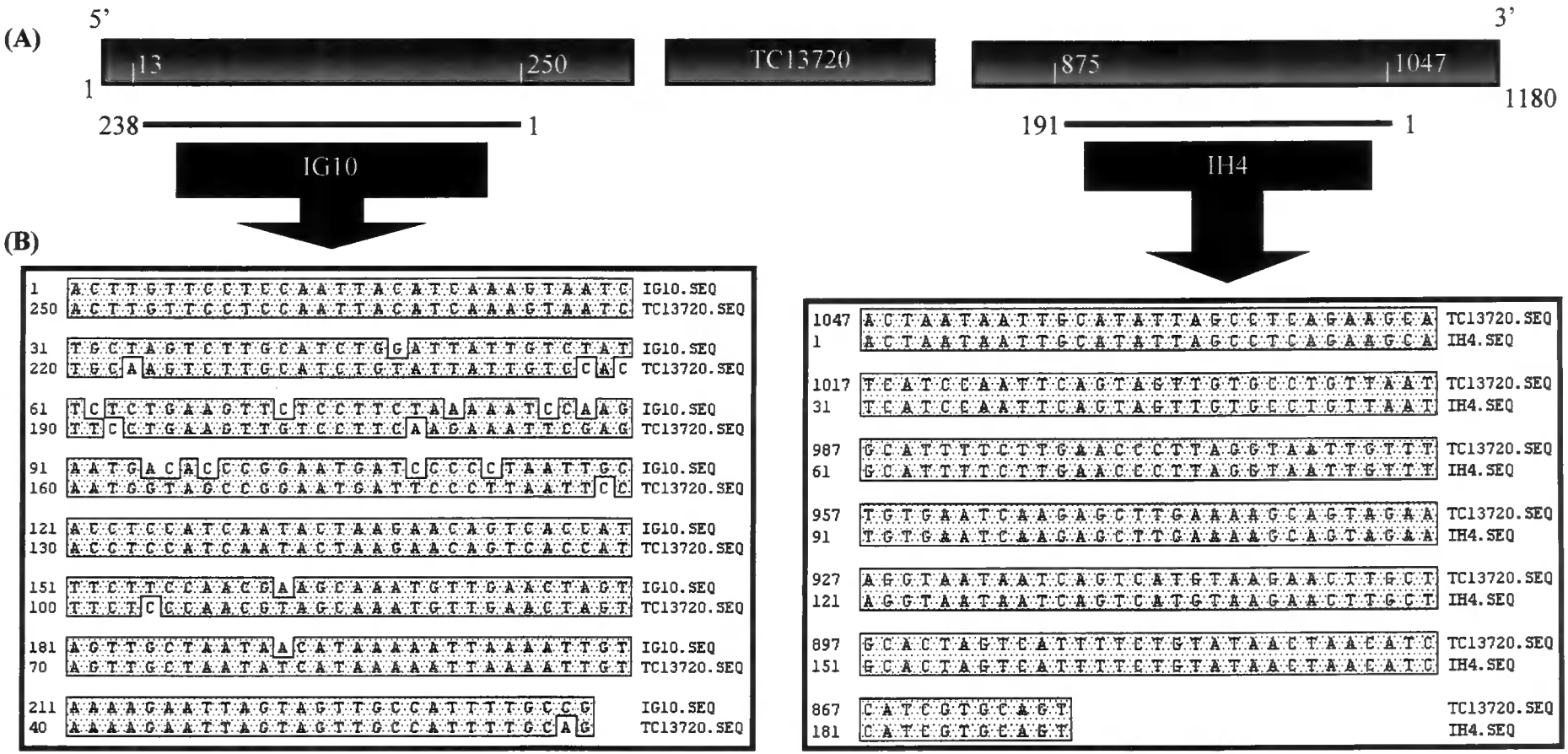


Figure 7-10: Clustal alignment of the nucleotide sequences of clones IG10 and IH4 and the patatin nucleotide sequence (TC13720) retrieved from the TIGR database. The block diagram (**A**) at the top represents the overlapped regions. The alignments (**B**) show the actual nucleotide sequence by the Clustal W method using Megalign. The shaded boxes represent identical residues.

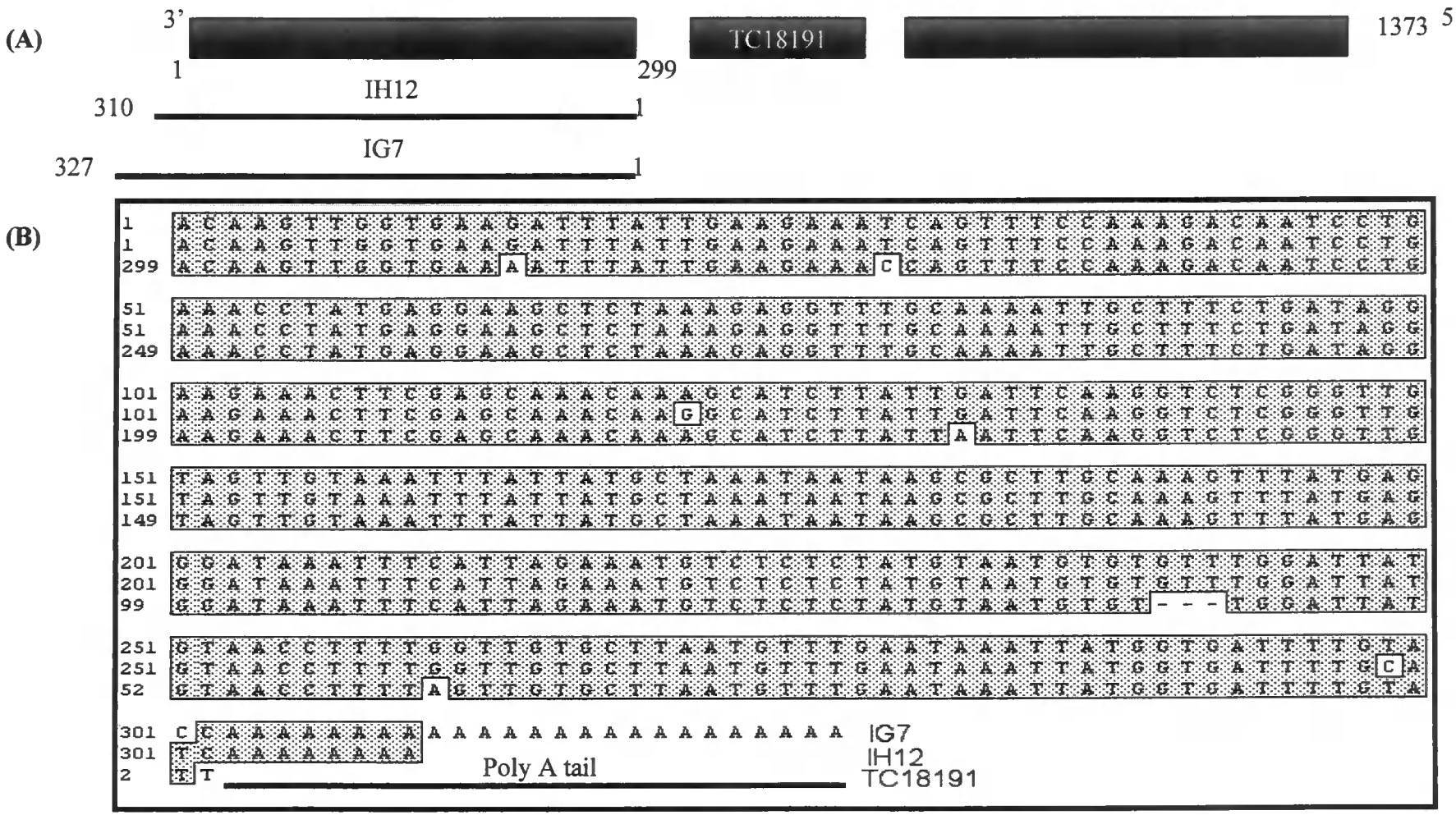


Figure 7-11 Clustal alignment of the nucleotide sequences of clones IG7 and IH12 with the patatin-like protein nucleotide sequence (TC18191) retrieved from the TIGR database. The block diagram (**A**) at the top represents the overlapped regions. The alignment (**B**) shows the actual nucleotide sequences aligned by the Clustal W method using Megalign. The shaded boxes represent identical residues and dashes (-) indicate gaps introduced to maximise alignment. The poly A tail is underlined.

It has been hypothesized that, besides being the main storage protein of potato tubers, patatin might also be involved in resistance responses induced during pathogen attack. The lipid acylhydrolase activity of patatin could in this context be important for the rapid degradation of cell membranes and thus rapid degradation of certain metabolites. Senda *et al.*, (1996) have shown that patatin is identical to a cytosolic phospholipase A2 (PLA2) from potato shedding new light on its possible physiological function. By treating potato tuber discs with an incompatible race of *P. infestans* or by treatment with fungal elicitor hyphal wall components (HWC), Kawakita *et al.*, (1993) showed that the activity of PLA2 increased to peak activity at 2-3 h following the treatment and indicated that PLA2 seems to be involved in signal transduction during the initiation of defence responses in potato tubers. Soybean cells have also been found to respond to treatment with bacterial and fungal elicitors by PLA2 activation (Chandra *et al.*, 1996).

Recent results demonstrated that, in tobacco leaves undergoing a hypersensitive reaction to tobacco mosaic virus, a strong increase in soluble PLA2 activity occurs at the onset of the appearance of necrotic lesions. This rapid PLA2 activation occurred just perior to the accumulation of 12-oxophytodienoic and jasmonic acids, two fatty acid-derived defence signals (Dhondt *et al.*, 2000). These results point to a possible role for patatin-like phospholipases in inducible plant defence responses. Membrane phospholipids in plant cells contain linoleic acid at the sn-2 position of glycerol. The liberation of linoleic acid by PLA2 might be an important step in the production of derivatives such as oxylipins, oxidized lipid-derived molecules, that have been shown to play significant roles in inducible plant defence responses against pathogens, either by directly deterring parasite multiplication, or as signals involved in the induction of sets of defence genes.

Patatin-like proteins have also been demonstrated in other plant species. Patatin-like

cDNAs have been cloned from various plants under different growth conditions such as cucumber seedlings (May *et al.*, 1998), tobacco leaves infected with tobacco mosaic virus (Dhondt *et al.*, 2000) and drought-stressed cowpea leaves (Matos *et al.*, 2001). Cucumber, tobacco and cowpea proteins were shown to display phospholipase A2 (PLA2) activity. Several other patatin-like homologs are published in sequence databases but their absolute identity and function remain putative.

In the current study, the abundant representation of patatin and patatin-like sequences during the potato-*P. infestans* interaction may lend support to a signalling role for these proteins in the signal transduction leading to resistance.

7.7.2.2 ADP-ribosylation factor 1

cDNA clone IA5 is 368 bp long. A database search for similarity to this sequence showed that it had 99% homology to the nucleotide sequence of potato ADP-ribosylation factor 1 (TC25479) in the TIGR database (figure 7-12).

ADP-ribosylation factor (ARF) is a highly conserved, low molecular mass (21 kDa) GTP-binding protein implicated in vesicle trafficking and signal transduction in yeast and mammalian cells. In recent years, a number of ARF proteins and genes have been identified from various eukaryotic organisms, including plants. Comparison of the ARF amino acid sequences identified has shown that all the sequences were highly similar to each other, indicating that ARF is a family of highly conserved proteins. Such conservation suggests that ARF's play a very important role in eukaryotic cell signalling (Kobayashi-Uehara *et al.*, 2001). In higher plants, cDNAs with high sequence similarity to mammalian ARF's have been isolated from *Arabidopsis* (Regad *et al.*, 1993), pea (Memon *et al.*, 1993), potato (Szopa and Mullerrober, 1994) and wheat

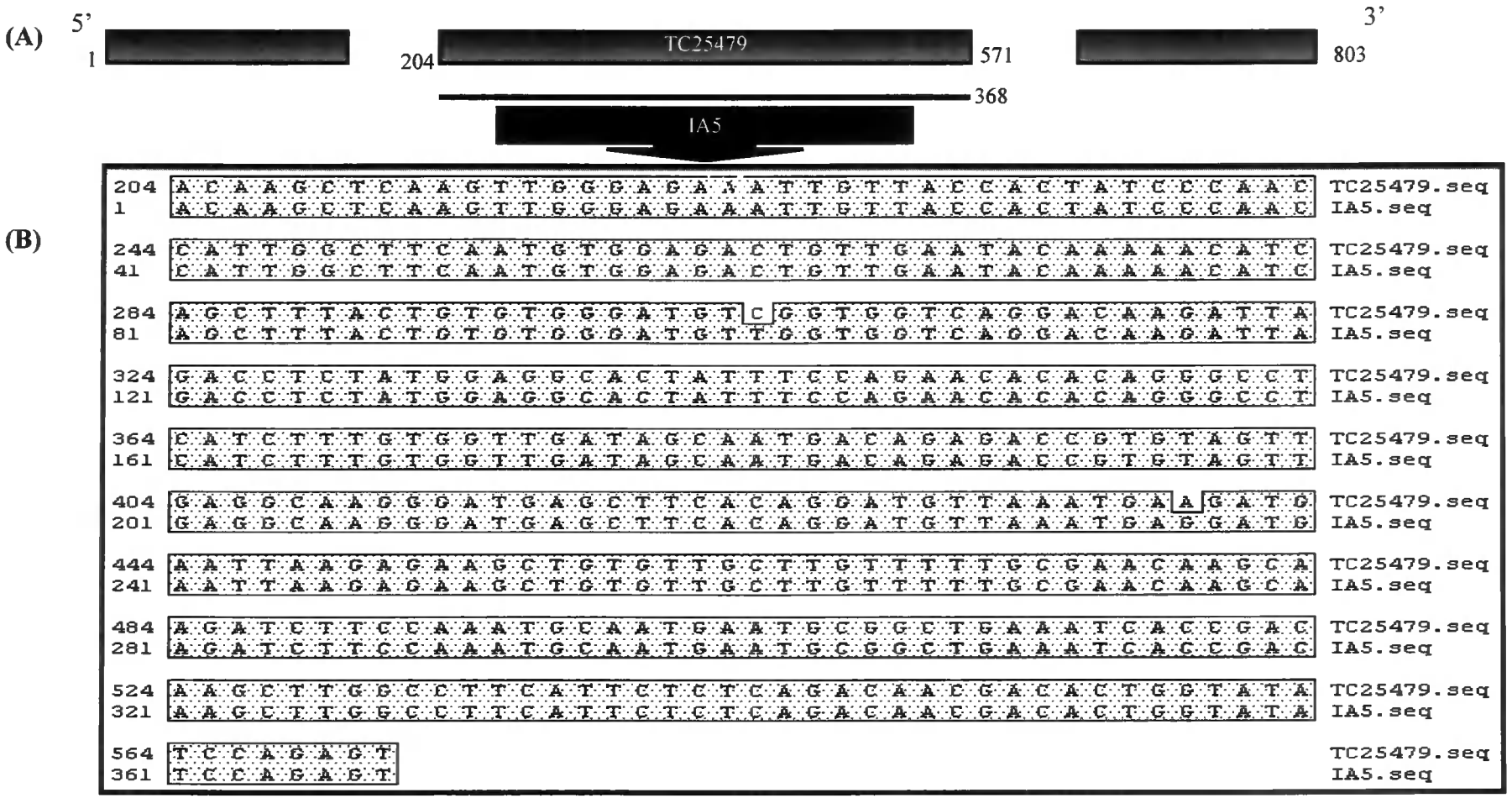


Figure 7-12: Clustal alignment of the nucleotide sequence of clone IA5 with the nucleotide sequence of potato ADP-ribosylation factor 1 (TC25479) retrieved from TIGR database. The block diagram (**A**) at the top represents the overlapped regions. The alignment (**B**) shows the actual nucleotide sequences aligned by the Clustal W method using Megalign. The shaded boxes represent identical residues.

(Kobayashi-Uehara *et al.*, 2001). However, little is known about the detailed functions of the plant ARF's.

Generally, ADP-ribosylation factor, a small G-protein, is a cellular regulator for phospholipase D (PLD; EC 3.1.4.4), which is one of the plant phospholipases that hydrolyse phospholipids, the structural elements of biological membranes. The activities of these enzymes not only have a profound impact on the structure and stability of membranes but also play a key role in regulating many critical cellular functions. The activation of phospholipases is involved in many cell-signaling cascades (see also patatin section 7.7.2.1). These enzymes often perform their regulatory functions through the generation of second messengers that transduce biotic and abiotic stress into physiological responses. Phospholipase D (PLD) has been identified recently as an important signalling enzyme in various organisms (for a review see Liscovitch *et al.*, 2000; Wang, 2000). Activation of PLD has been observed under a broad spectrum of biotic and abiotic stress conditions including water stress (Frank *et al.*, 2000), wounding (Ryu and Wang, 1996; Wang *et al.*, 2000), and pathogen challenge (Young *et al.*, 1996; van der Luit *et al.*, 2000).

For example, PLD gene expression in rice leaves was induced in response to *Xanthomonas oryzae* pv. *oryzae* and PLD was shown to be accumulated at the plasma membranes of cells at the point where resistant plants were infected. However, PLD was distributed evenly along the plasma membrane, and this distribution was maintained in the rice leaves undergoing susceptible interactions (Young *et al.*, 1996). This clustering along the plasma membrane could be related to the killing of the host cells and/or generating signalling messengers such as phosphatidic acid (PA) and its derivatives, free fatty acids and diacylglycerol (DAG). On the other hand, the change in

location could be the result of the hypersensitive response and might be involved in degrading cell membranes.

There are several possible mechanisms by which PLD is involved in stress responses:

I) PLD was found to release N-acylethanolamine (NAE) from N-acylphosphatidylethanolamine (NAPE) in fungal elicitor-treated tobacco cell suspensions. NAE accumulated in the medium 10min after xylanase or cryptogein elicitor treatment (Tripathy *et al.*, 1999). The activation of NAPE metabolism in plants appears to be associated mostly with cellular stress. In response to pathogen elicitors, NAPE is hydrolyzed by PLD, and the resulting medium-chain, saturated N-acylethanolamines (NAEs) are released by plant cells where they act as lipid mediators to modulate ion flux and activate defence gene expression (Chapman, 2000).

II) Recently, (Sang *et al.*, 2001) indicated that PLD plays a role in mediating superoxide production (oxidative burst) in plants through the generation of PA as a lipid messenger. PA promotes superoxide production by activating NADPH oxidase, which is a multicomponent enzyme composed of membrane-bound and cytosolic proteins. It becomes active when its four cytosolic proteins translocate to the membrane. A PA-dependent protein kinase mediates the functional reconstitution of this complex at the plasma membrane.

III) PLD plays important roles in plant responses to stresses through mediating the action and production of the stress-related growth factors such as abscisic acid (Fan *et al.*, 1997; Jacob *et al.*, 1999), ethylene (Lee *et al.*, 1998) and jasmonic acid (Wang *et al.*, 2000).

IV) PLD may also participate in defence responses by regulating the trafficking and

secretion of defence compounds. Recent studies suggest that mammalian PLD plays a role in regulated exocytosis (Jones *et al.*, 1999; Roth *et al.*, 1999). Although no reports address the role of PLD in protein secretion and vesicular trafficking in plants, several properties of plant PLD suggest future investigations in these areas.

The isolation of clone IA5 in the current study may highlight the importance of an indirect role for an ADP-ribosylation factor in the potato defence response to pathogen attack as a cellular regulator of PLD activity.

7.7.3 Sequences related to pathogenesis related proteins

Among the most frequently observed biochemical events that follow plant infection by pathogens is the production and accumulation of a family of proteins collectively known as pathogenesis-related proteins (PR-proteins) (see section 1.2.6); Including thaumatin-like proteins (PR-5), proteinase inhibitors (PR-6) and peroxidases (PR-9). Some of the cDNA clones that were obtained during this study contained sequences showing very strong homology to several pathogenesis- related proteins.

7.7.3.1 Clone IG9, peroxidase (PR-9)

cDNA clone IG9 is 339 bp long and showed very strong similarity (99%) to a potato peroxidase (TC16647; TIGR) as well as to the EST536542 sequence from *P. infestans*–challenged potato leaves (BI433781; NCBI) (100%), which although unidentified is presumably also a peroxidase enzyme (table 7-2). The alignment of the predicted protein sequence from this cDNA and the sequences retrieved from the TIGR (TC16647) and NCBI (BI433781) databases is shown in figure 7-13.

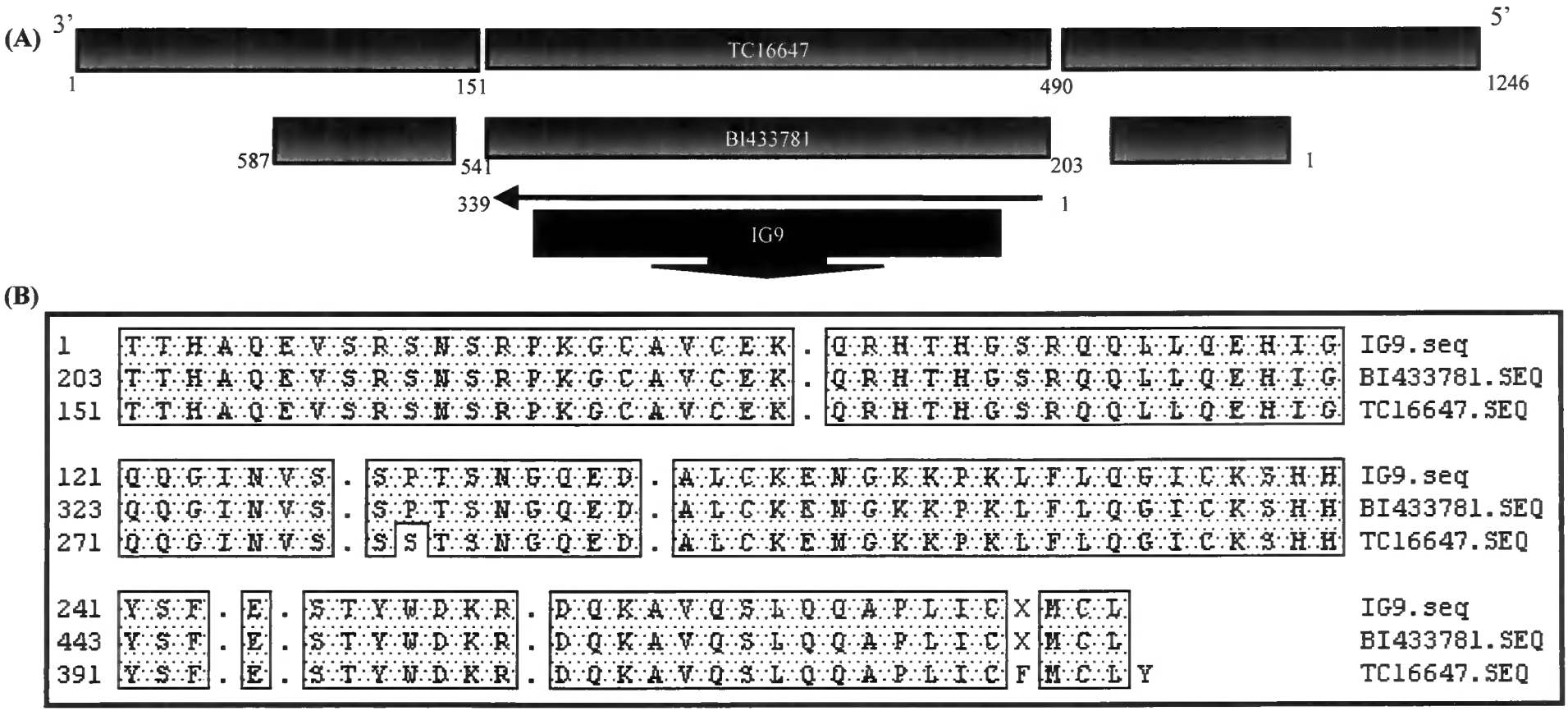


Figure 7-13: Clustal alignment of the translated nucleotide sequence of clone IG9 with the translated nucleotide sequences of EST536542 sequence from *P. infestans*–challenged potato leaves (BI433781), retrieved from the NCBI database and potato peroxidase (TC16647) retrieved from the TIGR database. The block diagram (**A**) at the top represents the overlapped regions. The alignment (**B**) shows the actual translated sequences aligned by by the Clustal W method using Megalign. The shaded boxes represent identical residues.

It has been reported that an increase in the activity of peroxidases is associated with early responses to pathogen infection (Cook *et al.*, 1995; Harrison *et al.*, 1995). Peroxidases are key enzymes in the plant cell wall-strengthening processes, which constitute one of the first lines of the plant defence against pathogen invasion. These processes include peroxidase-mediated oxidation of hydroxycinnamyl alcohols into free radical intermediates, phenol oxidation, polysaccharide cross-linking, extensin monomers cross-linking, lignification and suberization. All of which protect the plant tissues against the spread of pathogens.

Peroxidases have been proposed to enhance resistance by the construction of a cell wall barrier that slows down pathogen ingress and spread (Harrison *et al.*, 1995; Kawalleck *et al.*, 1995; Ostergaard *et al.*, 2000; Zimmerlin *et al.*, 1994). Peroxidases are also believed to be involved in several plant defence responses such as wound healing and in the production of antimicrobial compounds. For example, active oxygen species (AOS) generated within minutes after pathogenic interaction by an extracellular peroxidase are toxic to pathogens and are implicated in cell wall fortification (Thordal-Christensen *et al.*, 1997). On the other hand, these active oxygen species may also act as messengers to activate the plant defence responses that contribute to resistance (Levine *et al.*, 1994). Moreover, several papers reported the production of specific peroxidase isoforms during fungal infections of plants (Harrison *et al.*, 1995; Caruso *et al.*, 1999; Curtis *et al.*, 1997).

Recently, Collinge and Boller, (2001) isolated two genes using mRNA differential display, whose transcript levels increased during potato-*Phytophthora* interaction. One of these genes was a putative peroxidase, while the other was similar to putative

transcriptional activators from *Arabidopsis*. These *Arabidopsis* transcriptional activators were also shown to be induced by wounding.

Isolation of sequence homologous to peroxidase in the subtracted library may support the participation of this enzyme in durable resistance by slowing down the pathogen spread until the other defence responses are activated, by the strengthening the cell wall barrier and the production of antimicrobial compounds.

7.7.3.2 Clones ID9, IB12, IIG1, IIE1 and IH1, proteinase inhibitor (PR-6)

The most abundant sequences in the subtracted library, represented by five clones out of 60 (~ 8 %), showed strong homology to the potato proteinase inhibitors. Clones ID9, IB12, IIG1, IIE1 and IH1 are truncated cDNAs 261 bp long except IH1 clone which is 255 bp long. Searching the Genbank and TIGR nucleotide databases with these sequences revealed strong similarities to several plant proteinase inhibitors including potato aspartic (TIGR; TC27525) and kunitz-type (NCBI; STU30814 and D17330) proteinase inhibitors. The highest similarity was observed with the proteinase inhibitor sequences from potato (table 7-2). Alignment of the protein sequences of the cDNAs sequence with the sequences retrieved form the databases showed some residue mismatches, but it is unclear whether these are due to genuine differences between the protein sequences as these mismatches agreed at least in two different sequences (figure 7-14), or due to errors during constructing the library.

Proteinase inhibitors (PI's) belong to one group (family 6) of PR-proteins. However, several biological aspects of PI's distinguish them from other PR protein families. For example, PI's are structurally unrelated subclasses of proteins with different

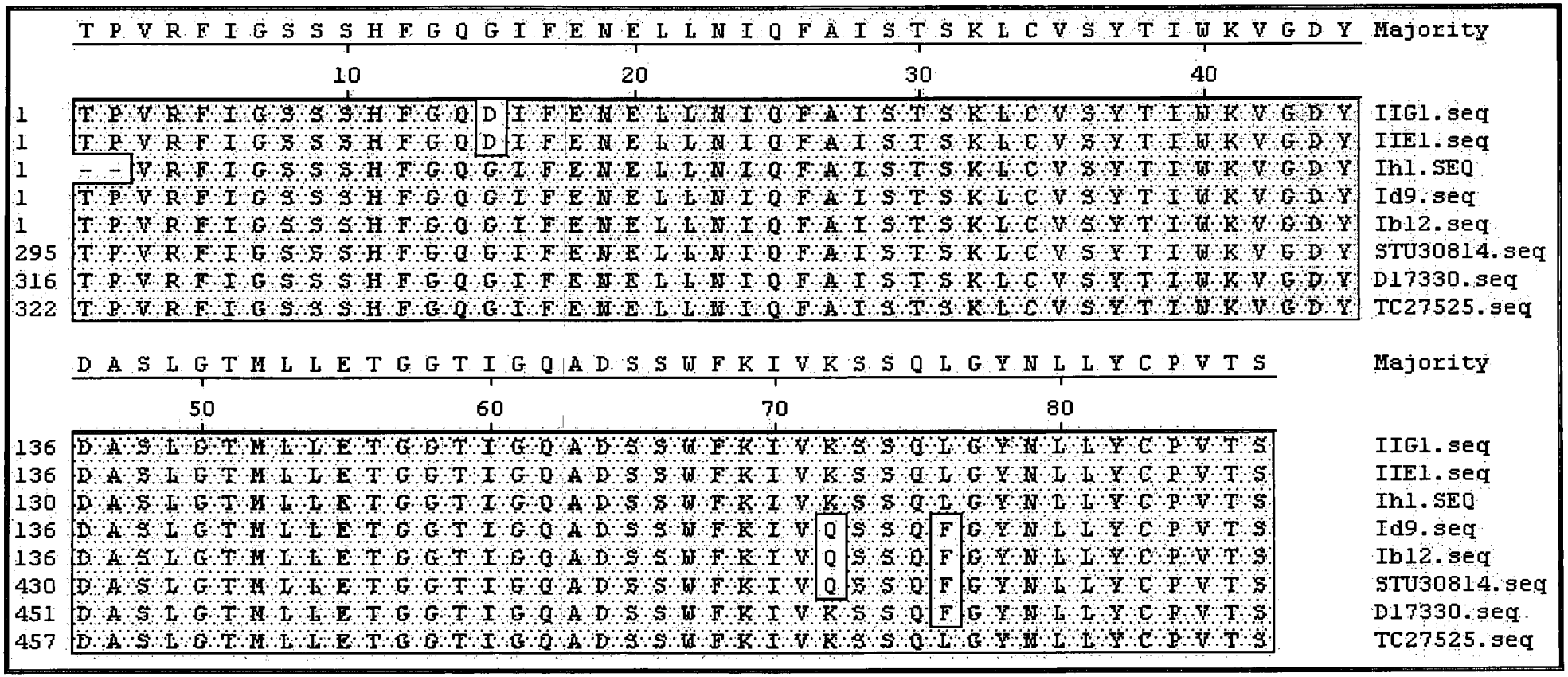


Figure 7-14 Clustal alignment of the overlapped regions of the translated nucleotide sequences of clones IIG1, IIE1, IH1, ID9 and IB12 with the translated nucleotide sequence of potato proteinase inhibitors (STU308414 and D17330) retrieved from the NCBI database and (TC27525) retrieved from the TIGR database. The shaded boxes represent identical residues.

mechanisms of regulation, but, in general, have the property of binding to proteinases. Furthermore, PIs control proteinase activity, a general biochemical function likely to be involved in many physiological processes during the development of a plant. Therefore, the involvement of PI's in defence may only represent one aspect of their function in the plant cell. Based on homology, number and location of disulphide bridges and reactive binding site position, there are ten structural groups (families) of plant proteinase inhibitors (Valueva *et al.*, 1998). Kunitz-type proteinase inhibitors, a member of the serine proteinase inhibitors, and aspartic proteinase inhibitors are examples of proteinase inhibitor that have been shown to be active on microbial or insect proteinases, and whose genes are induced in infected or wounded plants. Kunitz-type proteinase inhibitors are mostly single chain polypeptides of 20-24 kDa with four cysteines linked in two disulfide bridges with a single binding site. A group of proteins from potato tubers with molecular masses ranging from 20 to 25 kDa and having homology to Kunitz-type proteinase inhibitors, has been described (Ishikawa *et al.*, 1994a). It has also been shown that mechanical wounding of potato leaves results in the transcriptional activation of cysteine and aspartic proteinase inhibitor genes (Hildmann *et al.*, 1992). These inhibitors have also been shown to play a significant role in the natural defence mechanisms of the potato plant against insect and pathogen attack. For example, (Ishikawa *et al.*, 1994b) demonstrated that treatment of potato with methyl jasmonate induces the expression of two gene families encoding cysteine and aspartate proteinase inhibitors. Northern blot hybridisation of total RNA, isolated from potato leaves and tubers under non-stress conditions revealed that the gene transcripts encoding aspartic proteinase inhibitors normally occur mainly in potato tubers, but treatment of the potato plantlets by jasmonic acid, at concentrations of 50 -100 μM, dramatically induced the expression of the aspartic proteinase inhibitor gene transcripts

in leaves (Kreft *et al.*, 1997).

The abundant representation of proteinase inhibitor (~8.0% of the sequenced clones) in the current study, most likely indicates an important role for proteinase inhibitor in the establishment of durable resistance in potato-*P. infestans* system.

7.7.3.3 Clone IE9, thaumatin-like protein (PR-5)

IE9 is a truncated cDNA 315 bp long. Comparison of the nucleotide sequence of clone IE9 with nucleotide sequences in Genbank and TIGR databases revealed strong similarities (98%) to EST537212 from *P. infestans*-challenged potato leaves (NCBI; BI434451), and 97% to potato P23 protein (thaumatin-like protein; PR-5) (TIGR; TC27525). Alignment of the nucleotide sequence of this clone with the retrieved sequences from the TIGR (TC27525) and NCBI (BI434451) databases is shown in figure 7-15.

Pathogenesis-related proteins (PR-5) are a family of proteins that are induced by different pathogens in many plants and share significant sequence similarity with thaumatin, so PR-5 have been designated thaumatin-like proteins (TLP's). TLP's are not normally detected in leaves of young healthy plants, but they rapidly accumulate to high levels in response to biotic and abiotic stress in both dicot and monocot plants. For instance, a PR-protein named P23, a protein of 23 kDa, is accumulated in tomato leaves after infection with citrus exocortis viroid. P23 shows homology to tomato osmotin, which was found to be associated with osmotic stress in tomato (Rodrigo *et al.*, 1991).

Osmotin is a pathogenesis-related protein of group 5 (PR- 5) that displays antifungal activity *in vitro* and *in vivo*. For instance, Liu *et al.*, (1994) demonstrated that constitutive overexpression of osmotin in transgenic potato plants to a level of

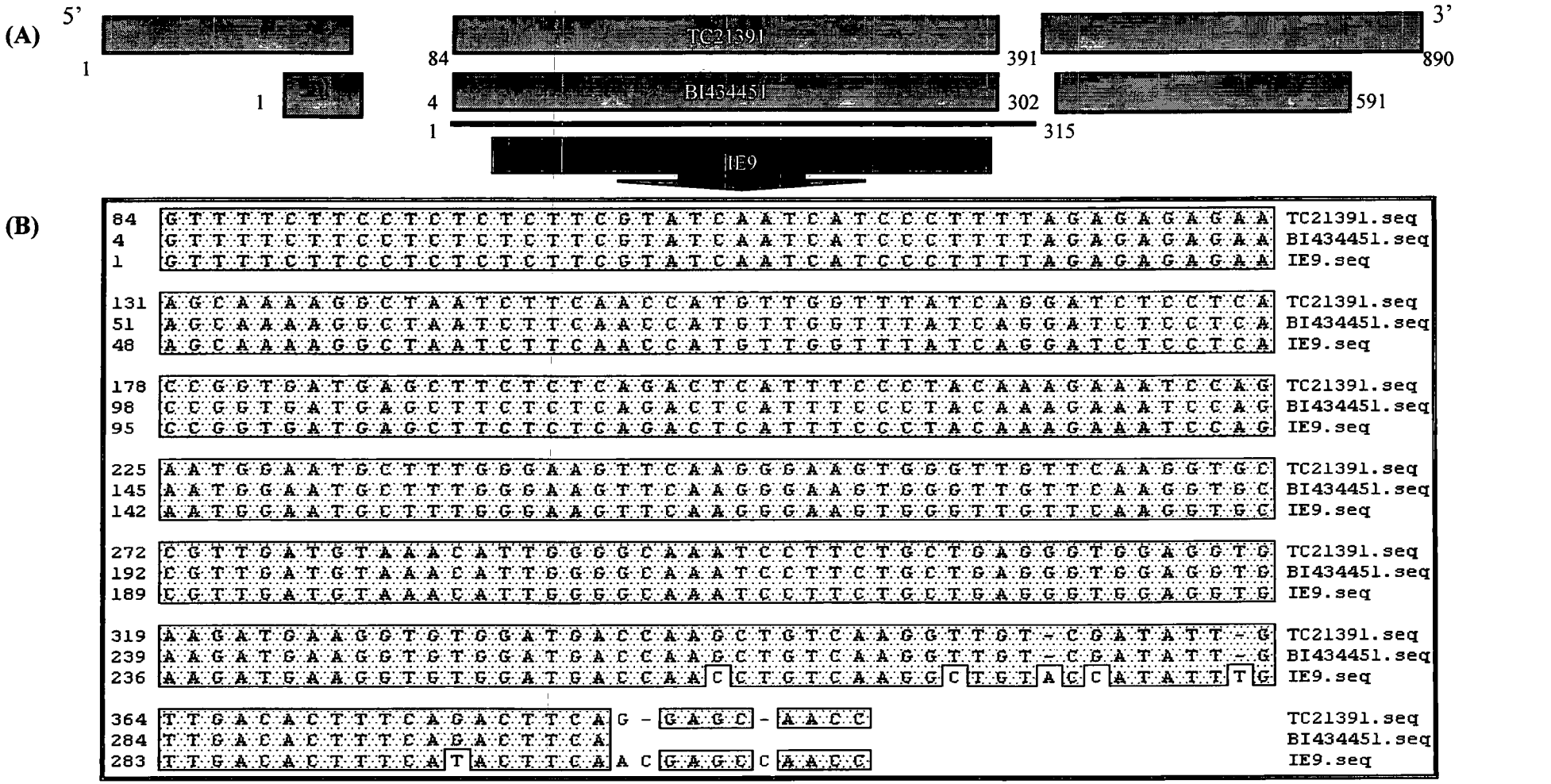


Figure 7-15 Clustal alignment of the nucleotide sequence of clone IE9 with the nucleotide sequences of potato EST537212 *P. infestans*-challenged leaves (NCBI; BI434451), and P23 protein (thaumatin-like protein; PR-5) (TIGR; TC27525). The block diagram (**A**) at the top represents the overlapped regions. The alignment (**B**) shows the actual nucleotide sequences aligned by the Clustal W method using Megalign. The shaded boxes represent identical residues.

approximately 2% of total cellular protein, delayed development of disease symptoms after inoculation with spore suspensions of *P. infestans*.

The site of accumulation of these proteins is important for their function. Basic (vacuolar) forms of PR-5 proteins have a C-terminal extension compared to the acidic forms which are secreted extracellularly. Liu *et al.*, (1996) demonstrated that transgenic potato plants overexpressing truncated PR-5 protein, without the 20 C-terminal amino acids, which is then secreted extracellularly, exhibited resistance to *P. infestans*.

Zhu *et al.*, (1995b) have characterised three cDNAs encoding osmotin-like proteins from potato cell cultures and found that infection with the fungus *P. infestans* activated strong expression of all three osmotin-like protein genes. The accumulation of osmotin-like proteins was detected only in *P. infestans*-infected tissues but not in plants treated with NaCl, low temperature, or wounding. Indicating this to be a pathogen-specific response.

Hu and Reddy, (1997) reported that the expression of ATLP-3 and ATLP-1 (*Arabidopsis* thaumatin-like proteins) genes, encoding PR5-like proteins, was induced by pathogen infection and salicylic acid. They, also, suggested that ATLP-3 may be involved in plant defence against fungal pathogens as the ATLP-3 protein showed antifungal activity, *in vitro*, against several fungal pathogens.

Lin *et al.*, (1996) carried out an extensive analysis of the induction of TLPs in oat seedlings infected with the stem rust fungus *Puccinia graminis*. They showed that four distinct TLP mRNAs were induced, some as early as 24h after infection. Plants infected with an incompatible isolate of the stem rust fungus pathogen accumulated higher levels of TLP mRNAs compared with those using compatible isolates. They also demonstrated that the expression of TLP genes in oats, especially the TLP-1 gene, is associated

with resistance reactions in response to infection by incompatible isolates of the stem rust fungus.

In the current study, the isolation of sequences homologous to TLP (PR-5) in the subtracted library was expected as it has been demonstrated that the expression of TLP increased after inoculation of potato plants with *P. infestans*, and the overexpression of TLP in transgenic potato resulted in delaying the development of disease symptoms after inoculation with *P. infestans* (Liu *et al.*, 1994; Liu *et al.*, 1996; Zhu *et al.*, 1995b).

7.7.4 Sequences related to ubiquitin-conjugating protein

Another one of the most abundant sequences found in the subtracted library encoded ubiquitin-conjugating protein. Three clones (AE3, IID2 and IIC2) showed very strong homology with the potato ubiquitin-conjugating protein (TC27621) in the TIGR nucleotide database. Clones AE3 and IID2 are truncated cDNA sequences (310 and 317 bp) containing the 3' end of the sequence. They showed 99% homology with the potato ubiquitin-conjugating protein (TC27621). Clone IIC2 (256 bp long) also showed 99% homology with the same gene but to a non-overlapping upstream sequence of the gene. The alignment of the translated sequence is shown in figure 7-16.

Ubiquitin is a widespread small protein found in all eukaryotes. It is involved in several important processes, including protein turnover, chromatin structure and in stress responses. Within the cell, ubiquitin is covalently linked to substrate proteins, often targeting them for degradation via the so-called ubiquitin pathway. This pathway has been demonstrated to be required for both the bulk degradation of cellular proteins and the targeted proteolysis of specific regulatory proteins. Proteolysis participates in many aspects of plant physiology and development. For example, it is responsible for:

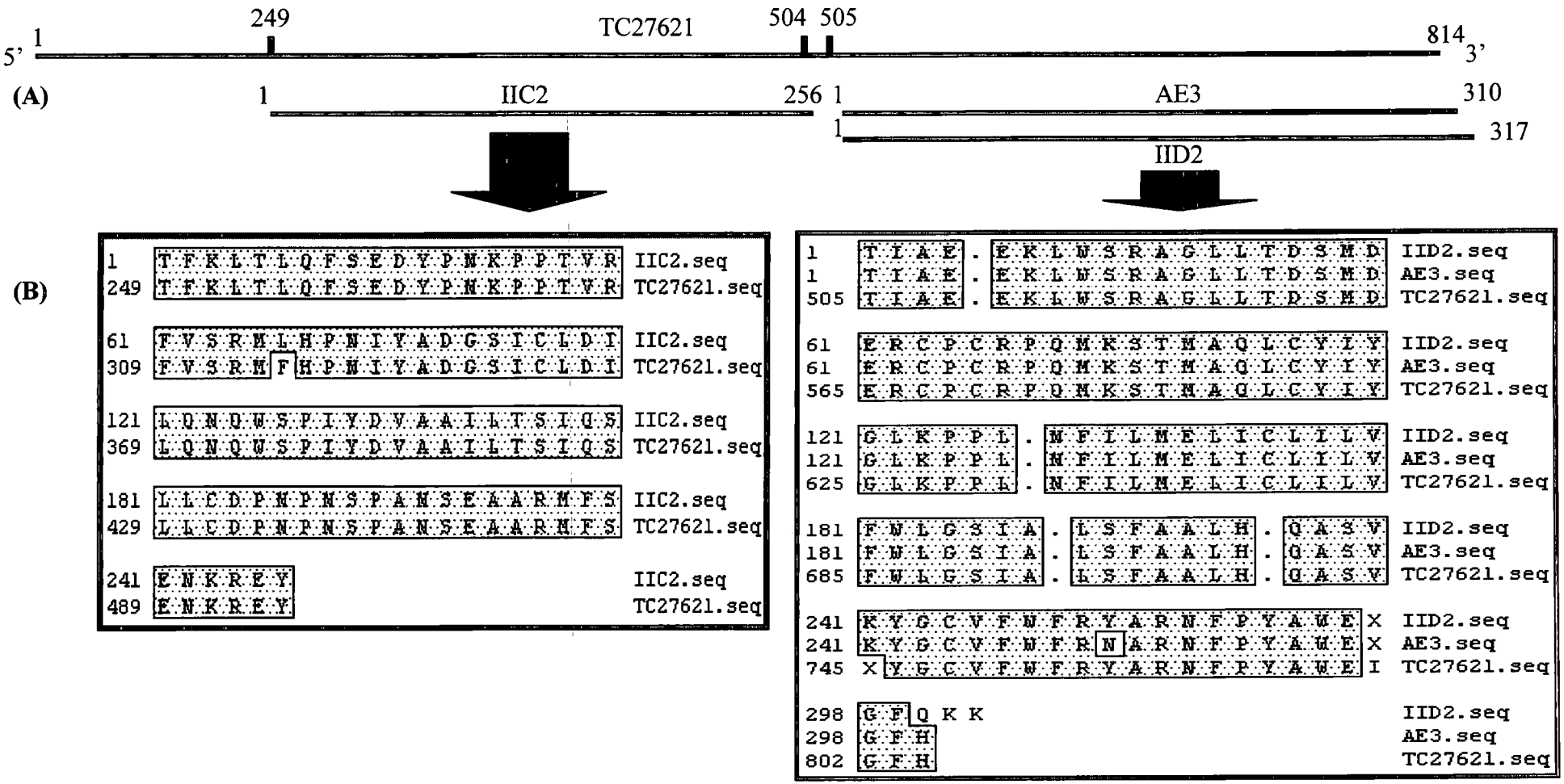


Figure 7-16 Clustal alignment of the translated sequences of clones AE3, IID2 and IIC2 and the translated nucleotide sequence of potato ubiquitin-conjugated protein (TC 27621) retrieved from the TIGR database. The block diagram (**A**) at the top represents the overlapped regions. The alignment (**B**) shows the actual translated sequences aligned by the Clustal W method using Megalign. The shaded boxes represent identical residues.

recycling amino acids needed to make new proteins; in response to stress by removing abnormal/misfolded proteins; in regulation of transcription; in controlling metabolism and development by reducing the abundance of key enzymes and regulatory proteins; and in programmed cell death of specific plant organs or cells (for a review see; Belknap and Garbarino, 1996; Vierstra, 1996 and vonKampen *et al.*, 1996).

Ubiquitin-dependent proteolysis of proteins occurs in two steps; first, ubiquitination-covalent attachments of ubiquitin to the ε-NH_2 group of an internal lysine residue of the target protein then degradation of the ubiquitinated protein complex in the proteasome. The covalent attachment of a ubiquitin monomer to the target protein requires three distinct enzymes: ubiquitin-activating enzyme, ubiquitin-conjugating enzyme and ubiquitin-protein ligase.

It has been suggested that the ubiquitin-proteasome system may play a crucial role in a process which switches the signalling pathway for diverse plant defence responses into a functional state, as it is known to participate in many basic cellular processes in both animals and yeast (Becker *et al.*, 2000).

More recently, the identification of the fifth subunit of ubiquitin ligase (Sgt1) as an essential component of R gene-mediated disease resistance suggested that the ubiquitin protein degradation pathway plays an important role in plant defence (Gray, 2002).

Rickey and Belknap, (1991) found that mechanical injury and heat shock induced the expression of several stress-responsive gene families in potato tubers. The steady- state levels of mRNA-encoding ubiquitin, HSP70, and phenylalanine ammonia-lyase (PAL) all increased within 45min of impact injury.

Basso *et al.*, (1996) demonstrated that inoculation of susceptible and partially resistant

potato cultivars with low doses of *P. infestans* sporangia led to a 5-fold accumulation of potato ubiquitin transcripts in both cultivars. These results established the connection between ubiquitin expression and defence reactions in plants and agree with the results from the present work. Thus it is highly probable that ubiquitin and its associated enzymes are important participants in establishing the resistance in potatoes.

7.7.5 Sequences related to oxidative burst

Under different environmental stresses, active oxygen species (AOS) such as superoxide radicals and hydrogen peroxide are produced at high levels. When hydrogen peroxide accumulates to levels of 10 μM, in chloroplasts, the enzymes of the Calvin cycle lose 50% of their activity. Superoxide dismutase (SOD) and ascorbate peroxidase (APX) protect these enzymes by scavenging active oxygen species reducing them to harmless products. In the reaction catalyzed by APX, ascorbate is oxidized to monodehydroascorbate (MDA). Rapid regeneration of ascorbate from its oxidized forms is required to support antioxidant capacity (scavenging of hydrogen peroxide by APX). During the regeneration of ascorbate in chloroplasts, some of the MDA is reduced to ascorbate by ferredoxin and the enzyme monodehydroascorbate reductase (MDAR) while some disproportionates to ascorbate and dehydroascorbate (DHA). DHA is reduced to ascorbate by glutathione-dependant dehydroascorbate reductase (DHAR) (EC 1.8.5.1) (Foyer and Mullineaux, 1998; Shimaoka *et al.*, 2000).

Two clones from the subtracted library (clones AF12 and IID1) showed homology to database gene sequences encoding enzymes associated with oxidative stress. Clone AF12 (296 bp long) showed 100% homology to potato superoxide dismutase (SOD) (NCBI; AF354748) in 264 bp overlapped region (Appendix C-1). While clone IID1

(277 bp long) showed 99% homology to GSH-dependent dehydroascorbate reductase (DHAR) (TIGR; TC25547) (Appendix C-2).

Superoxide dismutase (SOD), an enzyme involved in detoxification of superoxide radicals, plays a key role in the cellular defence against reactive oxygen species. Superoxide dismutase catalyses the dismutation of superoxide radical into O_2 and H_2O_2 thereby maintaining a low level of activated oxygen molecules in the cell.

It was found that overexpression of a manganese superoxide dismutase (MnSOD) in chloroplasts or mitochondria of transgenic tobacco plants could significantly reduce the amount of cellular damage which would normally occur by the generation of an oxidative burst (Bowler *et al.*, 1991).

Gupta *et al.*, (1993) studied transgenic tobacco plants expressing a pea gene encoding chloroplast-localized Cu/ZnSOD. They demonstrated that SOD is a critical component of the active oxygen-scavenging system of plant chloroplasts and indicated that modification of SOD expression in transgenic plants improved plant stress tolerance. Also, Allen *et al.*, (1997) demonstrated that increased activity of SOD in chloroplasts of transgenic tobacco plants generally leads to increased protection from membrane damage caused by exposure to the superoxide-generating herbicide methyl viologen (MV). In addition, they showed that overexpression of chloroplastic Cu/Zn SOD can lead to increased protection from photooxidative damage caused by growth under high light intensity and low temperatures.

Ho and Yang, (1999) found that mRNAs of defence-related genes, known to be differentially induced during the HR in Arabidopsis, including PR-1, glutathione S-transferase and Cu/Zn superoxide dismutase (SOD) increased significantly in the resistant *Arabidopsis thaliana* ecotype S96 leaves between 3 to 12 h after infiltration

with *R. solanacearum* strain Ps95 that causes bacterial wilt. The induction of these genes in the susceptible ecotype N913 was clearly delayed, indicating that these genes play an important role in the efficiency of plant defence.

Hernandez *et al.*, (2001) reported the effect of plum pox virus (PPV) infection on the antioxidative enzymes of apricot plants, including SOD and DHAR. They observed an increase in total SOD and DHAR activities in the inoculated resistance cultivar (Goldrich), while in the susceptible cultivar (Real Fine), inoculation with PPV brought about a decrease in SOD while DHAR activities were raised in comparison to non-inoculated (control) plants. The authors suggested a relationship between the SOD and DHAR activities and the apricot plants level of resistance to PPV.

7.7.6 Sequences related to heat shock proteins

Heat shock proteins (HSPs), which offer some protection from cellular damage, are present in both prokaryotic and eukaryotic cells. Some authors use the term ''Stress Protein'' instead of heat shock proteins as these proteins are induced not only under heat stress but also under a variety of other cellular stresses such as trace heavy metal exposure, organic pollutants, changes in temperature or osmolarity (water stress), oxidative stress, plant-pathogen interaction and exposure to ultraviolet radiation (Byth *et al.*, 2001; Lewis *et al.*, 1999).

There are several families of heat shock proteins basically classified according to their molecular weight, intracellular location, main inducer(s) and proposed function. According to their molecular mass there are three main groups; HSP90, covering the size range between 80 and 100 KDa; HSP70, covering sizes between 65 and 75 KDa and HSP60 and the small HSP's of sizes ranging from 16 to 40 KDa. The small HSP's

are most abundantly induced in plants by various stresses (Byth *et al.*, 2001; Lewis *et al.*, 1999).

Four clones, from the subtracted library, showed homology to different families of HSP. cDNA clone AA1 (412 bp long) showed 96% homology to tomato HSP90 (NCBI; AF123259), cDNA clone IG12 (382 bp long) showed 86% homology to tomato HSP70 (TIGR; TC21404), cDNA clone AF5 (358 bp long) showed 98% homology to potato small HSP18 (TIGR; TC23617), and cDNA clone AC2 (240 bp long) showed 83% homology to an EST from tomato callus similar to HSP80 (TIGR; AW033426) (Table 7-2; appendices C-3 to C-6).

Recently, an HSP90-based multiprotein complex, which plays an important role in signalling pathways in animal and yeast cells, has been identified in plant cells, but it remains to be seen if the HSP90 chaperone system plays a critical role in signalling pathways of plant cells, as it does in animal cells (Krishna, 2000).

It has been proposed that, at least in tomato, HSP70/HSC70 (inducible or constitutive 70-kDa heat shock protein) is induced by avirulent strains of *Ralstonia solanacearum* as part of the defence response to protect newly synthesised defence proteins and to maintain cellular homeostasis essential for the execution of a full defence response (Byth *et al.*, 2001).

Although, several components in plant resistance responses to pathogens are suppressed by heat shock (HS), it has been hypothesised that thermotolerance, marked by prior accumulation of HSP70/HSC70, protects the resistance response from heat-induced inhibition during simultaneous exposure to heat and to avirulent pathogens. This hypothesis was investigated in tomato by studying the effect of thermotolerance induced by a prior HS pulse, on phenylpropanoid metabolism activated by exposure to an

avirulent strain of *Ralstonia solanacearum* given simultaneously with a prolonged heat shock. This study indicated that thermotolerance associated with Hsp70/Hsc70 accumulation protects the enzymes of phenylpropanoid metabolism against heat-induced inhibition and in particular PAL enzyme activity during a second prolonged HS. In contrast, a prolonged HS without a prior HS pulse suppressed phenylpropanoid metabolism and promoted cell death (Kuun *et al.*, 2001).

In plants, some heat shock protein genes are inducible by oxidative stress. For example, it has been demonstrated that application of H_2O_2 and gamma irradiation to tomato cell suspension cultures induced the expression of a small heat shock protein (HSP22). Heat shock or a mild H_2O_2 pretreatment was also shown to lead to plant cell protection against oxidative injury (Banzet *et al.*, 1998).

Eckey-Kaltenbach *et al.*, (1997) characterised the first small heat shock protein cDNA clone from parsley (*Petroselinum crispum* L.), which has been shown to be induced by heat shock and by oxidative stress caused by exposure to ozone.

A conformational change has been observed during moderate heat stress and oxidation treatments (as judged by a shift to lower mobility in non-denaturing electrophoresis) in the structural properties of HSP21. This was demostrable both in purified recombinant form and in transgenic *Arabidopsis thaliana* plants engineered to constitutively overexpress HSP21 (Harndahl *et al.*, 1999). The authors suggested that the over-expression of the HSP21 in transgenic *Arabidopsis* may protect the plants from oxidative stress.

Taken together, these results demonstrate that in plants some HSP genes are inducible by oxidative stresses and pathogen challenge and play important role in protecting the

plant cell and response enzymes from oxidative damage and may participate in signalling pathways, which is very relevant in the current study.

7.7.7 Other stress and defence response related sequences

7.7.7.1 Clone IB4, metallothionein-like protein

The nucleotide sequence of clone IB4 (443 bp) showed 97% homology to a potato metallothionein-like protein (TIGR; TC25557) (table 7-2 and appendix D-1).

Plant metallothioneins are metal binding, low molecular weight cysteine-rich proteins. The general function of these proteins appears to be in the binding of metals through coordinate complexes with the thiol side groups thereby reducing a potentially toxic level of the metal in the cell. In recent years, metallothioneins (MTs) and metallothioneins-like proteins (MTL) have generated enormous interest due to their involvement in various physiological and pathological events. Differential expression of MTs and MTL proteins has been observed during embryogenesis and plant development as well as in response to exposure to external heavy metal concentrations (especially of Cu^{2+} and Fe^{2+}), and various stress factors such as heat shock, oxidative stress, wounding and plant pathogens (Kotrba *et al.*, 1999).

It has been proposed that antioxidant activity is an important function of MTs and MTL proteins in animal and plant cells, although the specific mechanisms of their antioxidant action are not known. For example, it has been reported that MTL protein genes of tomato are induced by oxidative stress (Giritch *et al.*, 1998). Choi *et al.*, (1996) reported that wounding and virus infection of tobacco plant resulted in the induction of an MTL protein gene.

The induction of MTL protein in our system (*P. infestant*-potato interaction) may support the involvement of these proteins in the resistant response to pathogen infection.

7.7.7.2 Clone IA4, auxin induced protein

Clone IA4 was 391 bp long and showed 93% homology in 137 bp overlapped region with a tobacco IAA-induced protein mRNA sequence in the NCBI database (AF123509) (appendix D-2A).

Indole-3-acetic acid (IAA) is the most abundant and widespread growth regulator in plants and mediates an enormous range of developmental and growth responses. Yamada, (1993) observed the attenuation of disease symptoms exhibited by plants infected with IAA-deficient mutants of *Agrobacterium tumefaciens*, so proposed the involvement of IAA in this plant-pathogen interaction. Furthermore, indole-3- ethanol, a storage form of auxins, was shown to inhibit zoospore germination and mycelium growth of different pathogenic fungi (Brown and Hamilton, 1992).

Recently, Noel *et al.*, (2001) demonstrated that pre-treatment of potato leaves with 10-μM IAA resulted in a 50 % reduction of *P. infestans* disease severity. Also, IAA was shown to exhibit antimicrobial activity directly *in vitro*. A 45 % inhibition of growth of the *P. infestans* mycelia was observed in V8-agar medium containing 1 μM IAA. The extent of protection and the percentage of mycelial growth inhibition were dose-dependent. These data support a putative role of IAA in the potato-*P. infestans* as a natural defence for pathogen spread and disease development.

7.7.7.3 Clone IIB2, formate dehydrogenase

The short nucleotide sequence of clone IIB2 (85 bp) showed 98% homology with formate dehydrogenase (FDH, EC 1.2.1.2) when searched against the nucleotide

sequence in TIGR database (TC19814) (appendix D-2B).

Formate dehydrogenase (FDH), a mitochondrial NAD-dependent enzyme, catalyses the oxidation of formate, which is a potential one-carbon source in higher plants and arises from various metabolic pathways, to CO_2. FDH has been shown to be induced under various stress responses as well as by treatment with chemical factors. For example, Hourton-Cabassa *et al.*, (1998) studied the effects of various environmental and chemical factors on FDH expression in potato leaves. They found that the abundance of FDH transcripts was strongly increased under various abiotic stresses including chilling, drought, hypoxia, dark, and wounding. They found that various chemical factors and metabolites such as formate, methanol, and ABA also induced the expression of FDH.

Recently, Li *et al.*, (2001b) reported the identification, and molecular characterisation of an *Arabidopsis* FDH cDNA clone. In studying the expression from the FAD gene they observed that the steady-state levels of FDH transcripts increased quickly (within hours) to high levels in response to various stresses.

The increase of FDH transcripts under various stresses and in our experiment may also support a putative role of FDH in the *P. infestans*-potato interaction as a natural defence for pathogen infection.

7.7.7.4 Clones AE6 and AH4

Clones AE6 and AH4 showed homology to ESTs isolated from *P. infestans*–challenged potato leaves. Clone AE6 (246 bp) showed 100% homology to EST537479 isolated from *P. infestans*-challenged potato leaf in Genbank nucleotide sequence database (NCBI; BI434718). Also this clone showed 100% homology to the water-stress induced tonoplast intrinsic protein (a channel protein in tonoplast) when searched against the

TIGR nucleotide sequence database (TIGR; TC18260) (see table 7-2; appendix D-3).

Clone AH4 (310 bp) showed 99% homology to EST534164 isolated from *P. infestans*-challenged potato leaf in the Genbank database (NCBI; BI431403) as well as 99% homology to chlorophyll a/b-binding protein type I precursor in the TIGR database (TIGR; TC23477) (see table 7-2; appendix D-4). It has been reported that the chlorophyll a/b-binding protein gene is induced in rice leaves 48h post-inoculation with the fungal pathogen *Magnaporthe grisea* (Rauyaree *et al.*, 2001).

7.7.7.5 Clones IG4, IIH5, AD2, AE1, IE7, ID2 and IC1

These seven clones (~ 12% of the selected subtracted clones) also showed homology to ESTs isolated form potato or tomato leaf treated with the *P. infestans* or treated with mixed elicitor. For the length, homologoes, identities and other information about these clones see table 7-2 and for the sequence alignment see appendices D-5 to D-11.

7.8 Conclusion

The suppression subtractive hybridisation (SSH) method was employed successfully to construct a cDNA library rich in the differentially expressed sequences that are induced during the compatible interaction between potato (variety Stirling) and the causal agent of late blight disease, *P. infestans*. Also it used to subtract the pathogene sequences that may have been presented in the treated plant tissues.

528 clones were randomly selected. At least sixty of these clones were sequenced and analysed in detail. The selection of these clones was mainly based on their hybridisation with the forward subtracted probe (i.e. up-regulated gene sequences) and in some cases with identified probes obtained from other labs. The sequence search for homology of

the sequenced cDNA's using the available databases revealed that all the cDNA's, except two, show high homology and probably identity with a diverse range of gene sequences. For the purpose of discussion these sequences have been organised into categories according to their putative function. Some sequences may warrant inclusion in more than one category. Among the categories identified were defence- and stress-related sequences that alone comprised 63% of the total sequences, signalling-related sequences, which comprised 8.5% of the sequences, and transcription-related sequences that made up 5% of the sequences. These specific categories are very relevant to the potato-*P. infestans* studies. Indeed several of the homologous sequences identified have come from similar biological systems, which were published during the course of this project. Also among these categories were the metabolism-related sequences which also have some relevance to the current studies because many changes in metabolism are likely to occur, especially in a compatible interaction between a plant and a pathogen. Similar results were obtained, recently, in different studies of a plant-pathogen interaction (Beyer *et al.*, 2001; Fristensky *et al.*, 1999; Rauyaree *et al.*, 2001).

Interestingly, among the sixty sequenced cDNA's, eighteen (30%) were novel potato gene sequences (see table 7.2). These sequences will eventually be submitted to the DNA databases. Three sequences were submitted to the Genbank database during this study. These sequences were superoxide dismutase (AC; AF354748), myo-inositol-1-phosphate synthase (AC; AF357837) and ubiquitin activating enzyme (AC; AF357838). A further 10 cDNA's were sequenced later in the project but were not subjected to detailed bioinformatics analysis- their putative identifies appear in table 8.1 (section 8).

Identification of cDNA's for many genes previously characterised from other species in this system provides corroboration of the involvement and potential importance of these

components in similar plant-pathogen situations. While the identification of such cDNA's is important, the value of the 'unknown class' of cDNA's, comprising a significant 15% of the subtraction cDNA's plus 6 clones (10%) categorised with the stress- and defence-related sequences, as they have homology to ESTs isolated from pathogen or elicitor treated leaves or cell cultures, and showed strong homology to sequences in the databases of unknown function, should not be underestimated. Results on the expression profile of these genes during the compatible interaction between potato and *P. infestans* may shed light on their potential defence function. These genes are also only a subset from the total library of more than 500 clones, of which 25% equals more than 125 clones still to be evaluated.

The abundant representation of some sequences such as proteinase inhibitors, patatins and heat shock proteins compared with others may indicate the importance of these genes in the resistance responses.

In section 8 cDNA microarray chips technique has been used in a preliminary study of the differences of transcribed genes profiles between the control Stirling untreated plants and Stirling plants treated with *P. infestans* during the establishment of durable resistance. DNA arrays have been constructed using the selected 60 sequenced clones along with identified and control cDNA clones, in order to get more detailed information about these differentially expressed gene sequences.

8 Expression profiling by microarraying

8.1 Introduction

Since changes in the physiology of a cell or an organism are associated with changes in the pattern of gene expression, analysis of gene expression is important in many fields of biological research. Several routine methods have been utilised to assess gene expression based on mechanisms of measuring the mRNA level such as Northern blotting, RT-PCR, differential display, and RNA dotblot analysis. However, each of these methods has its disadvantages, which make them unsuitable particularly if large numbers of expression products have to be analysed simultaneously. Recently, considerable improvement in sensitivity and throughput of expression screening has been obtained by the introduction of DNA microarray technology. The benefits of this approach over others for assaying gene expression are the huge numbers of genes that can be assayed simultaneously and the large number of transcript situations (RNA isolates) that can be easily investigated. The high initial cost of the precision robotic printing system, the expensive consumables and the demanding, time-consuming processing of large numbers of samples are disadvantages.

The Department of Biological Sciences was fortunate to receive funding for the purchase of a microarraying facility which was only completed and the instruments installed towards the end of 2001. It was a great opportunity for me to learn this new technique while being here and to study the levels of specific potato gene expression during the establishment of durable resistance to *P. infestans* by expression profiling. A selection of the characterised clones from the cDNA subtraction library was used along with various control sequences as immobilised probes printed on coated glass slides for

assaying the levels of transcription from individual genes.

The microarraying procedures described in the Materials and Methods were largely established during the first 6-months of 2002 by Dr Croy. The successful production of the first microarrays was based on the subtraction library clones produced and characterised in the present project. The relatively small number of clones used for these microarrays compared with conventional microarrays is offset by the fact that these clones had already been selected as upregulated genes at two levels - i) the production of the subtraction library and ii) screening of the library with forward and reverse subtraction cDNA probes. It was therefore anticipated that most if not all of these clones would be confirmed as upregulated.

Due to severe time limitations the experiments described here are essentially preliminary attempts and there was no opportunity to exploit the full time course of RNA samples available and necessary to describe fully the gene expression associated with the establishment of resistance to *P. infestans*.

8.2 Preparation of cDNA probes for arraying

Plasmids from the subtraction library clones along with a number of characterised potato clones, obtained from other sources as detailed in section 3.4, were prepared as described previously (section 4.3.8) or released from lysed cultures as described in section 4.5.1. The purified plasmids were used as templates for PCR amplifications of the cloned cDNA fragments as described in section 4.5.2. The amplified PCR products were purified (section 4.5.3) and then analysed simultaneously on 1% (w/v) agarose gels in TBE buffer (89mM Tris-borate, 2mM EDTA, pH8.3) using a 96-sample FAST gel 'stretch' system. Figure 8.1 shows a typical gel electrophoretic analysis of the PCR

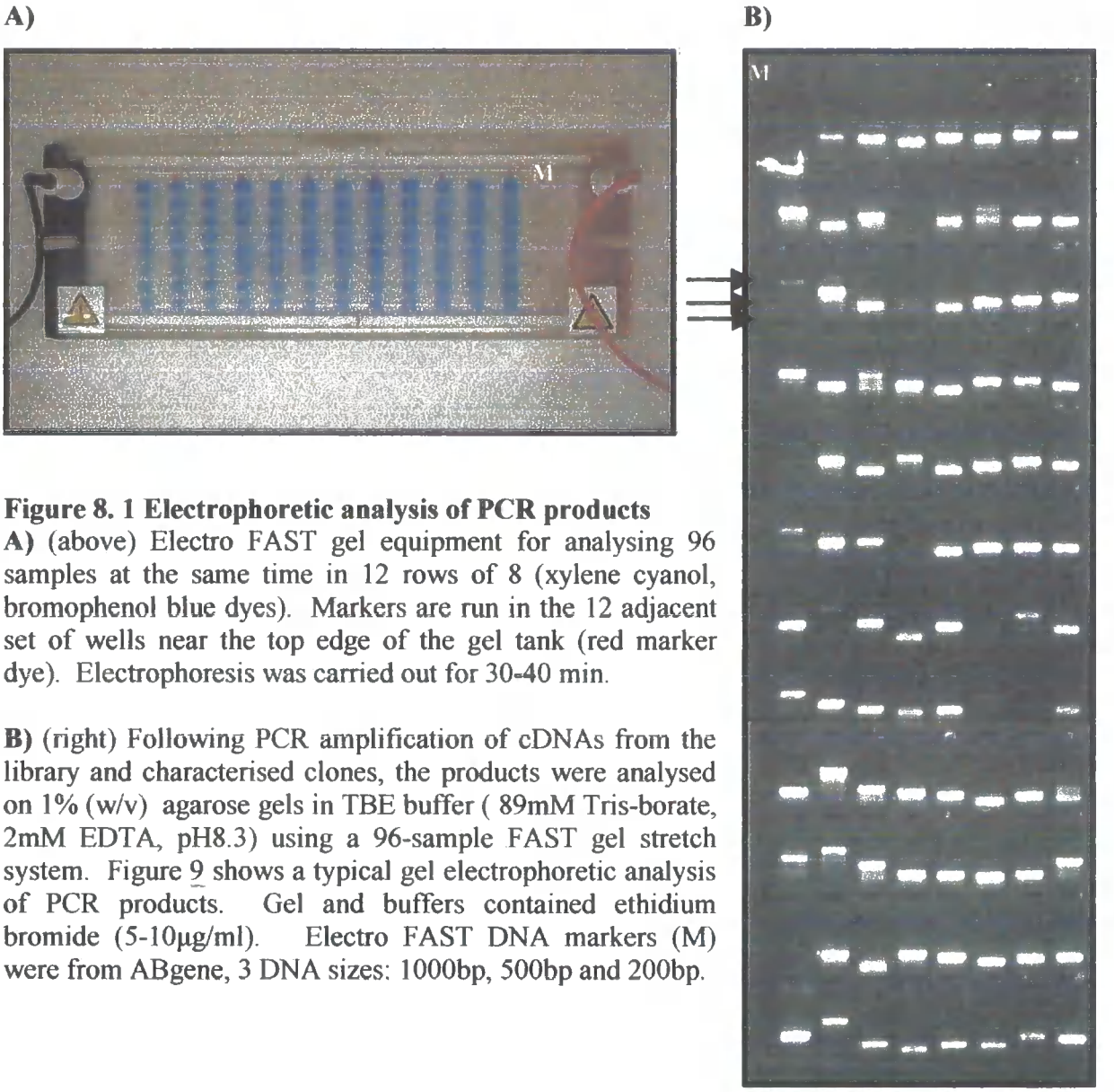


Figure 8. 1 Electrophoretic analysis of PCR products
A) (above) Electro FAST gel equipment for analysing 96 samples at the same time in 12 rows of 8 (xylene cyanol, bromophenol blue dyes). Markers are run in the 12 adjacent set of wells near the top edge of the gel tank (red marker dye). Electrophoresis was carried out for 30-40 min.

B) (right) Following PCR amplification of cDNAs from the library and characterised clones, the products were analysed on 1% (w/v) agarose gels in TBE buffer (89mM Tris-borate, 2mM EDTA, pH8.3) using a 96-sample FAST gel stretch system. Figure 9 shows a typical gel electrophoretic analysis of PCR products. Gel and buffers contained ethidium bromide (5-10µg/ml). Electro FAST DNA markers (M) were from ABgene, 3 DNA sizes: 1000bp, 500bp and 200bp.

products. The concentration of the amplified cDNAs was estimated either by fluorescence assay using Hoescht 33258 benzimidazole fluorescent dye or by ethidium bromide fluorescence following electrophoresis (section 4.5.3). Where necessary PCR products were concentrated by isopropanol precipitation and dissolved in smaller volumes or were printed using multiple transfers.

8.3 Construction of DNA microarrays

It was crucial to establish microarray formats, to identify the location of the cDNA probes on the microarray slides and to check the performance of the microarrayer before printing the cDNA probes. To achieve these, test arrays were printed with food dyes, diluted 1:1 with DMSO, on agarose coated slides using a Genomic Solutions GeneTAC G3 workstation equipped with a 48-pin printing tool as described in section 4.5.4. Figure 8.2 shows a test microarray slide using food dyes, showing half of the 8x8 matrices (patch pattern). The same patch pattern was used for arraying the purified PCR amplified probes along with black ink markers as shown in figure 8.3. Use of black ink (Indian ink) produced markers which lasted throughout the slide hybridisation processing and showed up during fluorescence scanning. This allowed the location of individual patches and cDNA probes. The cDNA probe samples were printed in 32 patches of 3 DNA samples each (=96 probes). To confirm that the cDNA's were arrayed successfully, the printed slide was stained with SYBR green I stain (section 4.5.4) to visualize the DNA spots as shown in figure 8.3B and C. The location of the printed cDNA probes on the microarray slides are indicated (figure 8.4) and the full identity of the probes is summarised in table 8.1.

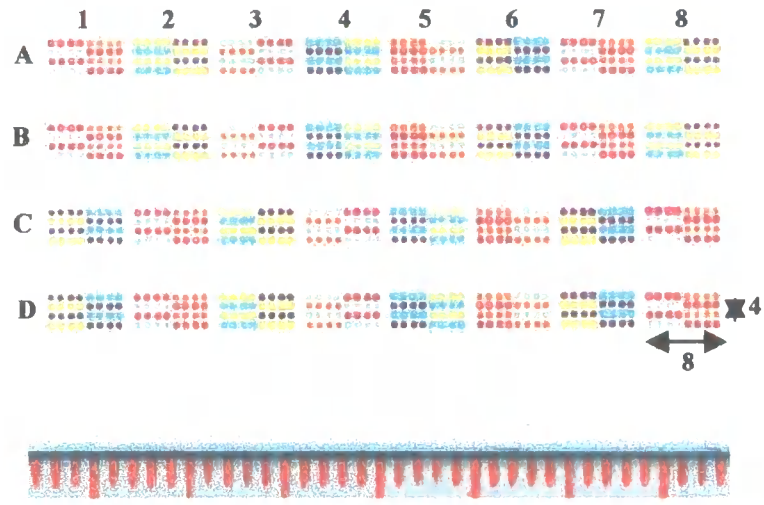


Figure 8.2 Test microarray using coloured food dyes showing the 8x8 matrix (patch pattern) used for arraying the gene probes. Each sample was printed in quadruplicate onto an agarose coated glass slide and represents 256 samples printed in 32 patches (4rows of 8 patches). The scale below is in millimetres. Each spot is ~400nm in diameter and 500nm apart. This microarray represents only half the normal patch size (8X4) since only 1X384 sample plate was printed.

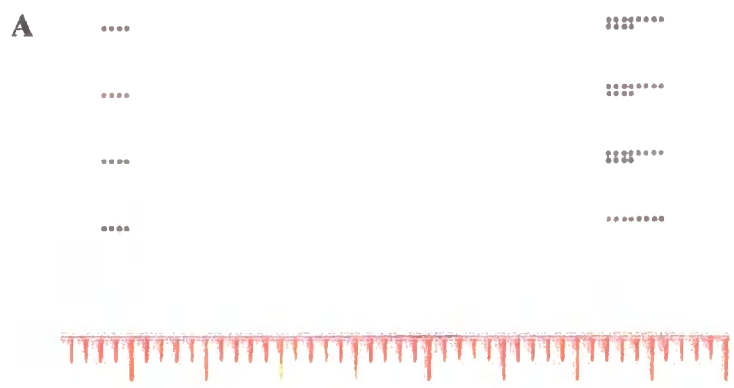


Figure 8.3 Example of a cDNA printed array comprising of 32 patches each of 3 DNA samples = 96 samples

A – Unstained array showing the positions of the black ink markers. The scale is in millimetres.

B – the array stained with SYBR green I stain

C – Closeup of a section of the stained array showing the stained quadruplicated cDNA's and markers.

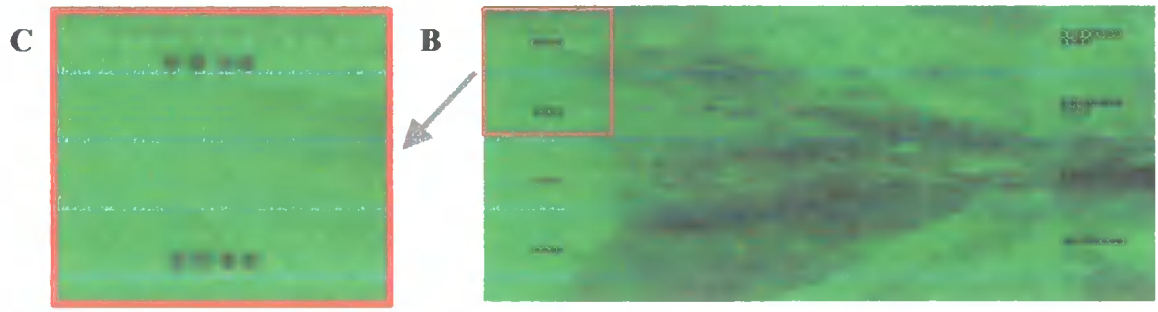


Table 8.1 - cDNA probes printed on slide microarrays

Row	Column	cDNA Library	Clone Number	Notes on library and any clone characterisation
A	1	potato subtraction cDNA library made from - cv Stirling tissue culture plants challenged with *P.infestans*	AF5	Heat shock protein - HSP18
	2		AF12	superoxide dismutase (SOD)
	3		AG1	PGPD14 {Petunia x hybrida}
	4		AG2	flavonol synthase
	5		AG5	unknown
	6		AH2	40S Ribosomal protein
	7		AH3	CCR4-associated factor 1
	8		AH4	Chlorophyll a/b-binding protein
	9		AD5	mixed clone
	10		AD12	PAL
	11		AE3	ubiquitin conjugating protein
	12		AE4	-
B	1		AE5	myo-inositol-1-phosphate
	2		AE6	ion channel protein
	3		AE7	arogenate dehydrogenase
	4		AF1	-
	5		AA1	heat shock protein - HSP90
	6		AA3	EST-cTOS cDNA clone
	7		AB9	**PAL**
	8		AC1	-
	9		AC2	heat shock protein - HSP 80
	10		AC5	HMGR- CoA reductase
	11		AD3	unknown
	12		AD4	putative splicing factor (60%)
C	1		IIC2	ubiquitin conjugating protein
	2		IIF3	**Kunitz proteinase inhibitor**
	3		IID1	dehydroascorbate reductase (GSH)
	4		IID2	ubiquitin conjugating protein
	5		IIE1	proteinase inhibitor
	6		IIG1	proteinase inhibitor
	7		IIG3	-
	8		IIH1	-
	9		IG11	similar to JD1 (tobacco)
	10		IH1	potato proteinase inhibitor
	11		IH4	patatin
	12		IH9	myo-inositol-1-P synthase
D	1		1H11	tomato EST – resistant
	2		IH12	patatin-like protein
	3		IIB1	cold inducible (Ci21A) gene
	4		IIB2	formate dehydrogenase
	5		IF2	**dormancy protein,auxin reg**
	6		IF8	**unknown - ethylene receptor?**
	7		IF11	dehydration responsive protein
	8		IG4	unknown - putative protein
	9		IG5	DNA-binding protein
	10		IG7	patatin-like protein
	11		IG9	peroxidase
	12		IG10	patatin
E	1	potato subtraction cDNA library made from - cv Stirling tissue culture plants challenged with *P.infestans*	ID2	tomato mixed elicitor
	2		ID4	-
	3		ID7	-
	4		ID9	potato proteinase inhibitor
	5		IE3	**major latex protein (ripening specific)**
	6		IE4	-
	7		IE5	promoter binding protein
	8		IE7	putative protein & elicitor
	9		IA4	IAA induced protein
	10		IA5	ADP-ribosylation factor I
	11		IB4	metallothionein-like proteien
	12		IB5	**unknown - catalase domain?**
F	1		IB12	proteinase inhibitor
	2		IC1	unknown function & elicitor
	3		IC2	**unknown - no clear sequence**
	4		IC9	chalcone synthase
	5		IH8	serine O-acetyltransferase
	6		IIH5	EST P. infestans-challenged leaf, potato
G	1		IG12	heat shock protein, HSP70
	2		AG7	*A. thaliana* ORF, tomato
	3		IE9	P23 protein, tomato
	4		IIH4	**PAL (*Agastache rugosa*)**
	5		AD1	cyt NADP-malic enzyme, tomato
	6		AD2	EST *P. infestans*-challenged leaf, tomato
	7		AE1	EST P. infestans-challenged leaf, tomato
	8		1E12	dehydration-response protein RD22
H	1	*standards*	**pRR8**	*chloride channel protein A.thaliana*
	2		**pRR12**	*heat shock tomato*
	3		**pRR19**	*alcohol dehydrogenase*
	4		**pRR20**	*alcohol dehydrogenase*
	5		**pLOX28**	*lipoxygenase*
	6		**pYP3**	*unknown tuber sequence*
	7		**pYP5**	*unknown tuber sequence*
	8		**pYP6**	*unknown tuber sequence*
	9		**pTEL15**	*potato extensin*
	10		**pTEL16**	*potato extensin*
	11		**SOD**	*superoxide dismutase*
	12		**AC**	*acidic chitinase*
			PPO32	*polyphenoloxidase*
			BC	*basic chitinase*
			PPO33	*polyphenoloxidase*
			AG	*acidic glucanase*
			NSE	*control 1 - NSE neurone specific enolase*
			BG	*basic glucanase*
			GAP43	*control 2 – growth-associated protien*
			PAL	*phenylalanine-ammonia lyase*
			pUC18	*control 3 - pUC18 plasmid*
			PR-1	*pathogenesis related protein*

Additional characterised clones IG1 = glutathione S-transferase; GST (tomato) IH2 = kunitz proteinase inhibitor

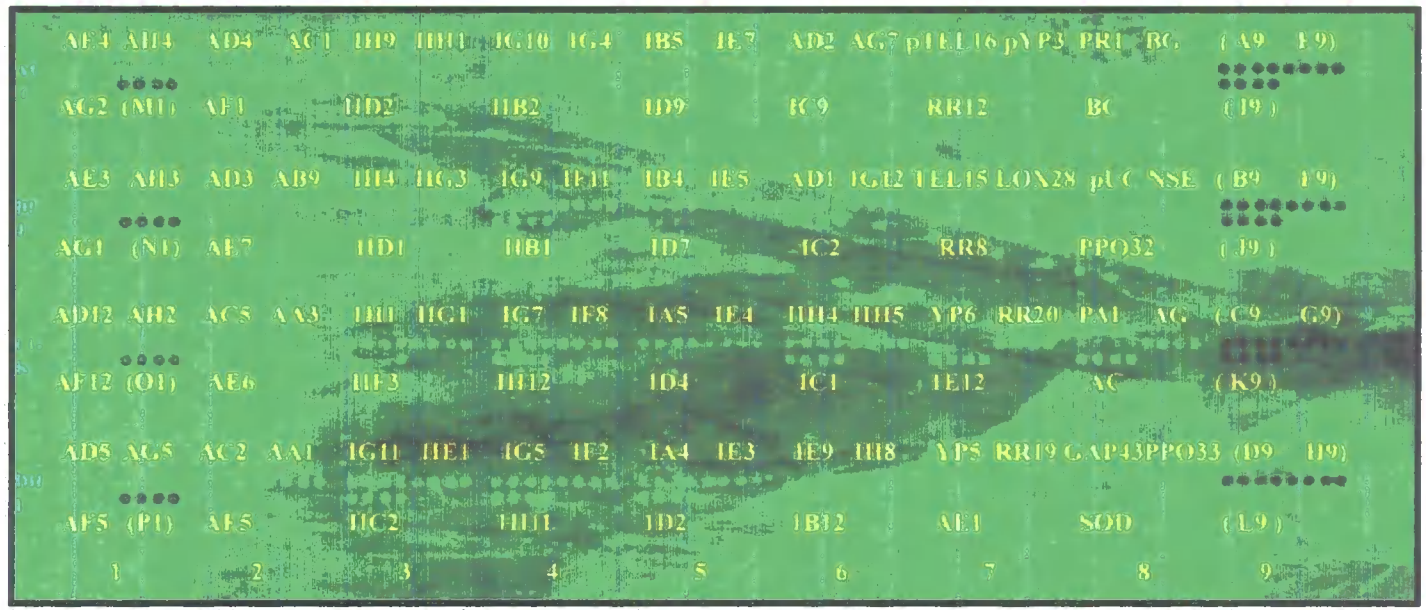


Figure 8.4 Location of the printed cDNAs on the SYBR green I stained microarray. See Table 8.1 for the full identity of the probes. The stained array was scanned in a Genomic System LSIV scanner using the cyanin 3 laser / filter. The array is flanked by black marker spots which were maintained throughout the hybridisation and washes and show up against the fluorescent background.

8.4 Hybridisation and results analyses

The cDNA microarray containing 76 potato subtraction library cDNA's produced and characterised in the present project, and 22 cDNA obtained from other groups was hybridised with a combined Cy3 and Cy5 fluorescent-labelled targets prepared from total RNA of a pooled potato tissues from treated and control Stirling plants during the development of durable resistant as described in section 4.5.5. Following hybridisation and washing, the microarray slide was scanned using a Genomic Solutions LSIV array scanner using lasers/filters optimised for detection of Cy3 (excitation 552nm; emission 565nm) and Cy5 (excitation 650 nm; emission 667nm). A scan of Cy5 fluorescence is shows in figure 8.5. Clones that are expressed at detectable levels in untreated Stirling leaf tissues (control) such as AG2, AG7, IB4 and IC2 are indicated. As expected, none of the negative control sequences showed a detectable level of fluorescence. Under the conditions used the background Cy3 fluorescence was very high and non-uniform which hides many of the patches (example is shown in figure 8.6). The possible reasons for this were drying out of labelled target solution, precipitation of target, inadequate/inefficient washing steps or binding of the targets to the slide coating. However, by selecting different areas of the array and adjusting the laser gain and background levels it was just possible to come to conclusions about expression from some of the genes (see figures 8.7A and B). This was only possible where at least three of replicate spots showed about the same level of fluorescence such as AD2, PR-1, BG, BC and IC9 (figure 8.7A). It was however not possible to use the automatic spot detection and quantitation option produced by the scanner analysis software. Spots revealed in the Cy3 (green) channel represent hybridisation with target sequences from *P. infestans* treated leaves and in the Cy5 (red) channel, hybridisation with target

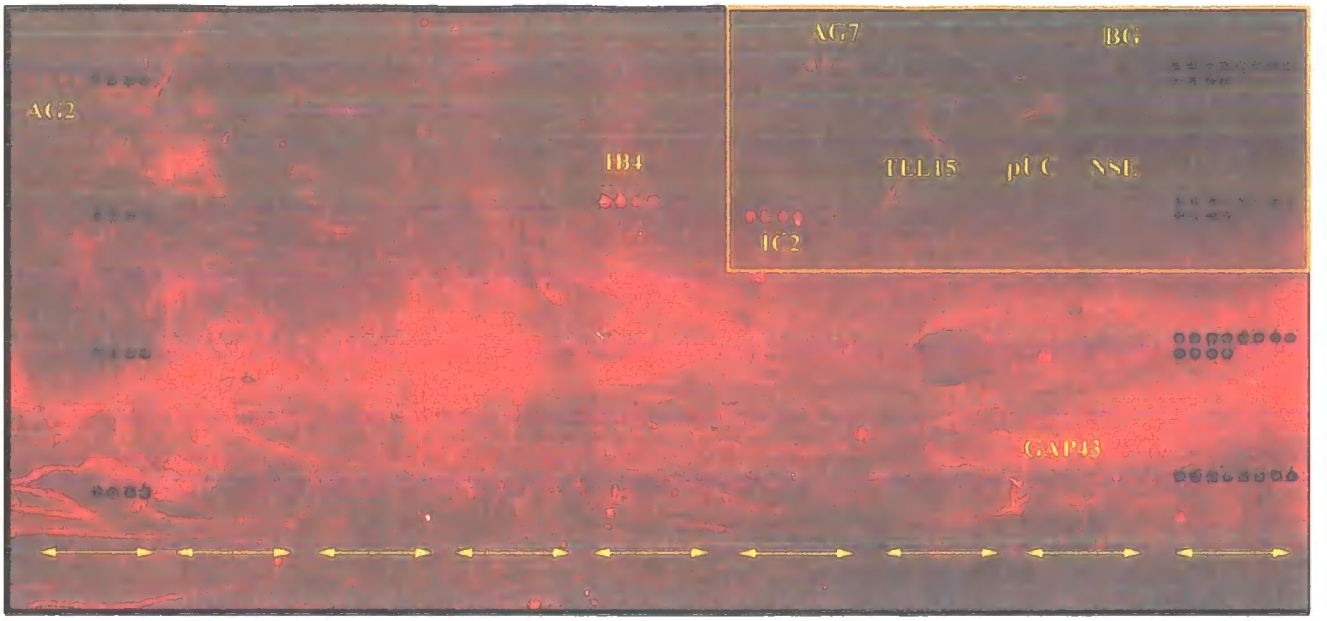


Figure 8.5 Scan of Cy5 fluorescence following microarray hybridisation. Whole DNA array equivalent to that shown in figure 8.4, hybridised with mixed Cy3/Cy5 labelled target cDNA's and imaged using Cy5 fluorescence showing those clones which are expressed at detectable levels in untreated Stirling leaf tissues (control) and the negative control sequences (pUC, NSE, GAP43). The hybridised array was scanned in a Genomic Systems LSIV scanner using the Cy5 laser/filter. Clones which appear to be expressed in the control plants are indicated. See table for identities of these clones. NSE, GAP43 = negative controls (animal specific sequences: NSE – neurone specific enolase; GAP43 – growth-associated protein). The array is flanked by black marker spots show up dark against the fluorescent background.

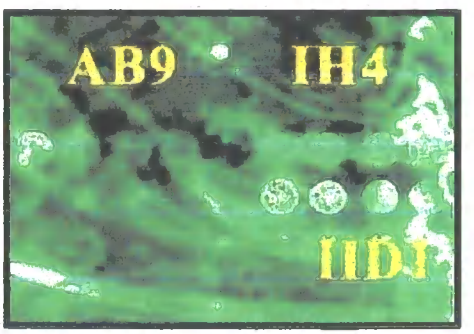
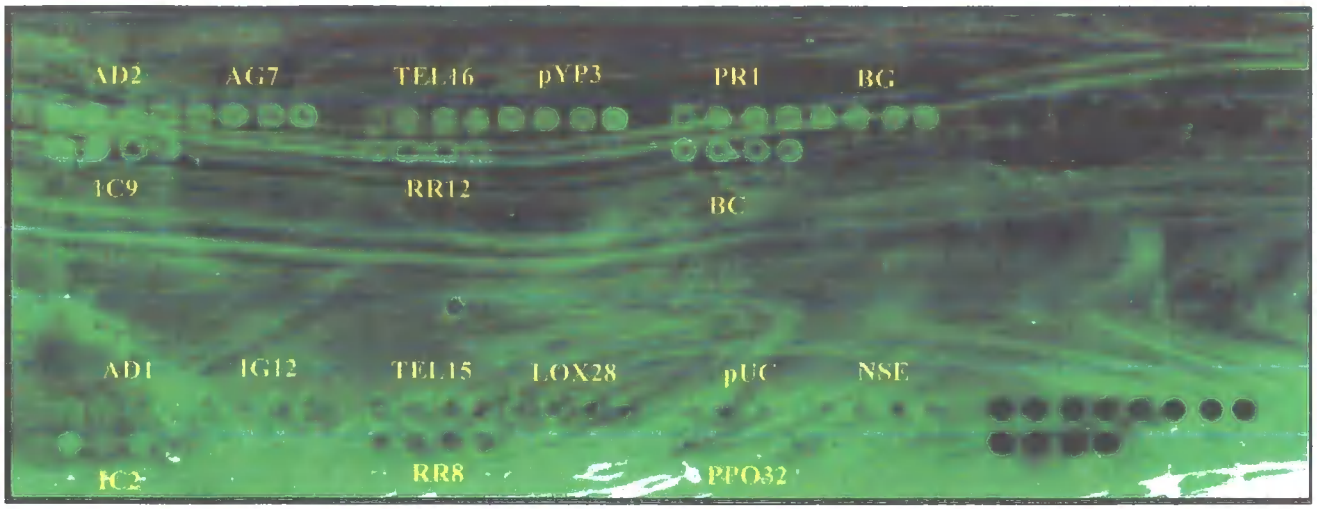


Figure 8.6 Example of the high non-uniform background fluorescence in Cy3 channel obscuring many of the hybridising spots

A)



B)

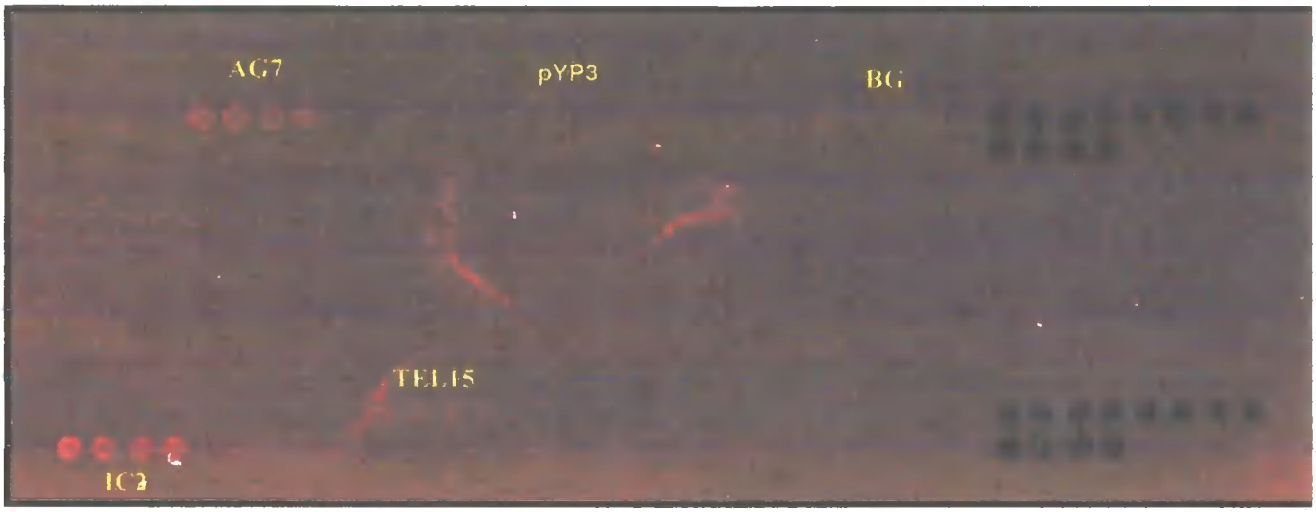


Figure 8.7 Scanned microarray

A) Example microarray cyanin 3 scan

Figure shows a high resolution scan of the selected area of the microarray indicated in figure 8.5 by the rectangle in the top right. Gene sequences in the patches covered in this part of the array are indicated (the clone designations are described in table 8.1). Hybridising spots represent gene sequences upregulated in the treated Stirling plants.

B) Example microarray cyanin 5 scan

Figure shows the corresponding area shown in Figure 8.7 A) scanned for cyanin 5 fluorescence. Gene sequences showing hybridisation with labelled control target cDNA are indicated (the clone designations are described in table 8.1)

Table 8.2 Preliminary data on gene expression assays from microarrays

Clone Number	Putative identity	Expression	Clone Number	Putative identity	Expression
AF5	Heat shock protein - HSP18	•?	ID2	tomato mixed elicitor	•
AF12	superoxide dismutase (SOD)	↑?	ID4	-	•
AG1	PGPD14 {Petunia x hybrida}	↑	ID7	-	↑
AG2	flavonol synthase	↓?	ID9	potato proteinase inhibitor	↑
AG5	unknown	•	IE3	**major latex protein (ripening specific)**	?
AH2	40S Ribosomal protein	•?	IE4	-	•
AH3	CCR4-associated factor 1	↑	IE5	promoter binding protein	↑
AH4	Chlorophyll a/b-binding protein	•	IE7	putative protein & elicitor	↑?
AD5	mixed clone	•	IA4	IAA induced protein	•
AD12	PAL	•?	IA5	ADP-ribosylation factor I	•?
AE3	ubiquitin conjugating protein	•	IB4	metallothionein-like proteien	→
AE4	-	•?	IB5	**unknown - catalase domain?**	↑
AE5	myo-inositol-1-phosphate	•	IB12	proteinase inhibitor	•
AE6	ion channel protein	•	IC1	unknown function & elicitor	•
AE7	arogenate dehydrogenase	↑	IC2	**unknown - no clear sequence**	→
AF1	-	↑	IC9	chalcone synthase	↑
AA1	heat shock protein - HSP90	•	IH8	serine O-acetyltransferase	?
AA3	EST-cTOS cDNA clone	•	IIH5	EST P. infestans-challenged leaf, potato	↑
AB9	**PAL**	↑	IG12	heat shock protein, HSP70	?
AC1	-	•	AG7	*A. thaliana* hypothetical protein, potato	→
AC2	heat shock protein - HSP 80	•	IE9	P23 protein, tomato	•
AC5	HMGR- CoA reductase	•?	IIH4	**PAL (*Agastache rugosa*)**	↑
AD3	unknown	↑	AD1	cyt NADP-malic enzyme, tomato	↑?
AD4	putative splicing factor (60%)	•	AD2	EST *P. infestans*-challenged leaf, tomato	↑
IIC2	ubiquitin conjugating protein	↑	AE1	EST P. infestans-challenged leaf, tomato	•
IIF3	**Kunitz proteinase inhibitor**	↑	IE12	dehydration-response protein RD22	↑
IID1	dehydroascorbate reductase (GSH)	↑	**pRR8**	*chloride channel protein A.thaliana*	?
IID2	ubiquitin conjugating protein	↑	**pRR12**	*heat shock tomato*	↑?
IIE1	proteinase inhibitor	↑	**pRR19**	*alcohol dehydrogenase*	↑
IIG1	proteinase inhibitor	•?	**pRR20**	*alcohol dehydrogenase*	↑
IIG3	-	↑	**pLOX28**	*lipoxygenase*	↑?
IIH1	-	↑	**pYP3**	*unknown tuber sequence*	↑
IG11	similar to JD1 (tobacco)	↑	**pYP5**	*unknown tuber sequence*	?
IH1	potato proteinase inhibitor	•	**pYP6**	*unknown tuber sequence*	↑
IH4	patatin	↑	**pTEL15**	*potato extensin*	?
IH9	myo-inositol-1-P synthase	↑	**pTEL16**	*potato extensin*	↑
IH11	tomato EST – resistant	↑	**SOD**	*superoxide dismutase*	↑
IH12	patatin-like protein	•?	**AC**	*acidic chitinase*	↑
IIB1	cold inducible (Ci21A) gene	↑	**PPO32**	*polyphenoloxidase*	?
IIB2	formate dehydrogenase	↑	**BC**	*basic chitinase*	↑
IF2	**dormancy protein, auxin reg**	↑	**PPO33**	*polyphenoloxidase*	↑
IF8	**unknown-ethylene receptor?**	•?	**AG**	*acidic glucanase*	↑
IF11	dehydration responsive protein	•	**NSE**	*control 1 - NSE neurone specific enolase*	•
IG4	unknown - putative protein	↑	**BG**	*basic glucanase*	→
IG5	DNA-binding protein	↑	**GAP43**	*control 2 - growth-associated protien*	•
IG7	patatin-like protein	•?	**PAL**	*phenylalanine-ammonia lyase*	↑
IG9	peroxidase	?	**pUC18**	*control 3 - pUC18 plasmid*	•
IG10	patatin	↑	**PR-1**	*pathogenesis related protein*	↑

Key:

↑	**up regulated**	↓	**down regulated**	→	**same level of expression**	•	**no expression detected**	?	**results not good enough to assess**

sequences from untreated (control) leaves. Spots showing up only in the green channel were taken to represent sequences upregulated in pathogen treated leaves, while spots showing up in both green and red channels was taken to mean that the sequences were expressed in both control and treated leaf tissues. The best examples of this are shown in figure 8.7 and the conclusions of the manual analysis are summarised in table 8.2.

8.5 Conclusions

The following conclusions are drawn from this piece of work:

The results from the single microarray experiment undertaken in the last period of this project were disappointing. It was clear that hybridisation of both probes was taking place to some of the sequences and not to the negative control sequences. However, under the conditions used the background Cy3 fluorescence was very high and non-uniform which obscured many of the patches.

It was impossible to assess the expression levels of certainty for some of the genes as a result of the very high and non-uniform background Cy3 fluorescence.

Most of the clones that were selected for sequencing in section 7 as containing upregulated gene sequences and where it was possible to detect them in the array, showed detectable levels of fluorescence with Cy3 channel but not with Cy5, which confirms that these gene sequences were most likely upregulated during the establishment of durable resistant.

Some of the inserts, confirmed to be upregulated, were homologous to entries in the databases with unknown function, which provides the opportunity for further studies in the future.

Most of the clones that were acquired from other groups and known to be upregulated under stress conditions including pathogenesis related proteins such as PR-1, glucanase and chitinase, and cell wall glycoproteins such as extensin, showed hybridisation with the labelled target from the treated Stirling plants.

The work described here provides the basis for a more extensive study using optimised microarray hybridisation and processing.

9 Final discussion and future work

In the current study, the durable resistance of the potato plants of the variety Stirling to *P. infestans* has been confirmed. Once detecting the pathogen, Stirling plants respond by delaying the infection process, compared with the moderately resistant variety Désirée, which succumbs entirely to infection with the same pathogen. Durable resistance is first manifested by the production of newly green viable shoots in the presence of the pathogen and also by the production of microtubers (see section 5). These shoots and the plants derived from them as well as the plants drived from the infected microtubers showed a strong general resistance response (durable resistance) to the compatible strain of *P. infestans* and to the potato pathogens *R. solani* and *F. sulphureum* even after two generations culture of the plants in the absence of the pathogen indicating that the resistance character is sufficiently long-lived to be useful as a practical protection against pathogen attack.

During the interaction between Stirling plants and *P. infestans* meristematic tissues developed into new resistant shoots. The bases of this phenomenon could be explained as a result of a systemic response to the pathogen infection (see section 1.2.5). Infection of the lower parts of the plant could activate the accumulation of high levels of antimicrobial compounds in distal uninfected parts of the plants through secondary messenger. Antimicrobial compounds such as phytoalexins and pathogenesis-related proteins as well as the induction of other defence genes could play a crucial role in preventing the distal parts of the plants from subsequent infection with the same pathogen as well as to other pathogens. During this study genes related to phytoalexin production such as HMGR, PAL and CHS, and genes related to pathogenesis-related

proteins such as thaumatin-like protein, peroxidase and proteinase inhibitors have been isolated (see section 7).

The induction of resistance in parts of the plant distant from the site of primary infection is believed to result from the translocation of a systemic signal produced in the vicinity of the primary infection, transported though the plant to uninfected tissues. This signal triggers the plant defence responses in these distant tissues against further pathogen attack before any pathogen challenges these tissues. The defence responses involved in SAR include a combination of physical changes such as cell wall lignification and callose deposition, and induction of various pathogenesis-relate proteins (for reviews see: Mauch-Mani and Metraux, 1998; Metraux, 2001; Ryals *et al.*, 1996 and Sticher *et al.*, 1997). Besides such a role for salicylic acid, recent biochemical and genetic studies confirm that hydrogen peroxide, nitric oxide and polypeptides may function as signalling molecules (Delledonne *et al.*, 1998; Klessig *et al.*, 2000; Neill *et al.*, 2002; Takayama and Sakagami, 2002). In the present study, the durable plants showed a fast production of ROS in response to the treatment with *P. infestans* culture filtrate elicitor, which could play an important role as signalling molecules in the activation of plant defence responses, and also in direct toxicity to invading pathogens (see section 6). Also these plants showed a tight deposition of callose surrounding the HR area, which deprive the pathogen of nutrients and limit pathogen growth to a small region of the plant (see section 6). These may indicate that durable plants are already activated to a level which only requires slightly more activation to inhibit pathogens growth.

An Alternative explanation for the establishment of durable resistance of the newly developed shoots is the possibility of rearrangement in the chromatin structure resulting in the activation of genes encoding specific resistance proteins or peptides. There are

many ways in which genes may be regulated in this way. For example, recent results have shown that chromatin conformation can be dictated by methylation, acetylation, or phosphorylation of specific amino acids of the histones. In turn, these histone modifications can regulate local and sometimes more global gene expression (Hetherington and Waterhouse, 2002).

In the present study, a subtracted cDNA library enriched for low abundance differentially expressed sequences that are induced during the compatible interaction between Stirling cultured potato plants and *P. infestans* was successfully constructed using the suppression subtractive hybridisation (SSH) method.

This method, which is based on a technique called suppression PCR and combines normalisation and subtraction in a single procedure, has been a powerful approach used to identify and isolate cDNAs of differentially expressed genes in animal systems (Diatchenko *et al.*, 1996). The normalisation step equalises the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver populations. It was reported that the SSH technique enriched for rare sequences over 1,000-fold in one round of subtractive hybridisation (Diatchenko *et al.*, 1996). Recently, several applications, using this method, have been reported in plant systems (e.g. Beyer *et al.*, 2001; Birch et al., 1999; Kim *et al.*, 1999; Caturla *et al.*, 2002).

During this project, this technique was not only used to subtract the common sequences between the tester (treated-Stirling) and the driver (control-Stirling) populations but also included subtraction of the pathogen sequences, which may have been presented in the treated plant samples, in order to avoid isolating clones carrying gene sequences of fungal origin. This was achieved by mixing the total RNA from cultured *P. infestans*

mycelium with the pooled total RNA from the control plant tissues to create the driver RNA population for the forward subtraction. This approach along with strategy for sampling plant tissue free from pathogen were judged very effective for preventing the isolation of any clone carrying gene sequences of pathogen origin in the subtracted library since no positive hybridisation of any library clones with probe prepared from the pathogen.

Recently, Beyer *et al.*, (2001) used the same idea to subtract constitutively expressed *P. infestans* sequences from potato plants challenged with zoospores of *P. infestans*, while constructing a library to screen for genes induced in potato during the interaction between potato and *P. infestans* using the SSH technique. They also eliminated the pathogenesis-related (PR) gene transcripts that accumulate to very high levels during infection from the resulting differential library by using control plant tissue in which PR genes were induced by treatment with BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid S-methylester), which induces the same set of PR genes. Thus this technique, unlike normal libraries in which the abundant sequences represent the bulk of the library clones, can be adapted to subtract abundant and any other unwanted gene transcripts and enrich only the desired transcripts which makes the screening much more easier in the final stages of the procedure (i.e. this method can be selective). This is a particularly useful strategy where the aim is to study expression patterns of a selected subset of genes (e.g. microarray expression profiling).

Constructing a subtracted cDNA library using the PCR-select subtraction kit is technically challenging but once the method is fully understood is relatively straight forward provided the initial mRNAs are good and undegraded. However, the screening process to identify the desired up-regulated clones can be problematic. For instance,

although using identified probes such as PAL and SOD revealed the correct sequence, others such as PR-1, AC, AG and PPO did not reveal the expected genes, nevertheless the selected clones showed hybridisation with these probes. The latter genes proved to be up regulated using the cDNA micro arraying technique.

The reverse subtraction and differential screening were very powerful and allowed the identification of the truly up-regulated cDNA clones representing gene transcripts expressed by the potato plant during the establishment of durable resistance. Interestingly, among the sixty sequenced cDNAs, eighteen (i.e. 30%) were novel potato gene sequences, which highlight the importance of such approaches for identifying new gene sequences.

The majority of the sequenced cDNAs had very strong homologies to sequence entries in the databases. These sequences were assigned to five main functional groups, according to their putative function. Some sequences may warrant inclusion in more than one category. These categories included: defence and stress, signalling, transcription, unknown and metabolism-related sequences. Defence and stress responses, signalling events and changes in transcription are known to be induced when the plant is colonised by a pathogen, so isolating such transcripts in the current study was not unexpected. Also many changes in metabolism are likely to occur, especially in a compatible interaction, as both plant and pathogen are competing with each other for the available resources. Changes in the metabolism are known to occurr in a similar plant-pathogen interaction and gene sequences representing these changes have been isolated (Beyer *et al.*, 2001; Fristensky *et al.*, 1999; Rauyaree *et al.*, 2001).

Not surprisingly, all the sequences with known identities were found to be of plant origin and not of pathogen origin because, as mentioned earlier, many precautions had

taken place while constructing the subtracted library to prevent isolating clones of fungal origin. Although all these precautions had taken place, it is more likely that some fungal gene transcripts that were up-regulated during the infection process may be present, which did not subtract. These types of gene transcripts are underrepresented in the library either because the *Phytophthora* biomass –if any were present- was too low compared to the plant biomass, or because there is a difference in GC content between the pathogen and the plant genomes. This difference in GC content could direct the subtractive procedure towards retrieval of potato sequences rather than the pathogen sequences. Owing to the relatively high GC content of *P. infestans* genomic DNA, Van der Lee *et al.*, (1997) had to modify AFLP DNA fingerprinting protocols to construct genetic linkage map based on polymorphic DNA markers.

In the cDNA subtracted library, the abundant representation of some sequences such as proteinase inhibitors, patatins, heat shock proteins and ubiquitin conjugate proteins compared with other sequences may indicate the importance of these genes in the resistance responses. Proteinase inhibitors have been shown to play a significant role in the natural defence mechanisms of the potato plant against insect and pathogen attack and have also been shown to induced by wounding (Hildmann *et al.*, 1992; Ishikawa *et al.*, 1994b). The discovery of differential expression in patatin genes is interesting. Recent investigation of the physiological function of patatin as phospholipase A2 may shed new light on its possible role in the signal transduction leading to plant resistance (Senda *et al.*, 1996). Heat-shock proteins have also been demonstrated to be induced under various stresses including oxidative burst and pathogen attack (Banzet *et al.*, 1998; Byth *et al.*, 2001). Ubiquitin genes are frequently associated with protein turnover. Potato is likely to be metabolically very active during the resistance response

to *Phytophthora* and thus the expression of ubiquitin was not unexpected. Moreover, it has been demonstrated that ubiquitin transcripts are indeed induced in potato in response to *Phytophthora*, mechanical injury and heat shock (Rickey and Belknap, 1991; Basso *et al.*, 1996). These reports established a connection between the expression of these genes and the defence reactions in plants and agree with the results from the present work. Thus it is highly probable that these genes are important participants in establishing resistance in potatoes.

Several sequences (15 sequences; i.e. 25% of the sequenced cDNAs) were homologous to sequences of unknown function. Six of them were categorised with the stress- and defence- related sequences as they have homology to ESTs isolated from pathogen or elicitor treated leaves or cell cultures, while the others (9 sequences; i.e. 15%) categorised as unknown function. The value of these unknown sequences should not be underestimated as our observation that these transcripts are induced during the interaction between potato and *Phytophthora* leading to the establishment of durable resistant may shed light on their possible defence function. Further studies and analyses of such unknown and novel genes may potentially contribute in understanding the basis of this type of strong resistance.

The other isolated subtracted cDNA sequences in the library that have not been analysed in the present studies will be useful in the future. Because the cDNA library was constructed from Stirling tissue culture plants treated with *P. infestans*, further characterisation and functional analysis of this collection of gene sequences will lead to a more comprehensive understanding of host–pathogen interactions and the identification of new important host resistance genes. The information derived may lead to an understanding of some aspects of Stirling resistance to *P. infestans*. One possible

way of using these sequences is through the use of the cDNA microarray (DNA chip) technology. Using this technique with specific arrays of gene-specific sequences, one can assay the expression of thousands of genes in a single experiment or series of experiments.

Initial attempts to use this technology to study the differences of transcribed genes profiles between the control Stirling untreated plants and Stirling plants treated with *P. infestans* using the selected sixty isolated cDNA's along with identified and control cDNA's gave rather unsatisfactory results. Although this technology looks promising for assaying the expression of thousands of genes, it proved to be very labour intensive and problematic. The normalisation of the cDNA's on the microarrays, choosing slides with the best coating, preparing the labelled target cDNA and the hybridisation conditions all proved to be very demanding.

Using oligonucleotides designed specifically to known genes (e.g. those identified during this study) and other genes that play an important role in resistance is a possible way forward. These commercially designed oligos provide a better chance for producing high quality normalised microarrays with minimal effort. Publicly available printed microarrays containing 5,000 or 10,000 sequence-verified potato ESTs are produced by TIGR (The Institute for Genomic Research), are expected to be ready in Summer 2002 and is another alternative to 'home made' arrays to monitor gene expression patterns in potato plants challenged with *Phytophthora*.

As microarraying facilities are not available in the Biochemistry Department, Faculty of Agriculture, Cairo University, Egypt, future work will rely on collaborations with other institutes to answer the questions raised in this project. The 'Inverse Northern' procedure may also be used for future studies. Although this technique required more

effort than the microarraying and the number of genes that can be studied is limited, the advantage of this technique is that the required facilities are available. Using this procedure, DNA gel blots of the PCR amplified cDNA inserts, can be prepared from the isolated clones and probes prepared from cDNA populations derived from the control and treated plant samples can be used for screening. Other interesting inserts can be identified using this technique. Moreover, instead of using a collective sample covering the whole period, the expression profiling during the establishment of durable resistance can be studied using probes prepared from each time point.

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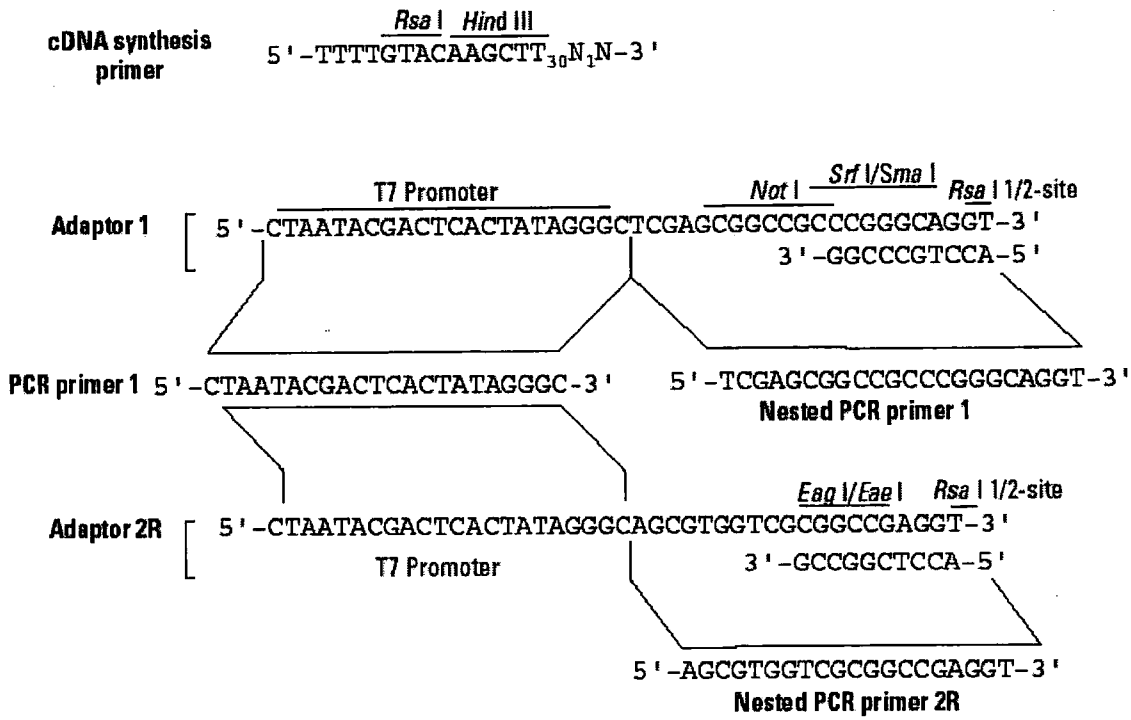
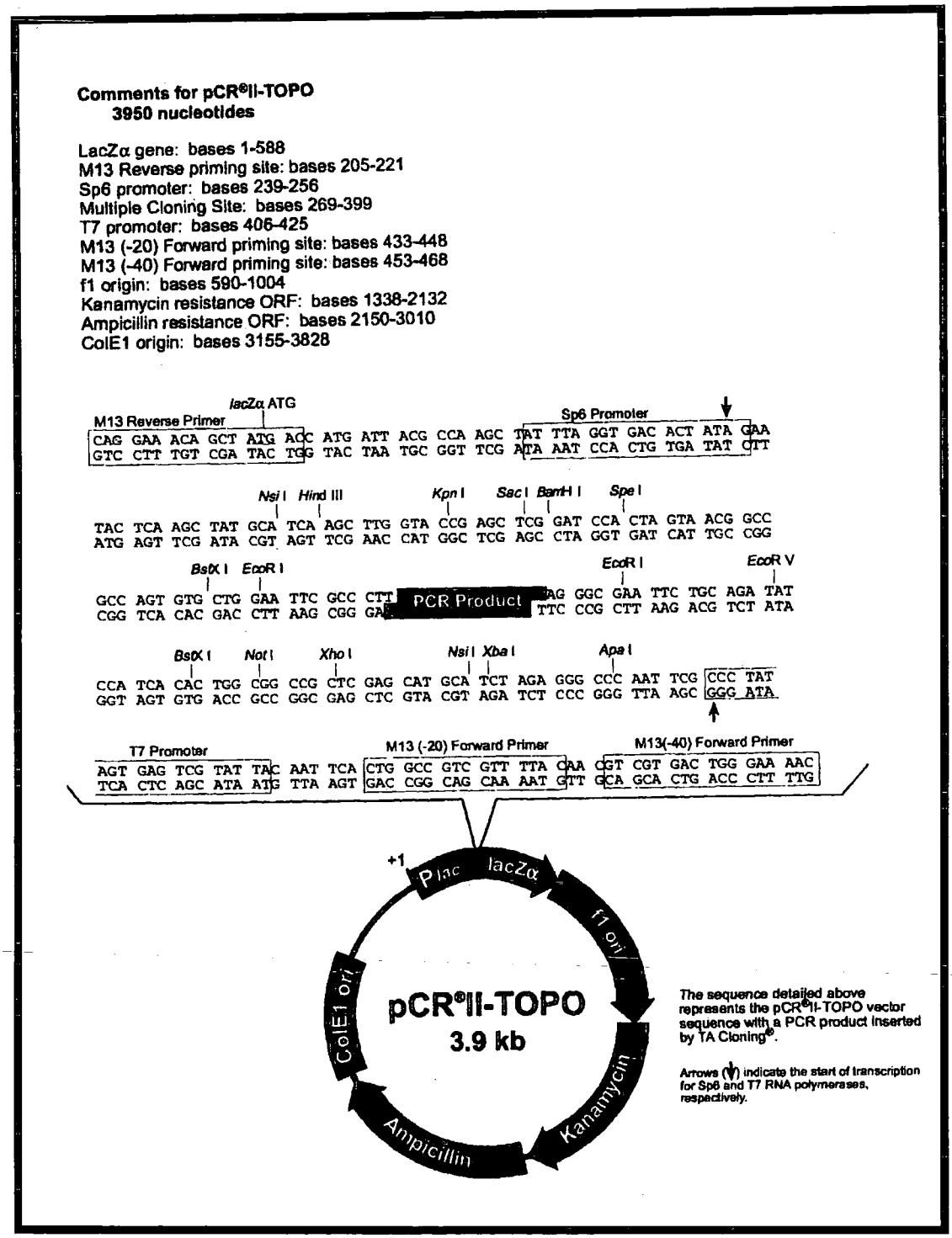
Appendix A: Adaptor and primer sequences



Sequences of the PCR-Select cDNA synthesis primer, adaptors, and PCR primers provided with the PCR-select subtraction kit (adapted from PCR-select subtraction kit user manual).

Appendix B: pCRII-TOPO Vector Map



The Map showing the features of the pCRII-TOPO plasmid vector and the sequence surrounding the cloning site. Restriction sites are labelled to indicate the actual cleavage site. The start of transcription for Sp6 and T7 polymerases is indicated by the arrows. This vector was used in the cloning of the subtracted cDNAs (section 4.3.18.1) (adapted from Topo™ TA cloning kit user manual; Invitrogen, The Netherlands).

Appendix C: Sequences related to oxidative burst and HSP

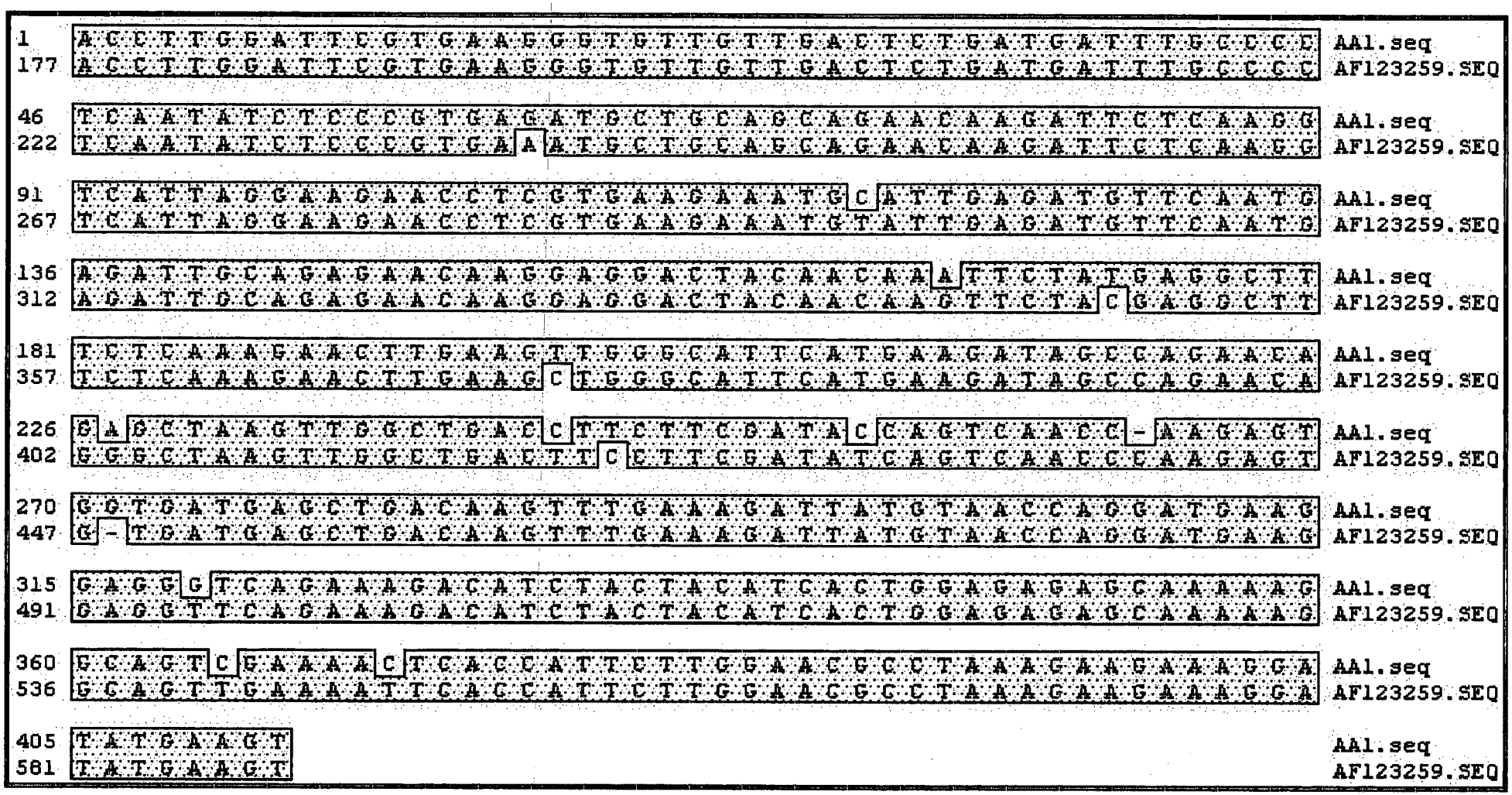
C-1 The nucleotide sequence alignment of clone AF12 (296 bp) with potato superoxide dismutase (SOD) (NCBI; AF354748).

```
1   ACCATCTTCTCCAACTGTGATGTTACCAAGATCACCAGCATGACG  AF12.SEQ
264 ACCATCTTCTCCAACTGTGATGTTACCAAGATCACCAGCATGACG  AF354748.SEQ

46  CACCTCATCTTCAGGAGCACCATGCTCCTTACCAGCAGGATTGTA  AF12.SEQ
219 CACCTCATCTTCAGGAGCACCATGCTCCTTACCAGCAGGATTGTA  AF354748.SEQ

91  ATGTGGTCCTGTTGACATGCAGCCATTTGTGGTATCACCAAGGGC  AF12.SEQ
174 ATGTGGTCCTGTTGACATGCAGCCATTTGTGGTATCACCAAGGGC  AF354748.SEQ

136 ATGGACATGGAAGCCATGAAGTCCAGGTTTTAGGCCAGATATATT  AF12.SEQ
129 ATGGACATGGAAGCCATGAAGTCCAGGTTTTAGGCCAGATATATT  AF354748.SEQ

181 TCCATTAACTGTGGTTGGAGCATCTCCATCTTGAGTGAAGAGGAT  AF12.SEQ
84  TCCATTAACTGTGGTTGGAGCATCTCCATCTTGAGTGAAGAGGAT  AF354748.SEQ

226 GGTGCCACAAACACCTTCACTGCTGCTAAGGACGGCAAC        AF12.SEQ
39  GGTGCCACAAACACCTTCACTGCTGCTAAGGACGGCAAC        AF354748.SEQ
```

C-2 The nucleotide sequence alignment of clone IID1 (277 bp) with GSH-dependant dehydroascorbate reductase (DHAR) (TIGR; TC25547).

```
1    ACTTCTGACATTGGAGGAAAAGAAAGTGACTTACAAGAAGCACTT  IID1.seq
141  ACTTCTGACATTGGAGGAAAAGAAAGTGACTTACAAGAAGCACTT  TC25547.SEQ

46   GATCAATGTTAGTGACAAGCCCAAATGGTTCTTGGAGGTGAACCC  IID1.seq
186  GATCAATGTTAGTGACAAGCCCAAATGGTTCTTGGAGGTGAACCC  TC25547.SEQ

91   GGAAGGGAAAGTTCCCGTGATCAATTTTGGTGACAAATGGATCCC  IID1.seq
231  GGAAGGGAAAGTTCCCGTGATCAATTTTGGTGACAAATGGATCCC  TC25547.SEQ

136  GGATTCTGATGTCATTGTTGGGATTATTGAAGAGAAATACCCAAA  IID1.seq
276  GGATTCTGATGTCATTGTTGGGATTATTGAAGAGAAATACCCAAA  TC25547.SEQ

181  TCCCTCTCTCTCCTTGCTCCCCCTGAATTTGCCTCTGTGGGCTCAAA  IID1.seq
321  TCCCTCTCTCTCATTGCTCCCCCTGAATTTGCCTCTGTGGGCTCAAA  TC25547.SEQ

226  AATATTTCCTACCTTCGTCTCGTTTCTGAAGAGCAAGGATTCTAG  IID1.seq
366  AATATTTCCTACCTTCGTCTCGTTTCTGAAGAGCAAGGATTCTAG  TC25547.SEQ

271  TGACGGT                                        IID1.seq
411  TGACGGT                                        TC25547.SEQ
```

C-3 The nucleotide sequence alignment of clone AA1 (412 bp long) with tomato HSP90 (NCBI; AF123259)



C-4 The alignment of the overlapped region of clone IG12 (382 bp) nucleotide sequence with potato HSP70 (TIGR; TC21404)

C-5 The alignment of the overlapped region of clone AF5 (358 bp long) nucleotide sequence and potato small HSP18 (TIGR; TC23617).

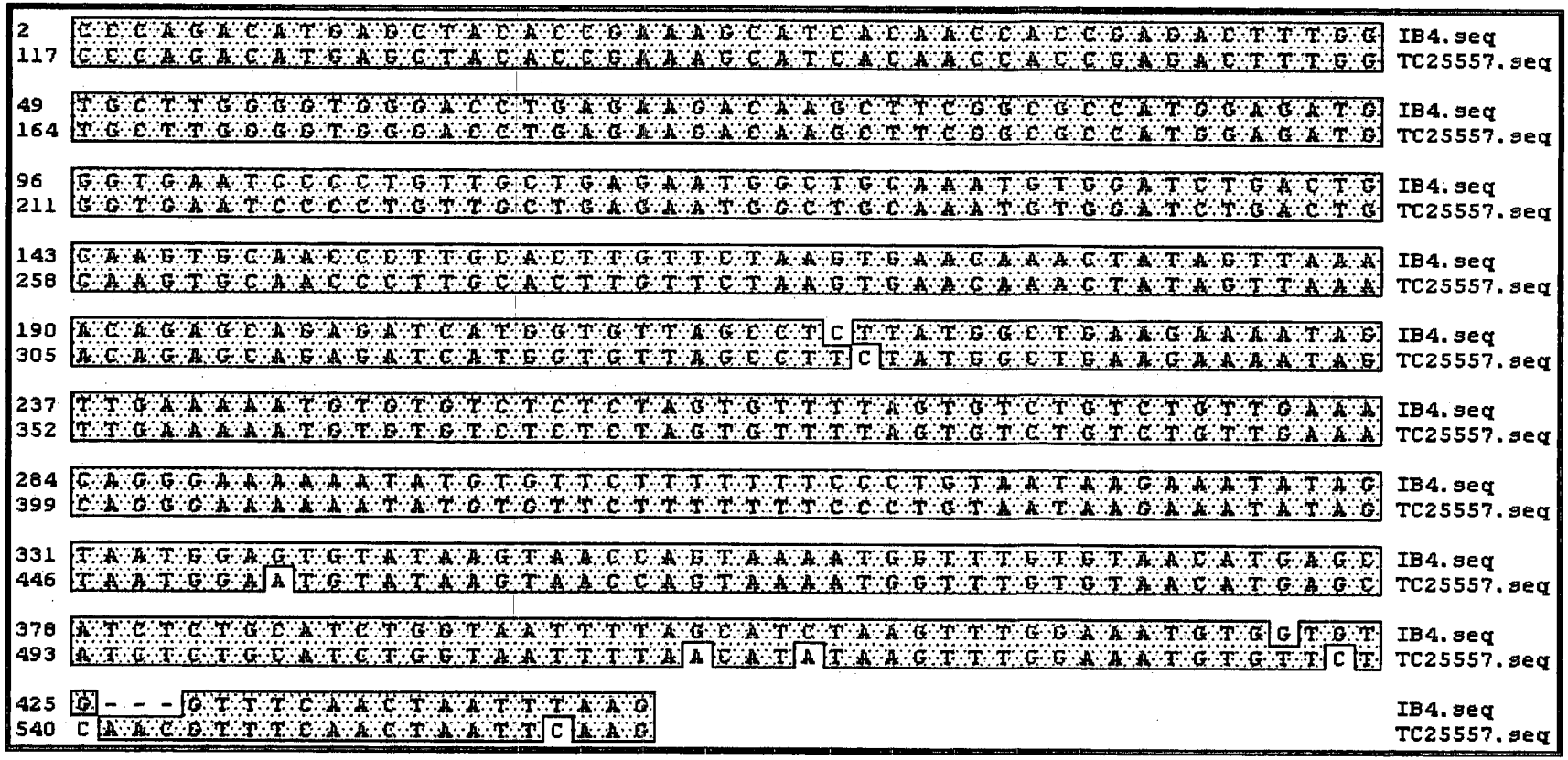
```
1   ACCGGGGGATTAAGAAAGAGGAAGTGAAAGTTGAAGTTGAAGAAGG  AF5.SEQ
248 ACCGGGGGATTAAGAAAGAGGAAGTGAAAGTTGAAGTTGAAGAAGG  TC23617.seq

46  AAGGATTTTGCAGATTAGTGGTGAGAGGAGTAAAGAACAAGAGGA  AF5.SEQ
293 AAGGATTTTGCAGATTAGTGGTGAGAGGAGTAAAGAGCAAGAGGA  TC23617.seq

91  GAAGAATGATCAATGGCACCGTATGGAGAGGAGTAGTGGTAAGTT  AF5.SEQ
338 GAAGAATGATCAATGGCACCGTATGGAGAGGAGTAGTGGTAAGTT  TC23617.seq

136 TGTAAGGAGGTTTAGGTTGCCGGAGAATGTGAAAATGGAGGAAAT  AF5.SEQ
383 TGTAAGGAGGTTTAGGTTGCCGGAGAATGTGAAAATGGAGGAAAT  TC23617.seq

181 TAAGGCGGCGATGGAGAATGGAGTGCTTACTGTAACTGTTCCTAA  AF5.SEQ
428 TAAGGCGGCGATGGAGAATGGAGTGCTTACTGTAACTGTTCCTAA  TC23617.seq

226 AGAAGAAGAGAAGAAACCTGAAGTGAAAGCCATTGATATTTCTGG  AF5.SEQ
473 AGAAGAAGGGAAGAAACCTGAAGTGAAAGCCATTGATATTTCTGG  TC23617.seq

271 TTGAAAAAAAGTTGCAGATCAAGTGTTTGACGAAAGTCCTCTGTT  AF5.SEQ
518 TTGAAAAAAAGTTGCAGATCAAGTGTTTGTCGAAAGTCCTCTGTT  TC23617.seq

316 AAAAATGGAGCANTACAGTCTATTCA                      AF5.SEQ
563 AAAAATGGAGCAATACAGTGTATTTA                      TC23617.seq
```

C-6 The alignment of the overlapped region of clone AC2 (240 bp long) nucleotide sequence and EST from tomato callus similar to HSP80 (TIGR; AW033426).

```
AC2:          1 ACGATCCTAAAATGATACTATTATATGGGTTTGCTTACACAACATGACACTACTAACTCG 60
                ||||||||||||||||| ||    ||  | | ||||||||||||||||||||| |||||
AW033426:    35 ACGATCCTAAAATGATA-TACGG-ATTTGCT-GCTTACACAACATGACACTACTGACTCG 91

AC2:         61 --A---CAAAACA-CA--GTATTCAACAGAAAGAAAAAGAAAAAACAACTATTTAGGGTA 112
                  |   ||||||| ||  |||||||||||||| ||||| |||| || ||| ||||||||
AW033426:    92 GAAGAACAAAACAACACAGTATTCAACAGAAAGGAAAAGGAAAA-CACCTACTTAGGGTA 150

AC2:        113 TCTAGAATCTCGATATGAACGGCATTGAGATGACACCAAGTGC-TTAGTCCACTTCCTCC 171
                || |||| ||| |||||||| ||||  ||||||| |  | ||| ||||||||||||||||
AW033426:   151 TCAAGAAACTCAATATGAACCGCATATAGATGACGCATAATGCCTTAGTCCACTTCCTCC 210

AC2:        172 ATCTTGCTTTCCTCACCCATTTCCTCTAGAGGAGGCATATCATCAACATCCTCACCAGCT 231
                ||||||||||||||||| |  ||||| |||||||||||||||||||||| ||||||||
AW033426:   211 ATCTTGCTTTCCTCACCAACATCCTCCAGAGGAGGCATATCATCAACATCCACACCAGCT 270

AC2:        232 TCCTCTTCT 240
                |||| ||||
AW033426:   271 TCCTGTTCT 279
```

Appendix D Other stress and defence response related sequences

D-1 The nucleotide sequence alignment of clones IB4 (443 bp) and the metallothionein-like protein type 2 b (TIGR; TC25557).



D-2 The nucleotide sequence alignment of clone IA4 with IAA 4.1 mRNA sequence (NCBI; AF123509) (A), and clone IIB2 with formated dehydrogenase sequence (TIGR; TC 19814) (B).

```
19  gtaccttgggagatgtttgttgattcatgcaagcgtttaaggataatgaaaggatcagag 78   IA4.seq
    |||||||||||||||||||||||||||||||| || ||||||||||||||||||||||||
631 gtaccttgggagatgtttgttgattcatgcaaacgcttaaggataatgaaaggatcagag 690  AF123509


79  gctattggactagcaccaagagcagtggagaaatgcaaaaacaggagctgaacttaatgt 138  IA4.seq
    || ||||||||||||||||||||||||||||||||| ||||| ||||||| || |||
691 gcaattggactagcaccaagagcagtggagaaatgcaagaacagaagctgaaattgatga 750  AF123509


139 tccaattaattgttcattg 157
    |||||||| ||||||||||
751 tccaattacttgttcattg 769
```

A

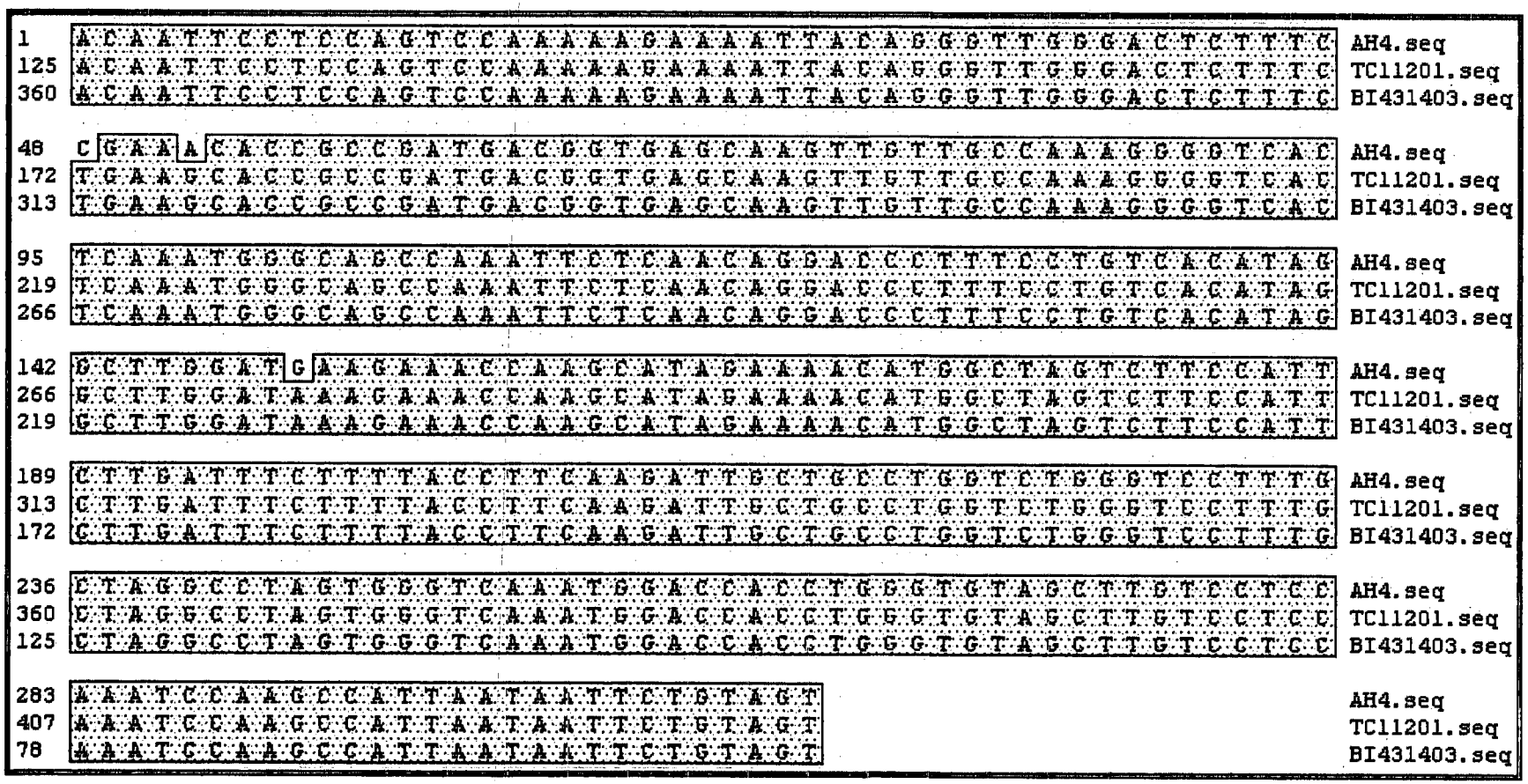
```
 85 ACATTAATAATTTAGCGATACTGAGGTGCCAATTCCCCATCCTTGACAATGTAATTTTCA 26   IIB2
    |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
291 ACATTAATGATTTAGCGATACTGAGGTGCCAATTCCCCATCCTTGACAATGTAATTTTCA 350  TC19814

 25 GCAGGGAAATCCTCACCCTTGAAGT 1      IIB2
    |||||||||||||||||||||||||
351 GCAGGGAAATCCTCACCCTTGAAGT 375    TC19814
```

B

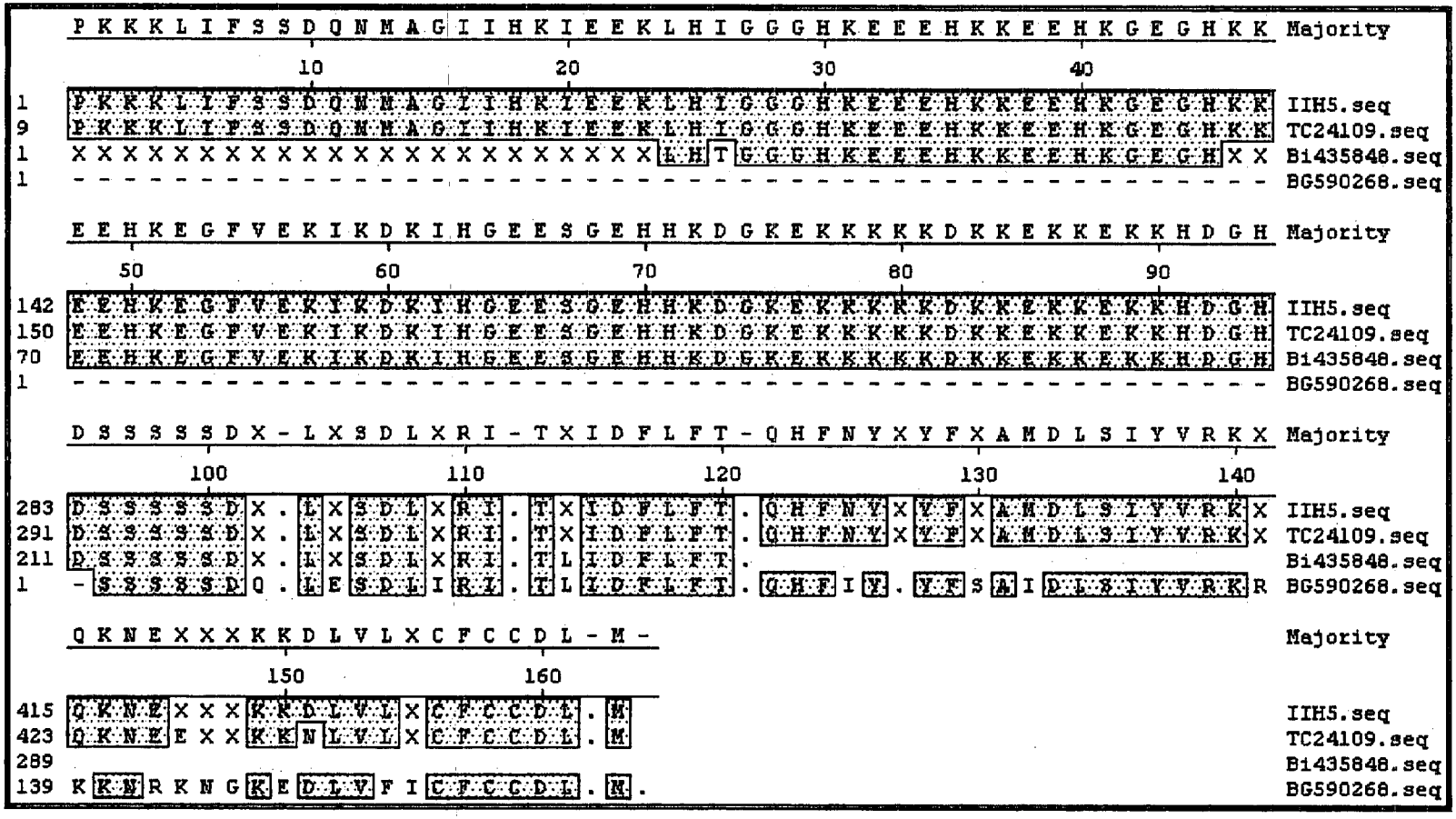
D-3 The nucleotide sequence alignment of clone IE6 (246 bp) with *P. infestans*-challenged leaf (NCBI; BI434718) and water-stress induced tonoplast intrinsic protein (TIGR; TC18260).

```
1   ACAGTAGACCAAAGCAAAGATCATACCACCAAAAGCCCAAGCAATCC  AE6.seq
336 ACAGTAGACCAAAGCAAAGATCATACCACCAAAAGCCCAAGCAATCC  BI434718 .SE
604 ACAGTAGACCAAAGCAAAGATCATACCACCAAAAGCCCAAGCAATCC  TC18260.seq

48  CTTGAATACCCACTGAAGAACATAGACTATCAGATCTCTTAAGACCC  AE6.seq
289 CTTGAATACCCACTGAAGAACATAGACTATCAGATCTCTTAAGACCC  BI434718 .SE
651 CTTGAATACCCACTGAAGAACATAGACTATCAGATCTCTTAAGACCC  TC18260.seq

95  ATAACAGTCAAGATTGTGATATACAAGAACAAGAAAGTTGCCATGAA  AE6.seq
242 ATAACAGTCAAGATTGTGATATACAAGAACAAGAAAGTTGCCATGAA  BI434718 .SE
698 ATAACAGTCAAGATTGTGATATACAAGAACAAGAAAGTTGCCATGAA  TC18260.seq

142 CTCTGCAATACCAGCCCTGTAAAAGGACCATGATGACAACTCCCCTG  AE6.seq
195 CTCTGCAATACCAGCCCTGTAAAAGGACCATGATGACAACTCCCCTG  BI434718 .SE
745 CTCTGCAATACCAGCCCTGTAAAAGGACCATGATGACAACTCCCCTG  TC18260.seq

189 GTTCAAACAATGGAGCTGGTGGTGGCTCTTTGTAATCTTTGTCTGTT  AE6.seq
148 GTTCAAACAATGGAGCTGGTGGTGGCTCTTTGTAATCTTTGTCTGTT  BI434718 .SE
792 GTTCAAACAATGGAGCTGGTGGTGGCTCTTTGTAATCTTTGTCTGTT  TC18260.seq

236 TGAGCTGATGT                                      AE6.seq
101 TGAGCTGATGT                                      BI434718 .SE
839 TGAGCTGATGT                                      TC18260.seq
```

D-4 The nucleotide sequence alignment of clone AH4 (310 bp) with Chlorophyll a/b-binding protein type I precursor (TIGR; TC23477) and EST534164 *P. infestans*-challenged leaf (NCBI; BI431403).



D-5 The alignment of the translated nucleotide sequence of clone IIH5 (474 bp) with unknown function sequence (TIGR; TC24109), EST538609 *P. infestans*-challenged potato leaf (NCBI; BI435848) and EST498098 *P. infestans*-challenged potato leaf (NCBI; BG590268).



D-6 The nucleotide sequence alignment of clone IG4 (322 bp) with EST536811 *P. infestans*-challenged potato leaf (NCBI; BI434050)

```
1    ACGATCCATCAACTATCATGTTCTTCTTCAGGAACAAGCACATCATG  IG4.seq
222  ACGATCCATCAACTATCATGTTCTTCTTCAGGAACAAGCACATCATG  BI434050.seq

48   ATTGATCTTGGCACAGGAAACAATAACAAGATCAACTGGGCACTCAA  IG4.seq
269  ATTGATCTTGGCACAGGAAACAATAACAAGATCAACTGGGCACTCAA  BI434050.seq

95   AGATAAACAGGAGTTCATTGATATTGTTGAGACAGTGTATCGCGGTG  IG4.seq
316  AGATAAACAGGAGTTCATTGATATTGTTGAGACAGTGTATCGCGGTG  BI434050.seq

142  CACGAAAGGGTCGTGGTCTAGTTATTGCACCAAAAGATTACTCTACC  IG4.seq
363  CACGAAAGGGTCGTGGTCTAGTTATTGCACCAAAAGATTACTCTACC  BI434050.seq

189  AAGTATCGTTACTGAGTGTCTGTTGATATTGTATTTTGTTTGTGGAA  IG4.seq
410  AAGTATCGTTACTGAGTGTCTGTTGATATTGTATTTTGTTTGTGGAA  BI434050.seq

236  TATGCTGCACTTTATGACTGAACTGTAACATACGGTTGCTAAGTGGT  IG4.seq
457  TATGCTGCACTTTATGACTGAACTGTAACATACGGTTGCTAAGTGGT  BI434050.seq

283  TTCCATTAGACCGATTCTGTTGCTTTCAAGATCCAAGTGT         IG4.seq
504  TTCCATTAGACCGATTCTGTTGCTTTCAAGATCCAAGTGT         BI434050.seq
```

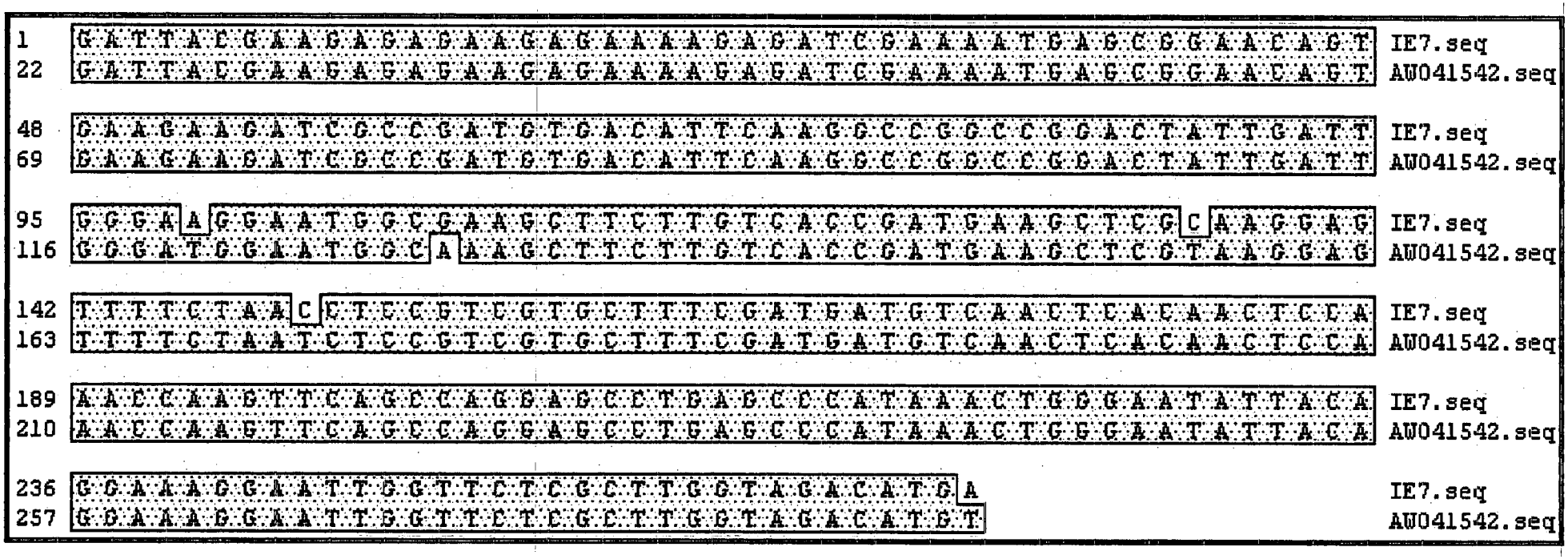
D-7 The nucleotide sequence alignment of clone AD2 (289 bp) with EST497292 *P. infestans*-challenged leaf (NCBI; BG589450)

```
1    ATTCTGGAGGGAAATTCACAGCACTGAAGGAGCTTGAAGCATTGGAT  AD2.seq
439  ATTCTGGAGGGAAATTCACAGCACTGAAGGAGCTTGAAGCATTGGAT  BG589450.seq

48   GTTAGTGTGAAGAAGATATACCCAGTCATACAGGATGGAGTAGACCC  AD2.seq
486  GTTAGTGTGAAGAAGATATACCCAGTCATACAGGATGGAGTAGACCC  BG589450.seq

95   TATTGACGCGGCAGAGTTAGAAATTCTAACTTCAGATCTGACTAAAA  AD2.seq
533  TATTGACGCGGCACAGTTAGAAATTCTAACTTCAGATCTGACTAAAA  BG589450.seq

142  AAACAGAATTTTTTTTCGGTAGGACTAGATCTCCTAGCAAAGGAGACA  AD2.seq
580  AAACAGAATTTTTTTTCGGTAGGACTAGATCTCCTAGCAAAGGAGACA  BG589450.seq

189  GATAAGTTCTTTCAAATCCTTCTAACTGGACGTGATGCTTTGCTTTG  AD2.seq
627  GATAAGTTCTTTCAAATCCTTCTAACTGGACGTGATGCTTTGCTTTG  BG589450.seq

236  TAACCTTAGAGCTGGTAGTGCAGTCGTT                      AD2.seq
674  TACCCTTAGAG-TGGTAGTGCAGTCGTT                      BG589450.seq
```

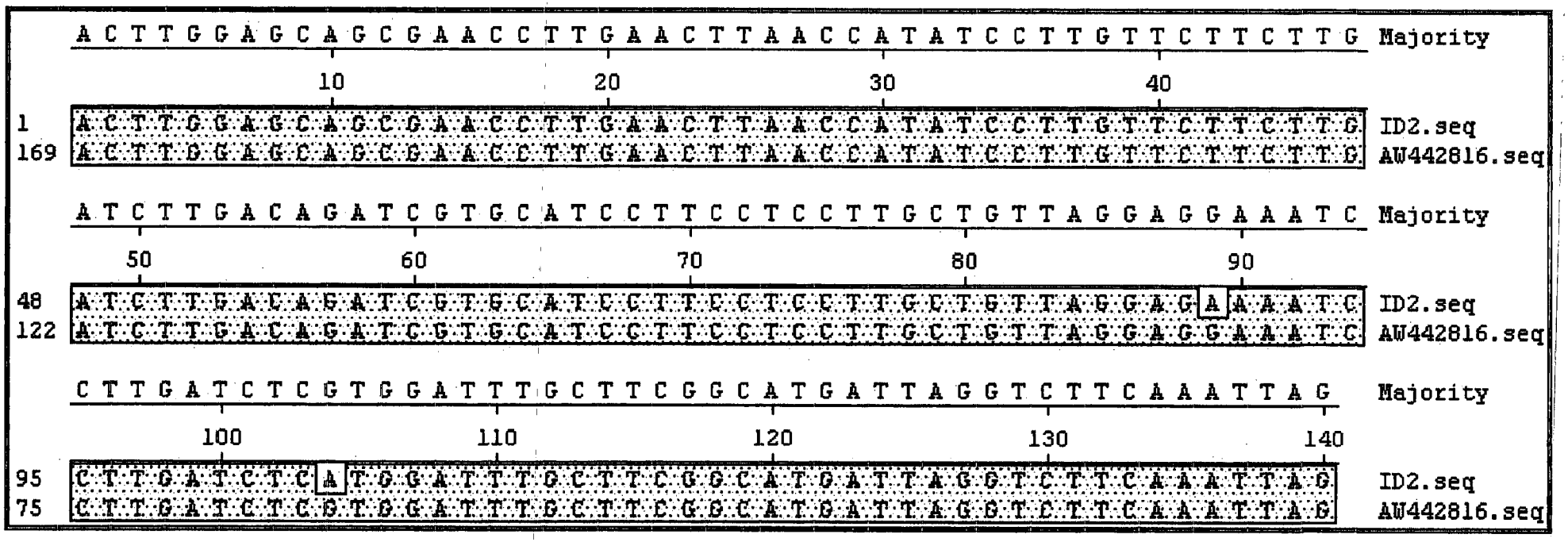
D-8 The nucleotide sequence alignment of the overlapped region of clone AE1 (296 bp) with EST536915 *P. infestans*-challenged leaf (NCBI; BI434154).

```
50   ATCTGCCTAACACATATGTAATATAGGTCCAACGTATTCAACTGAAC  AE1.seq
101  ATCTGCCTGACACAT--GTAATATAGGTCCAACGTATTCAACTGAAC  BI434154.seq

97   TCAACATTTTTCCTATGCGGAAATCAAACATAATATGTGAGAAATTA  AE1.seq
56   TCAACATTTTTCCTATGCGGAAAACAAACATAATATGTGAGAAATTA  BI434154.seq

144  CTATATTTC                                        AE1.seq
9    CTATATTTC                                        BI434154.seq
```

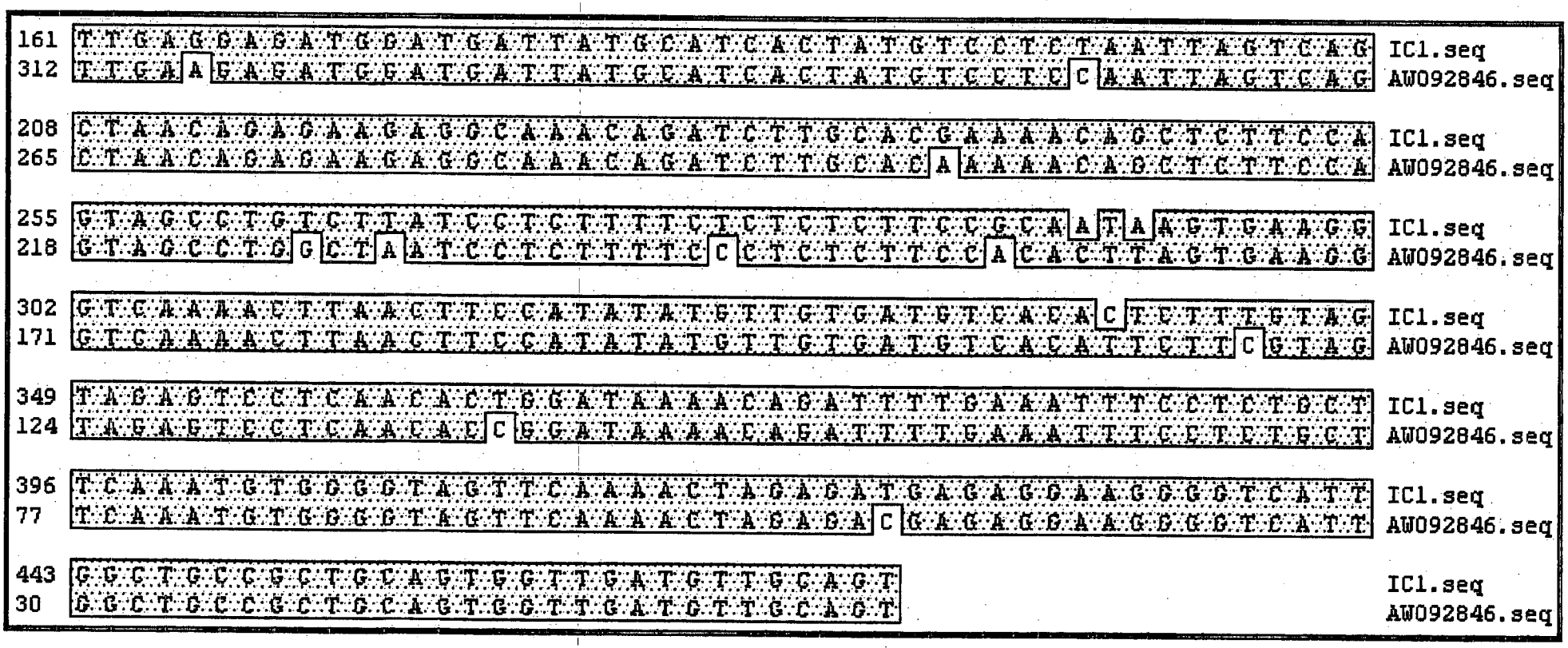
D-9 The nucleotide sequence alignment of clone IE7 (268 bp) with EST284406 from tomato treated with mixed elicitor (NCBI; AW041542



D-10 The nucleotide sequence alignment of clone ID2 (165 bp) with EST307746 from tomato treated with mixed elicitor (NCBI; AW442816)



D-11 The nucleotide sequence alignment of clone IC1 (503 bp) with EST286026 from tomato treated with mixed elicitor (NCBI; AW092846).



D-12 The nucleotide sequence alignment of clones IF11 (370 bp) and IE12 (217 bp) with the dehydration-responsive protein RD22 precursor (TIGR; TC21411).