

**Utilising model systems and crop species to
discover and characterise novel genes in plant
disease resistance**

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For my parents

Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or other university.

Eleanor M Gilroy

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Publications Arising From This Work

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Gilroy, E.M.¹, Faivre-Rampant, O.¹, Hrubikova, K., Hein, I., Millam, S., Loake, G.J., Birch, P.R.J., Taylor, M.A. and Lacomme, C. (2004) Potato Virus X-Induced Gene Silencing in Leaves and Tubers of Potato. *Plant Physiology* **134**, 1308-1316.

Tani, H., Chen, X., Nurmberg, P., Grant, J.J., SantaMaria, M., Chini, A., Gilroy, E.M., Birch, P.R.J. and Loake, G.J. (2004) Activation Tagging in Plants: A Tool for Gene Discovery. *Integrative and Functional Genomics*, **4**, 258-266.

Avrova, A.O., Taleb, N., Rokka, V.M., Heilbronn, J., Campbell, E., Hein, I., Gilroy, E.M., Cardle, L., Bradshaw, J.E., Stewart, H.E., Fakim, Y.J., Loake, G.J. and Birch, P.R.J. (2004) Potato oxysterol binding protein and cathepsin B are rapidly up-regulated in independent defence pathways that distinguish *R* gene-mediated and field resistance to *Phytophthora infestans*. *Molecular Plant Pathology*, **5**, 45-56.

Birch, P.R.J., Avrova, A.O., Armstrong, M., Venter, E., Taleb, N., Gilroy, E.M., Phillips, M.S. and Whisson, S.C. (2003) The potato-*Phytophthora infestans* interaction transcriptome. *Canadian Journal of Plant Pathology*, **25**, 226-231.

Abstract

This project was a collaboration between groups within the *Arabidopsis* research community and the crop-based research community through which the best resources of both could be utilised to study aspects of resistance to biotrophic pathogens. An objective was to work towards transferring information between models and crop plant.

The model plant, *Arabidopsis* is most amenable for discovering novel genes involved in disease resistance through large-scale mutagenesis and functional screening. Activation tagged *Arabidopsis* lines were screened for disease susceptibility to *Pseudomonas syringae* pv *tomato* (DC3000). Two mutant lines, designated activated disease susceptibility (*ads*) 1 and *ads*2, were confirmed to be susceptible and candidate gene(s) affected by the activation tag were identified.

On the other hand, functional analysis of genes in crop species, which currently lack sequenced genomes, relies on post-transcriptional gene silencing (PTGS) approaches that require sequence information prior to experimentation. Suppression subtractive hybridisation isolated a cathepsin B EST from *Phytophthora infestans* challenged potato undergoing resistance gene-mediated hypersensitive response (HR). *Nicotiana benthamiana*, a model plant for studying the HR, is a close relative of potato and an established virus-induced gene silencing system. Therefore, *N. benthamiana* was utilised to investigate a potential role for cathepsin B in plant disease resistance. Silencing of cathepsin B resulted in suppression of the *Erwinia amylovora*-induced HR which was confirmed using mammalian cathepsin B inhibitors.

A final aim was the development of a PTGS system in *Solanum tuberosum* to allow functional investigation for current and future defence-related genes identified from models and crop plant species. A PVX-based VIGS system was successfully developed for cultivated, tetraploid potato cultivars and for wild diploid *Solanum* species.

Abbreviations

4 x 35S	tetramer of <i>CaMV 35S</i> enhancer element
ACC	1-aminocyclopropane-carboxylic acid
<i>ADRI</i>	acquired disease resistance
<i>ads</i>	activated disease susceptibility
At	<i>Arabidopsis thaliana</i>
AVR	avirulence
BAC	bacterial artificial chromosome
BAR	Basta resistance
bp	basepairs
BLAST	basic local alignment search tool
BTH	benzo(1,2,3) thiadiazole
CathB	cathepsin B
CC	coiled coil
CFU	colony forming units
CHEF-gel	contour-clamped homogenous electric field gel
cm	centimetre
Col-0	<i>Arabidopsis</i> ecotype Columbia
CP	coat protein
CYP1	cysteine protease (RD21-like)
DAB	3,3-diaminobenzidine
DNA	deoxyribonucleic acid
<i>Eam</i>	<i>Erwinia amylovora</i>
EST	expressed sequence tag
ET	ethylene
<i>GFP</i>	green fluorescent protein
GM	genetically modified
GST	glutathione <i>S</i> -transferase
hpi	hours post-inoculation
HMW	high molecular weight
H ₂ O ₂	hydrogen peroxide
HR	hypersensitive response
HSP	heat shock protein
JA	jasmonic acid
kb	kilobase
KB	King's broth
kDa	kilodaltons
LRR	leucine rich repeats
LZ	leucine zipper
µg	micrograms
µl	microlitres
MAPK	mitogen activated protein kinase
Me-JA	methyl jasmonate
MES	2-[N-Morpholino]ethanesulfonic acid
MS	Murashige and Skoog media
<i>nahG</i>	salicylate hydroxylase gene
<i>NB</i>	<i>Nicotiana benthamiana</i>

NBS	nucleotide binding site
nm	nanometre
NO	nitric oxide
OD	optical density
PCD	programmed cell death
PCR	Polymerase Chain Reaction
<i>PDS</i>	phytoene desaturase
<i>PR</i>	pathogenesis-related
<i>Pst</i> DC3000	<i>Pseudomonas syringae</i> pv <i>tomato</i>
PTGS	post transcriptional gene silencing
PVX	potato virus X
QTL	quantitative trait locus
<i>R</i> gene	resistance gene
<i>RD21</i>	responsive to dehydration 21
REF	rubber elongation factor
RNA	ribonucleic acid
ROIs	Reactive oxygen intermediates
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SDW	sterile distilled water
SGS	SGT1-specific
SGT1	suppressor of G2 allele of SKP1
SSH	suppression subtractive hybridisation
<i>St</i>	<i>Solanum tuberosum</i>
TAIL-PCR	Thermal asymmetric interlaced PCR
TAIR	The <i>Arabidopsis</i> Information Resource
TIR	Toll/interleukin 1 receptor
TMV	tobacco mosaic virus
TPR	tetratricopeptide
TRV	tobacco rattle virus
VIGS	virus-induced gene silencing

1. Introduction

1.1. *Basic Overview of Plant Defence Responses*

One of the most aggressive and costly battles between agriculture and nature is the continuous arms race of pests and pathogens versus resistant plant varieties and new pesticides. This constant selective pressure perpetuates or increases the reliance of agriculture on intensely utilised and potentially damaging chemical treatments. Attempts to engineer durable resistance in crop plants have mainly failed due to the rate at which pathogens can evolve to escape recognition, complexity of the intersecting disease-resistance pathways and the sheer diversity of pathogen-host genotypic compatibility (Stuiver and Custers, 2001).

Many plant defences are pre-formed barriers that provide permanent protection against pathogen entry such as the cell wall, cuticle and root pericycle (Keen, 1992). Other plant defences are inducible and are only activated during pathogen infection e.g. enhanced strengthening of existing barriers, production of anti-fungal proteins and synthesis of anti-microbial agents (Delaney *et al.*, 1994). Plants scan their inter- and intra-cellular environment using an early warning system that includes a large battery of extremely polymorphic resistance (*R*) gene products (Martin, 1999; Stahl *et al.*, 1999). An invading plant pathogen can be detected by releasing avirulence (*Avr*) gene products, through a direct or indirect interaction with corresponding plant *R* gene products (Alvarez, 2000). Comparisons between known plant *R* gene products have revealed some widely conserved sequences, including leucine rich repeats (LRRs), nucleotide-binding sites (NBS) and serine-threonine kinase protein domains that are characteristic components of signalling pathways (Heath *et al.*, 2000a). In spite of this, no links between structure of *R* genes and classes of pathogen for which they convey resistance have been uncovered (Heath *et al.*, 2000a). However, this specific gene-for-gene interaction (Flor, 1956) between pathogen and host is associated with the induction of plant defence gene expression and activation of a genetically programmed cell death

mechanism known as the hypersensitive response (HR) at the site of infection (Lam *et al.*, 2001).

A rapid HR is triggered in response to an almost immediate burst of signalling molecules such as reactive oxygen intermediates (ROIs), Nitric Oxide and Calcium in combination with ion fluxes across plasma membranes. A clearly defined area of cell death develops to restrict an avirulent biotrophic pathogen to the site of attempted infection by destroying its nutrient source before it has energy to proliferate (Dong, 1998). This key signalling event in response to non-host and avirulent pathogens normally concludes between 24 and 48 hours post-inoculation. Experimental data from genetically controlled cell suicide (apoptosis) in animal models has provided plant scientists with an indication of the types of genes likely to be involved in the plant HR e.g. cysteine proteases (del Pozo and Lam, 1998; D'Silva *et al.*, 1998; Avrova *et al.*, 1999) and reactive oxygen intermediate (ROIs) producing mammalian neutrophil NADPH oxidase, gp91^{phox} (Grant and Loake, 2000; Torres *et al.*, 2002; Yoshioka *et al.*, 2003). However, specific examples of functionally conserved genes in plant and animal programmed cell death (PCD) have been rare and many of the associated components of these conserved proteins have not been identified in plants.

Following induction of the HR, a secondary, uniform and persistent increase of basal defence levels is established and can be described as primed for future attacks by any pathogens. This type of resistance is referred to as systemic acquired resistance (SAR) and establishes around five days post-infection. This defence response is dependent on the signalling molecule, salicylic acid (SA) which is necessary to induced expression of a number of SAR marker genes known as pathogenesis-related (*PR*) genes. In this state, the plant may induce HR to otherwise virulent pathogens.

There are also SA- and therefore HR-independent pathways that are required to induce defence responses against necrotrophic pathogens and/or wounding. An HR response is not necessary or effective against wounding and HR is actually suppressed when the plant can recognize necrotrophs. These pathways are characterised by requirement of

the signalling molecules jasmonic acid (JA) and ethylene (ET) and induced expression of an alternative subset of *PR* genes (Dong, 1998).

The plant therefore relies on early and informative pathogen recognition to tailor its most effective defence strategies to the type of pathogen encountered using a range of molecular and physiological processes; a number are shared with other eukaryotes but the majority are plant-specific. Scientists strive to understand this complex web of signals and proteins and I will attempt to explain and discuss the current knowledge of plant disease resistance in more detail throughout the following sections of this introduction.

1.2. Genetic Strategies for Crop Improvement

At present, consumers in the developed world are more aware of the damage caused to our environment through current agricultural approaches but remain suspicious of scientific advances targeted at alleviating these practices. The main concerns surrounding genetically modified (GM) crops include the toxicity of novel or natural defence gene products, the increased vigour and invasiveness of crops and the potential for gene flow from crops to native flora and fauna (Dale *et al.*, 2002; Smyth *et al.*, 2002). The true environmental impact of GM crops cannot be generalized as it varies depending on crop species, the engineered trait and the presence of sexually compatible relatives (Smyth *et al.*, 2002). Since the “Green Revolution”, plant-breeding programs have successfully produced crops with higher productivity and superior adaptations to the demands of their environment (Lee, 1998; Hoisington, 1999). Plant breeding shall remain an essential component of crop production systems in the future. However, disadvantages such as increasing genetic uniformity and decreasing genetic diversity in modern cultivars and hybrids threaten yields in the long term (Lee, 1998). Modifying specific gene targets in established crops, rather than selective plant breeding, can introduce traits of interest without associated detrimental inbreeding defects or linked inheritance of undesirable phenotypes (Cook, 1998). Once effective transgene containment strategies are broadly

applicable, genetic modification will provide a more efficient and accurate technique of engineering solutions to the demands of agriculture (Messing and Llaca, 1998; Daniell, 2002).

A direct or indirect interaction between the plant's *R* gene product and the pathogen's *Avr* gene product initially triggers the HR. Introgressing known *R* genes into crops to harness the vigour of the HR against the most common or devastating pathogens has lacked durability in the field as pathogens quickly evolve by accumulating mutations or shedding genes to escape recognition (Ellis *et al.*, 2000). Although rare, durable *R* genes have been identified e.g. *Xa21* in rice confers resistance to all isolates of *Xanthomonas oryzae* (Rommens and Kishore, 2000). Nevertheless, effective solutions may involve fine-tuning downstream branches of defence signal transduction pathways usually triggered by an R-AVR interaction i.e. systemic acquired resistance (SAR) that results in broad-spectrum resistance throughout the plant (Rommens and Kishore, 2000; Stuiver and Custers, 2001). Altering complex defence pathways can have positive and negative effects e.g. the natural mutant *mlo* in barley confers broad-spectrum resistance to powdery mildew (*Blumeria graminis* f.sp. *hordei*) but concomitantly increases susceptibility to another fungal pathogen, *Magnaporthe grisea* (Stuiver and Custers, 2001; Brown, 2002). In addition, activating large branches of normally inducible defence pathways to be constitutively active can have significantly detrimental effects e.g. application of the synthetic SAR elicitor benzothiadiazole (BTH) results in decreased plant vigour and grain yields (Heil and Baldwin, 2002; Heil and Bostock, 2002). To generate broad-spectrum resistance to the pathogen species that cause most harm, thorough dissection and comprehension of defence signalling networks is required to effectively manipulate key signalling regulators and the pathways they control.

1.3. Pathogen Recognition

The most common outcome of most plant-microbe interactions is incompatibility, inducing resistance which can be manifested at several stages of the interaction

(Peterhansel *et al.*, 1997). Firstly, preformed structural barriers e.g. pectin (Rogers *et al.*, 2000) or constitutively produced toxic compounds e.g. phytoanticipins (VanEtten *et al.*, 1994) may prevent pathogen infection. Secondly, metabolites manufactured by the plant can be insufficient for supporting pathogen growth (Hammond-Kosack and Jones, 1996). The third and most common scenario is known as non-host resistance when an entire plant species conveys resistance, usually including an HR, to a specific pathogen (Kamoun, 2001; Mysore and Ryu, 2004). Finally, if foreign proteins are recognized by any one of the plethora of R proteins, the plant actively defends itself by deploying an inducible range of chemicals, proteins and physiological processes tailored to overcome the type of pathogen encountered (Heath, 2000a).

1.3.1. Resistance Genes

Since the first *R* gene was characterised ten years ago (Jones *et al.*, 1994), many more genes that convey resistance to races within a pathogen species that possess a corresponding *Avr* gene have been characterised and classified to reveal six major classes (Figure 1.3.1). *R* gene classes are distinguished by the presence or absence of widely conserved sequences including leucine rich repeats (LRRs), nucleotide-binding sites (NBS) and serine-threonine kinase domains (Heath, 2000a). *R* genes are unevenly distributed throughout the plant genomes studied and family members are often found as clusters (Jones, 2001) and are thought to have resulted from gene duplication and diversifying selection (Holub, 2001). For example, resistance to *Peronospora parasitica* 4 (*RPP4*) maps to the resistance to *Peronospora parasitica* 5 (*RPP5*) locus where there are seven other *RPP5* homologues (Dixon *et al.*, 1998; van der Biezen, 2002). The *RPP1* locus is another example, where three tightly linked NBS-LRRs, each with a unique N-terminus, convey resistance to different isolates of *P. parasitica* suggesting they recognise different avirulence determinants (Botella *et al.*, 1998). In addition, quantitative (also referred to as field) resistance in potatoes has been mapped to *R* gene clusters suggesting that quantitative trait loci (QTL) responsible for polygenic resistance may encode *R* gene relatives (Gebhardt and Valkonen, 2001).

The leucine-rich repeats (LRR) domain is found in four classes of *R* genes and has been shown to be responsible for pathogen recognition synonymous with mammalian Toll/interleukin1 receptor (TIR)-LRR proteins which interact with specific ligands through protein-protein interactions at the LRR domain (Belkhadir *et al.*, 2004). A clear example of plant LRR domain function is described in Bryan *et al.*, (2000), when a single amino acid change in the LRR of rice R protein, Pi-ta, resulted in the loss of resistance to *Magnaporthe grisea* possessing Avr-Pita. The sub-cellular compartment in which the LRR is situated generally corresponds with the location targeted by the corresponding AVR protein (Bonas and Lahaye, 2002). The R proteins that detect Type III delivered AVR proteins typically possess cytoplasmic LRRs e.g. RPM1 (resistance to *P. syringae* pv *maculicola* 1) which recognises *P. syringae* AvrRpm1 (Grant *et al.*, 2000). The LRR domains that detect non-invasive fungi are generally extracellular e.g. Tomato *Cladosporium fulvum* 9 (CF-9) perceives Avr9 released from *C. fulvum* (Jones *et al.*, 1994; Hammond-Kasack *et al.*, 1998).

The nucleotide binding site (NBS) domain possesses three conserved kinase motifs in common with animal ATP- and GTP-binding proteins and a C-terminal ARC domain (apoptosis, *R* gene products and CED-4) which, as the name suggests, is conserved in apoptosis regulators and could control similar functions as a protein-binding molecular switch (Figure 1.5.2) (Belkhadir *et al.*, 2004, Li *et al.*, 1997a). An alternative supposition is that the NBS may act as a phosphoprotein based on homology with prokaryotic His-Asp phosphoproteins and evidence that RPP5 interacts with At-RSH1 (RelA/SpoT homolog, involved in bacterial (p)ppGpp mediated stress signalling), in a yeast-2-hybrid screen (van der Biezen *et al.*, 2000). The NBS-LRR proteins are subdivided into 2 groups according to N-terminal features, those with coiled coil (CC) domains and those with cytoplasmic Toll/interleukin1 receptor (TIR) domains (Bonas and Lahaye, 2002). The exact functions of CC and TIR domains remains unclear, although evidence suggests that these domains interact with signalling partners that cause conformational changes that positively or negatively regulate the NBS-LRR and subsequent defence pathways. For example, most CC-NBS-LRRs require NDR1 (non-race specific disease resistance) for signal transduction and RPM1's coiled coil domain

was shown to bind the regulator protein, RIN4 (RPM1-interacting Protein 4) (Takahashi *et al.*, 2003). RIN4 has been shown to positively regulate RPM1-mediated resistance on one hand yet also be a negative regulator of basal defence responses on the other (Mackey *et al.*, 2002). The majority of TIR-NBS-LRRs are dependent on EDS1 (enhanced disease susceptibility 1) and PAD4 for downstream pathway induction (Falk *et al.*, 1999; Jirage, 1999).

Two classes of *R* gene have neither NBS nor LRR domains. Nevertheless, evidence suggests that they utilise the same pathways as LRR-containing proteins (Fluhr, 2001). The protein kinase PTO is dependent on NBS-LRR protein PRF for *R*-gene signalling (Bogdanove and Martin, 2000) and, surprisingly, RPW8 (resistance to powdery mildew 8) protein (with elongated CC domains) is EDS1-dependent implying it has TIR-like function (Dangl and Jones, 2001). In spite the amount of experimental and sequence data, no links between structure of *R* gene products and classes of pathogen to which they convey resistance have been uncovered; highly homologous potato genes *Rx* and *Gpa2*, for example, convey virus and nematode resistances respectively (Van der Vossen *et al.*, 2000).

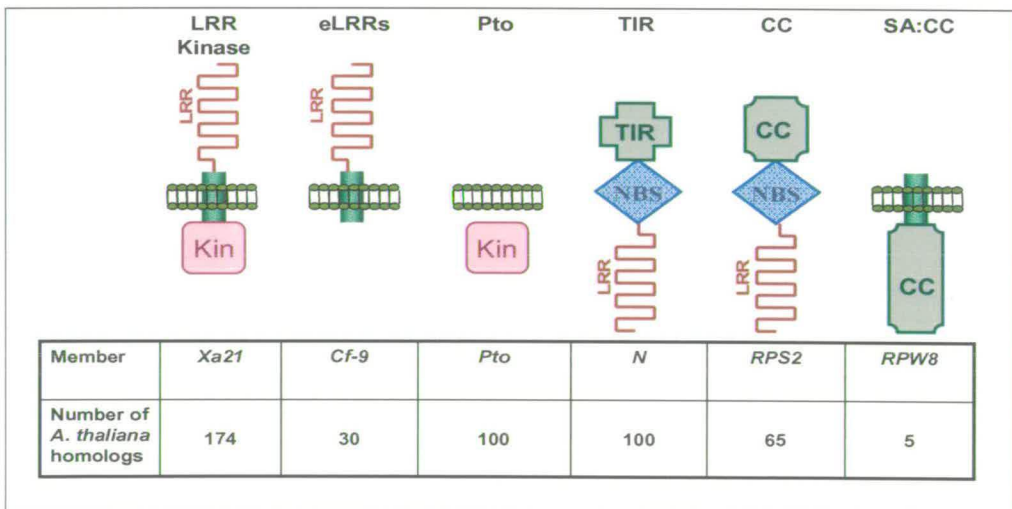


Figure 1.3.1: Classification of *R* genes by protein domains.

Example of each and number in *A. thaliana* genome. LRR, Leucine-rich repeats; Kin, Protein kinase; eLRR, extracellular LRR; Pto, *Pseudomonas* tomato resistance 1; NBS, Nucleotide-binding site; TIR, Toll and Interleukin-1 receptor; CC, Coiled coil; SA:CC, signal anchored coiled coil. Adapted from Dangl and Jones (2001) and Jones (2001).

1.3.2. Avirulence Genes

By and large, plant pathogens require varying degrees of specialisation to avoid recognition and/or to elude inducible defence responses initiated against them by particular plant species, determining their host ranges. Whatever the infection strategy, pathogens secrete or deliver a diverse range of proteins evolved to evade or manipulate their preferred host's defence responses (White *et al.*, 2000). However, these proteins or products of their activities can act as bait for R proteins, branding them avirulence (AVR) proteins and consequently enforcing a tight restriction on each pathogen's genotypic compatibility. In spite of this, pathogens maintain some *Avr* genes which trigger resistance in a wide range of potential hosts, indicating that these proteins may be essential for the infection process (e.g. Bean Dwarf Mosaic Virus nuclear shuttle protein, BV1) (Garrido-Ramirez *et al.*, 2000) or substantially enhance virulence in susceptible plants (e.g. *AvrBs2*) (Wichmann and Bergelson *et al.*, 2004). One exception to this rule is *AvrBs1* which carries a fitness cost to *Xanthomonas axonopodis* pv. *vesicatoria* (Wichmann and Bergelson *et al.*, 2004). AVR proteins are diverse in structure and often unlike any other proteins in databases, making elucidation of their molecular function more challenging. Table 1.3.2 includes some examples of AVR proteins with known functions.

Table 1.3.2: Examples of AVR proteins. Examples of AVR proteins from a range of plant pathogens and their role in pathogenicity. Adapted from Abramovitch and Martin, 2004)

Pathogen	Protein	Structure	Targets	Phenotype	Reference
<i>P. syringae</i> pv <i>tomato</i> DC3000	AvrPtoB	Unknown	HR	Suppresses PCD in plants and yeast	Abramovitch <i>et al.</i> , 2003
	AvrPto	3 helix bundle	Cell wall-based defence	Inhibits papillae formation; alters expression of cell wall-associated genes	Hauck <i>et al.</i> , 2003
	AvrRpt2	Cysteine protease	<i>PR</i> gene expression	Suppresses <i>PR</i> gene expression; susceptibility to <i>Pst</i> DC3000; RIN4 disappearance	Lim and Kunkel, 2004; Chen <i>et al.</i> , 2004; Mackey <i>et al.</i> , 2003
<i>S. lycopersici</i>	Tomatinase	Glycosyl Hydrolase	Preformed antimicrobials and HR	Degrades saponin causing HR suppression	Bouarab <i>et al.</i> , 2002;
<i>P. sojae</i>	Avr1b	secreted protein	unknown	unknown	Shan <i>et al.</i> , 2004
Potato Virus Y	NlaPro	Nuclear Inclusion protease	Process host protein target	Protease activity essential for triggering resistance	Mestre <i>et al.</i> , 2003; Rouis <i>et al.</i> , 2001

1.3.3. R-AVR Molecular Interactions

Since the acceptance of Flor's gene-for-gene hypothesis (Flor, 1971), the shortfall in the expected number of examples supporting direct R-AVR interactions, like AvrPto-Pto (Bogdanove and Martin, 2000), was originally thought disappointing. However, the majority of experimental evidence highlighted similar but more sophisticated molecular interactions, leading to the "Guard Hypothesis", which describes R proteins not only scanning for specific foreign proteins but also aberrant forms of the plant's own protein complexes (Dangl and Jones, 2001). These aberrant complexes are formed when AVR proteins attempt to modify key host housekeeping or signalling proteins for their own means (Marathe and Dinesh-Kumar, 2003). The most intensively studied example of this centres around the negative regulator, RIN4 (Mackey *et al.*, 2002). It has been shown that some *P. syringae* Type III effectors target RIN4 for post-translational modification, possibly enhancing its ability to suppress basal defence responses (Mackey *et al.*, 2002). The NBS-LRR proteins RPM1, directly, and RPS2, indirectly, interact with RIN4, thus detecting its phosphorylation or degradation by AvrRpm1, AvrRpt2 and AvrB (Mackey *et al.*, 2003). In addition, the molecular chaperone HSP90 (heat-shock protein 90) has been shown to associate with and modulate RPM1 (Hubert *et al.*, 2003) and to be essential for RPS2 (resistance to *P. syringae* 2)-mediated resistance, consequently linking an SGT1/RAR1/HSP90 complex and *R* gene-mediated resistance to *P. syringae* in *Arabidopsis* (Takahashi *et al.*, 2003) (discussed in more detail in 1.4.1.2). If this level of complexity is assumed for most R-AVR molecular relationships, it may explain the frustrating speed at which other plant-pathogen interactions are being understood in terms of their interacting transcriptomes and proteomes i.e. potato and *P. infestans* (Birch *et al.*, 2003).

1.3.4. Non-specific Elicitor-Induced Resistance

The term elicitor is currently used to refer to pathogen-derived molecules which are either directly or indirectly released during an infection that induce any type of plant defence response, including phytoalexin accumulation (Keen, 1975; Keller *et al.*, 1999). Non-specific elicitors can trigger a non-specific form of plant resistance (Reglinski *et*

al., 1994), which may encompass some of the *R* gene triggered resistance responses (Mysore and Ryu, 2004). For example, the ROI burst and subsequent HR are linked with both host and non-host resistance (although the timing differs) to various fungi and oomycetes (Able *et al.*, 2003; Vleeshouwers *et al.*, 2000; Kamoun *et al.*, 1999). Elicitors can be divided into two main groups depending on whether the elicitors originate from the pathogen or the plant (Ebel, 1998). Exogenous elicitors are either pathogen-derived structural components, also known as pathogen-associated molecular patterns (PAMPs) (e.g. flagellin) or secreted proteins (e.g. harpins) released during colonisation of the plant (Zipfel *et al.*, 2004; Mysore and Ryu, 2004). Endogenous compounds are plant-derived degradation products of pathogen enzymes e.g. pectic polysaccharides and oligogalacturonides (Cote and Hahn, 1994). These non-specific elicitors are thought to be major inducers of non-host resistance and unlikely to centre on any one, universal elicitor as the ultimate warning of pathogen invasion for all non-host species (Ebel, 1998). Nevertheless, elicitor-induced defence responses, sharing strong similarities with those elicited by *R* genes, allows the plant to act rapidly against colonisation by a pathogen (Kamoun *et al.*, 1999).

1.4. Early Signal Transduction Events

The induction of the appropriate defence pathways is thought to be controlled, at least in part, by the structure of the *R* protein involved in pathogen recognition (Loake, 2001). Initially, however, a series of common cellular responses is rapidly triggered, including calcium influxes, alkalization of extracellular space, protein kinase activation, generation of reactive oxygen intermediates (ROIs) and nitric oxide (NO) production (Numberger and Scheel, 2001). In spite of this, events that link pathogen recognition to defence gene expression are still poorly understood and therefore a general model cannot explain downstream signal transduction and resistance mechanisms for every plant-pathogen interaction. Nonetheless, despite the variety and intricacy of pathogen recognition events, many common plant responses have been identified and shall be discussed below.

1.4.1. Early Signalling Cascades

1.4.1.1. NDR1 and EDS1

Following recognition of biotrophic pathogens, there are at least two mutually exclusive parallel defence-signalling pathways in *Arabidopsis* that are activated by NBS-LRR genes distinguished by their TIR or CC domains (van der Biezen *et al.*, 2002). Mutations in *EDS1* block resistance mediated by TIR-NBS-LRR proteins e.g. RPP2 (resistance to *Peronospora parasitica*) and RPS4 but not CC-NBS-LRR proteins e.g. RPM1 (Aarts *et al.*, 1998). *EDS1* encodes a protein with sequence similarity to the catalytic domains of eukaryotic lipases that generate lipid signals (Loake, 2001). It is required for runaway cell death in *lsl1* plants with a dysfunctional HR but not the associated ROI production (Rustérucchi *et al.*, 2001). *EDS1* is required for SA accumulation, although applications of SA cause up-regulation of *EDS1* mRNA transcripts placing it upstream of SA production in SAR and also part of SA's potentiating feedback loop (Glazebrook, 2001). *PAD4* (phytoalexin-deficient 4) is another lipase-like protein required for TIR-NBS-LRR mediated resistance, although a functional *EDS1* protein is required for *PAD4* mRNA accumulation (Feys and Parker, 2000). Conversely a mutation in *PAD4* only partially compromises *EDS1* expression (Feys and Parker, 2000). In addition, *EDS1* and *PAD4* have been shown to directly interact in a yeast 2-hybrid screen in both healthy and challenged cells (Feys *et al.*, 2001). Although exact roles remain to be determined, *EDS1* and *PAD4* may hydrolyse lipids to pass the *R* gene-mediated signal onto the SA-dependent pathway (Feys *et al.*, 2001).

NDR1 (non-race specific disease resistance) is a small protein with 2 transmembrane domains that is necessary for signal transduction mediated by CC-NBS-LRR proteins e.g. RPM1 and regulation of HR (Aarts *et al.*, 1998). Superoxide also fails to accumulate in *P. syringae* challenged *ndr1* mutant plants (Loake, 2001). These mutants lose race-specific resistance to strains of *P. syringae* and *P. parasitica* but not resistance mediated through *EDS1* (Feys and Parker, 2000). *R* genes have been identified that are independent of both *EDS1* and *NDR1* e.g. *RPP7*, *RPP8* and *RPP13*, indicating an additional pathway may exist (Glazebrook, 2001; Bittner-Eddy and Beynon, 2001). CC-

NBS-LRR proteins were initially found to require an additional protein, PBS2 (*avrPphB* susceptible 2) however, it is also required by the EDS1-dependent pathway, revealing a potential convergence point (van der Biezen *et al.*, 2002). Positional cloning has since revealed this gene to be *AtRAR1* (*Arabidopsis* required for Mla12 resistance) (Tornero *et al.*, 2002).

1.4.1.2. RAR1, SGT1 and HSP90

The protein, RAR1 was identified in a barley mutant screen in which the mutant displayed an impaired ROI burst and HR when challenged with powdery mildew fungus (Freialdenhoven *et al.*, 1994). Further studies of *AtRAR1* in *Arabidopsis* have not shown it to be affiliated with any one R protein class or essential for EDS1-, PAD4- or NDR-dependent mechanisms (Muskett *et al.*, 2002; Liu *et al.*, 2002). RAR1, characterised by two Zn²⁺ binding domains known as a CHORD (cysteine and histidine-rich domain), is predicted to be cytosolic (Shirasu *et al.*, 1999) and possesses a CHORD and SGT1 specific (CS) domain (Muskett *et al.*, 2002). Interestingly, loss of function mutations in RAR1 resulted in the loss of detectable NBS-LRR protein, RPM1 and may therefore be required for its stability (Tornero *et al.*, 2002; Hubert *et al.*, 2003).

The protein SGT1 (suppressor of G2 allele of SKP1) was originally identified in yeast as a SKP1 interacting protein which placed it in the SKP1/Cdc53/F-box protein (SCF) ubiquitin ligase complex (Kitagawa *et al.*, 1999). SGT1 protein contains a tetratricopeptide repeats domain, two variable domains, CS domain and a SGT1-specific domain (Shirasu *et al.*, 1999). In *Arabidopsis*, the SGT1 homologues, SGT1a and SGT1b, were identified as RAR1-interacting proteins using a yeast-2-hybrid system and can both complement yeast *sgt1* mutants (Azevedo *et al.*, 2002). This subsequently links components of the kinetochore assembly and ubiquitin ligase complex with disease resistance (Azevedo *et al.*, 2002; Peart *et al.*, 2002; Steensgaard *et al.*, 2004). It was characterised further in several independent studies, utilising virus-induced gene silencing and *Arabidopsis* knock out mutants, highlighting SGT1 as a key molecular component of R gene-mediated and non-host resistance (Austin *et al.*, 2002; Tör *et al.*, 2002; Liu *et al.*, 2002; Peart *et al.*, 2002). Interestingly, silencing of *Nicotiana* SKP1

compromised *N* gene-mediated resistance to TMV (Liu *et al.*, 2002). In addition, yeast SGT1 was found to interact with and to be phosphorylated by the calcium-binding protein, calcyclin, at its C-terminal domain which suggests that plant SGT1 activity may possibly be regulated by calcium signalling (Nowotny *et al.*, 2003).

HSP90 is an ATP-dependent chaperone protein and is one of the most abundant proteins in eukaryotic cells (1-2 % in non-stressed conditions) (Sreedhar *et al.*, 2004). From studies in mammalian systems, HSP90 has been demonstrated to interact with more than 100 proteins involved in cell proliferation, differentiation and apoptosis (Pratt *et al.*, 2004) including tyrosine e.g. Wee1 (Aligue *et al.*, 1994) and serine-threonine protein kinases e.g. MAPKs (Piatelli *et al.*, 2002). In *Arabidopsis*, HSP90 interacts with many NBS-LRR proteins, including RPS2, RPM1 and N (Takahashi *et al.*, 2003; Hubert *et al.*, 2003; Liu *et al.*, 2004b). Like RAR1, HSP90 also appears to have an important role in stabilizing some NBS-LRR proteins as shown for Rx (Lu *et al.*, 2003). In addition, HSP90 mutations have been shown to affect different *R*-gene mediated pathways e.g. RPS2 and RPM1 and therefore HSP90's role in plant disease resistance has been predicted to be more than a general protein chaperone (Takahashi *et al.*, 2003; Lu *et al.*, 2003). HSP90 has also been implicated in pathogen signalling as demonstrated by its mRNA up-regulation in the pathogen *P. infestans* when *in planta* (Avrova *et al.*, 2003).

Interactions between these three proteins were confirmed when HSP90 was shown to interact with both RAR1 and SGT1 (Peart *et al.*, 2002). SGT1 interacts directly with HSP90 through its Tetratricopeptide repeat (TPR) domain and RAR1 through its CHORD II domain (Takahashi *et al.*, 2003). In addition, yeast-2-hybrid analysis highlighted a strong interaction between SGT1 and RAR1 (Azevedo *et al.*, 2002) predicted to be through the CS domains that they both share (Shirasu and Schulze-Lefert, 2003). However, RAR1 and SGT1 are not dependent on each other for function in all cases (Shirasu and Schulze-Lefert, 2003), for example, SGT1 has a broader role in non-host disease resistance (Peart *et al.*, 2002). Therefore, HSP90 is thought to stabilize R proteins, RAR1 is recruited in some cases and then SGT1 subsequently modulates downstream pathways (Takahashi *et al.*, 2003).

1.4.1.3. Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are highly conserved proteins involved in sensing a wide array of external stimuli, subsequently activating the appropriate downstream molecular responses by controlling transcriptional events, regulating enzyme activities and phosphorylation of cytoskeleton elements (Champion *et al.*, 2004). MAPKs are mostly arranged into cascades of at least three divisions, as the *Arabidopsis* genome reveals twenty MAPKs, ten MAPK kinases (MAPKKs) genes, eighty MAPKK kinases (MAPKKKs) (AGI, 2000; Asai *et al.*, 2002; Jonak *et al.*, 2002). In plant defence, MAPKs have shown to be activated by signalling molecules such as SA and NO (Xing *et al.*, 2002). However, MAPK's have also been shown to modify responses such as the oxidative burst (Grant *et al.*, 2000), activation of *PR* gene transcription factors (Ekengren *et al.*, 2003; Kroj *et al.*, 2003), activation of the WRKY family of transcription factors (Kim and Zhang, 2004) and triggering cell death in susceptible *P. syringae* infection (del Pozo *et al.*, 2004). MAPKs are thought to be convergent points of signalling downstream of widely varying triggers, supported by *Nicotiana* MAPKs, wound-inducible protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK), which are activated by oomycete elicitor and the race-specific interactions involving Avr9-Cf-9 and TMV-N (Romeis *et al.*, 1999; Zhang and Klessig, 1998; Zhang *et al.*, 1998). In addition, the *Arabidopsis* homologues MAPK3 and MAPK6 are activated by flagellin as part of the non-specific elicitor-induced cascade (Asai *et al.*, 2002). Moreover, a tyrosine phosphatase, which is reversibly inactivated by H₂O₂, correlated with the activity of MAPK6, provides a link between the ubiquitous production of ROIs after elicitor recognition and the MAPK signalling cascade (Gupta and Luan, 2003). Conversely, the MAPKK, SIMKK, has been shown to bind and activate more than one MAPK, suggesting that MAPK cascades could also provide divergence points in signalling pathways (Cardinale *et al.*, 2002). MAPK's probable role in other defence pathways was highlighted by the mutant, *AtMPK4* (*Arabidopsis* mitogen-activated protein kinase 4), as it was revealed to be deficient in JA-dependent gene induction and displayed increased resistance to susceptible pathogens and may therefore be a suppressor of early biotrophic defence responses (Petersen *et al.*, 2000).

The complexity within MAPK cascades, the crosstalk with alternative signalling mechanisms, in combination with the extent of downstream functions they perform, has almost certainly provided scientists with a huge challenge for the future.

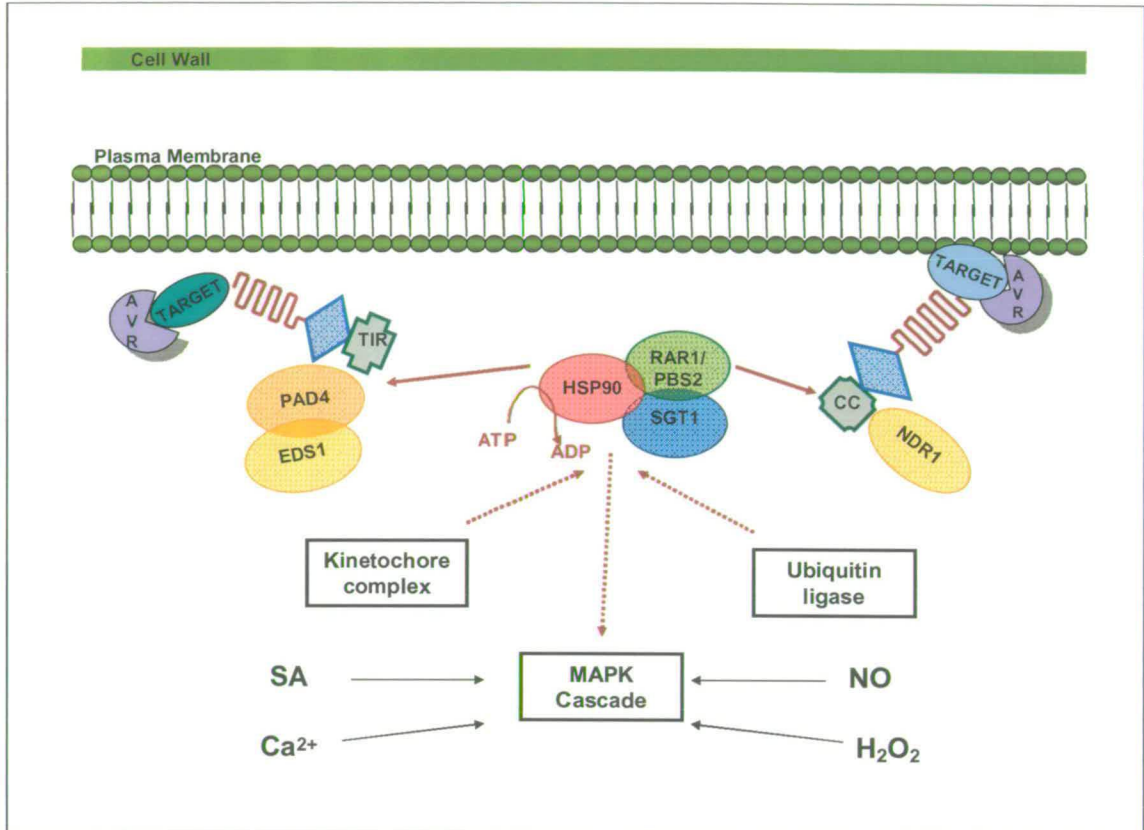


Figure 1.4.1: Summary of the key early signal transduction events after R-AVR interaction. AVR, Avirulent protein; TIR, TIR-NBS-LRR resistance protein; CC, CC-NBS-LRR resistance protein.

1.4.2. Ion Fluxes

1.4.2.1. Calcium

Influxes of extracellular calcium have been shown in response to biotic and abiotic stress (Pei *et al.*, 2000; Lee and Rudd, 2002). Production of ROIs, MAPK activation and defence gene activation have all been shown to be compromised when cytosolic Ca²⁺ levels are artificially reduced (Scheel *et al.*, 1998). *Arabidopsis* encodes 34 calcium-dependent serine/threonine protein kinases (CDPKs) that are thought to be activated by phosphorylation which allows them to sense calcium influxes caused by a variety of stress-inducing conditions (Romeis, 2000). Almost instantaneously after R protein

activation, plasma membrane calcium channels are activated, causing cytosolic calcium concentrations to increase in a biphasic manner (Gelli and Blumwald., 1997; Grant and Mansfield, 1999; Lecourieux *et al.*, 2002). R proteins, such as RPM1 and Cf-9, are proposed to link pathogen recognition and activation of calcium channels due to their membrane association (Grant and Mansfield, 1999). Moreover, calcium signalling may also activate NADPH oxidase through binding to unique EF motifs in the N-terminal region of *Arabidopsis* gp91^{phox} homologue (*AtRBOHA*) (Grant and Loake, 2000; Kadota *et al.*, 2004).

CDPKs are one of the largest families of calcium binding proteins and are presumably involved in a diverse range of signalling roles, as the *Arabidopsis* genome contains thirty four CDPKs (Lee and Rudd, 2002). The activation of the protein NtCDPK1 has been demonstrated upon non-race-specific elicitation with chitin fragments (Yoon *et al.*, 1999). Moreover, the gene, *NtCDPK2*, is activated following Avr9 elicitation of Cf-9 carrying tobacco (Romeis *et al.*, 2000) and, through studies of this interaction, some CDPKs have been shown to be activated by phosphorylation and crucial for induction of the HR (Romeis *et al.*, 2000; Romeis *et al.*, 2001). Many CDPKs are membrane-associated as predicted by their myristoylation motif e.g. NtCDPK2 and are thought to be activated by phosphorylation (Romeis *et al.*, 2001). The investigation of the *Arabidopsis* orthologue of *NtCdpk2* revealed its interaction with 14-3-3 proteins and activation of a tonoplast chloride channel, providing some evidence of downstream targets (Romeis *et al.*, 2001). Current evidence suggests that CDPKs are predicted to signal together with, but are not dependent on, MAPKs for HR induction (Romeis, 2001). Nevertheless, many protein targets of calcium and CDPKs leave their roles in signalling cascades to be elucidated.

1.4.2.2. Potassium

Potassium (K⁺) channels have been identified in an assortment of subcellular membranes, most commonly in the vacuole, and are shown to be involved in a variety of mechanisms such as stomatal regulation and ion uptake (Czempinski *et al.*, 2002). Interestingly, Huang *et al.*, (1997) showed that inward K⁺ transport could be regulated

by structural changes of actin filaments in guard cells. In addition, the efflux of K^+ is shown to be necessary for progression of mammalian apoptosis (Bortner *et al.*, 1997). A net loss of K^+ resulted from inhibition of inward K^+ channels after five minutes during the Avr9/Cf-9 interaction and increased activity of the outward K^+ channels in tobacco (Blatt *et al.*, 1999). In addition, Avr9 induced K^+ channel activities could be blocked by inhibitors of serine/threonine kinases highlighting a role of protein phosphorylation in K^+ -related signal transduction (Blatt *et al.*, 1999).

1.4.2.3. Protons

Proton translocation across membranes, resulting in alkalinisation of the apoplast, has been implicated in plant defence by a number of studies. Over-expression of an active form of bacterial H^+ -ATPase resulted in spontaneous lesions and *PR* gene expression in unchallenged tobacco (Mittler *et al.*, 1995; Pontier *et al.*, 2002). In support of this observation, the activator of the plasma membrane H^+ -ATPase, fusicoccin, increased the number of HR-cells in a compatible barley-powdery mildew interaction (Zhou *et al.*, 2000). The opposite phenotype was observed using ion channel antagonists which prevented ROI production and defence gene activation in parsley cells (Jabs *et al.*, 1997). In addition, 14-3-3 proteins, involved in signalling and protein import are associated with the membrane H^+ -ATPase (Baunsgaard *et al.*, 1998; Roberts, 2000). The resulting alkalinisation of the apoplast of epidermal cells has been suggested to trigger production of H_2O_2 in mesophyll cells which subsequently induces cell death of the epidermal cells (Zhou *et al.*, 2000).

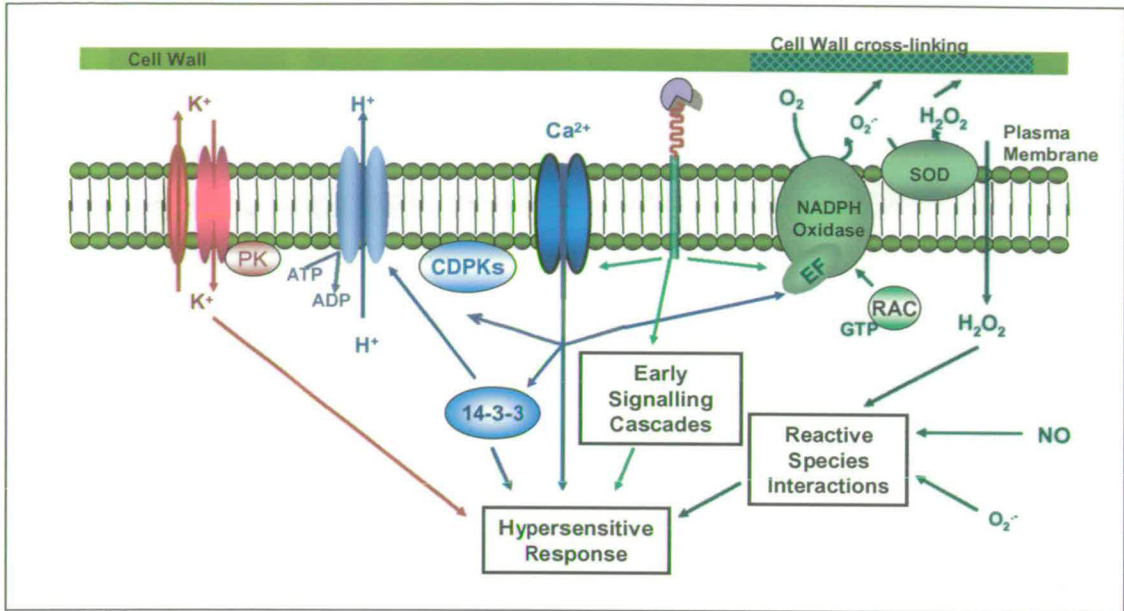


Figure 1.4.2: Ion Fluxes at plasma membrane post-pathogen recognition

Schematic representation of general ion fluxes known to take place at the plasma membrane. EF, Calcium binding domain; CDPK, Calcium-dependent protein kinases; PK, protein kinase; SOD, Superoxide dismutase; RAC, p21^{rac} homolog. Summary of literature discussed in section 1.4.2

1.4.3. Oxidative Burst

The rapid generation of ROIs in response to infection or pathogen elicitor treatment is known as the oxidative burst and is a key feature of inducible defence responses (Bolwell *et al.*, 2002). Generation of ROIs was first observed during an incompatible *P. infestans*-potato interaction but not during a compatible interaction (Doke *et al.*, 1983). The oxidative burst comprises a biphasic production of reactive oxygen intermediates (ROIs), such as superoxide ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}) and hydrogen peroxide (H_2O_2) following ($O_2^{\cdot-}$) dismutation, at the site of attempted pathogen infection (Hammond-Kosack and Jones, 1996; Scheel, 1998; Grant and Loake, 2000). The first peak of ROI production occurs between five minutes and one hour after biotic or abiotic stress and, following pathogen recognition, a second larger and sustained race-specific burst (Shirasu and Schulze-Lefert, 2000). The rapidity of ROI production suggests that it is independent of transcription and most probably regulated by secondary messengers such as Ca^{2+} and H^+/K^+ and by proteins such as kinases and G-proteins (Yang and Yang, 1997; Cessna and Low, 2001). During abiotic stress, studies of ROI generation have

been mainly concentrated on mitochondria, chloroplasts and peroxisomes, whereas studies of biotic stress have mainly focused on activities at the plasma membrane and cell wall (Robertson *et al.*, 1995; Lamb and Dixon, 1997; Asada, 1999; del Rio *et al.*, 2002). The general sources of ROI are summarised in Table 1.4.3. Initially, the cytotoxicity of ROIs were assumed to function by causing irreparable damage to DNA, proteins and lipids of the invading pathogen and the infected plant cells (del Rio, 2002). However, further research into the oxidative burst has revealed their more intriguing signalling properties (Overmyer *et al.*, 2003).

Superoxide ($O_2^{\cdot -}$) is itself cytotoxic but has a short half-life and cannot cross membranes and therefore its major functions are thought to be as a signal for HR induction and as the substrate for other ROI generating reactions. The major source of superoxide is thought to be at the plasma membrane by an NADPH oxidase complex ($O_2 + NADPH \rightarrow O_2^{\cdot -} + NADP^+ + H^+$) analogous to that of mammalian neutrophils (Grant and Loake, 2000). In the mammalian system, two of five complex components are membrane-bound (gp91^{phox}, p22^{phox}) and three are initially cytosolic (p47^{phox}, p40^{phox} and p67^{phox}), which associate into the full complex after phosphorylation, aided by a small GTP-binding protein, p21^{rac2} (Lal *et al.*, 1999; Diekmann *et al.*, 1994). However, NADPH oxidase is only partially conserved in plants as homologues of the cytosolic components p47^{phox}, p40^{phox} and p67^{phox} have not been identified in the *Arabidopsis* genome (AGI, 2000). Furthermore, the eight *Arabidopsis* gp91^{phox} homologues contain two calcium binding domains (EFs), not present in mammalian NADPH oxidase, suggesting that cytoplasmic Ca^{2+} is a potential regulator of the plant oxidative burst (AGI, 2000; Keller *et al.*, 1998). However, a p21^{rac} homologue has been identified in rice (*OsRac1*) that binds GTP and causes constitutive production of ROI and spontaneous lesions (Shirasu and Schulze-Lefert, 2000). In spite of the focus on NADPH oxidase, the presence of $O_2^{\cdot -}$ -generating proteins in peroxisomes, cell-wall-bound H_2O_2 -generating peroxidases and amine oxidases in the apoplast imply that there are multiple sources of ROI (Allan and Flur, 1997; del Rio *et al.*, 2002; Kawano, 2003). In *Arabidopsis* and potato $O_2^{\cdot -}$ accumulation is detectable and has been directly related to cell death signalling (Jabs *et al.*, 1996; Doke *et al.*, 1996). However, in tobacco and tomato, there is no detectable

production of $O_2^{\cdot -}$, although the use of diphenylene iodonium (an inhibitor of $O_2^{\cdot -}$ production) greatly reduced H_2O_2 accumulation and HR lesion development (Schraudner *et al.*, 1998; Wohlgenuth *et al.*, 2002). Nevertheless, whether $O_2^{\cdot -}$ or H_2O_2 accumulation is most responsible for PCD signalling remains unclear (Overmyer *et al.*, 2003).

Hydrogen peroxide (H_2O_2) is produced by the dismutation of $O_2^{\cdot -}$ by the extracellular superoxide dismutase (SOD) (Scheel, 1998) and, unlike $O_2^{\cdot -}$, can diffuse into the cell through membranes and its transport from cell-to-cell may even be facilitated by water channels (Henzler and Steudle 2000). Although H_2O_2 is also directly toxic to pathogens, experimental evidence insinuates that it has more significant roles in triggering the HR (Dat *et al.*, 2003), inducing gene expression (Vandenabeele *et al.*, 2003; Hein *et al.*, 2004) and initiating strengthening modifications to physical barriers (Grant and Loake, 2000; Neill *et al.*, 2002). Analysis of *Arabidopsis AtRbohD* and *AtRbohF* knockouts confirmed that NADPH oxidase is required for H_2O_2 generation in the apoplast during incompatible interactions (Torres *et al.*, 2002). In addition, blocking H_2O_2 production using SOD inhibitors was shown to compromise HR-related cell death (Beers and McDowell, 2001). However, treating *Arabidopsis* suspension cells with H_2O_2 increased subsequent H_2O_2 production by mitochondria (Tiwari *et al.*, 2002). In another study, prolonged activation of MAPKs in *Arabidopsis* resulted in generation of intracellular H_2O_2 preceding cell death (Ren *et al.*, 2002). In addition, a recently identified *Arabidopsis* serine/threonine kinase (OXI1) has been shown to be activated by H_2O_2 and required for full activation of MAPK3 and MAPK6 after treatment with ROIs or elicitor (Rentel *et al.*, 2004). It is therefore possible that increases in intracellular H_2O_2 concentrations may activate MAPKs that can stimulate the mitochondria to produce more H_2O_2 in order to amplify PCD (Neill *et al.*, 2002). While Ca^{2+} binding domains were found within NADPH oxidase, there is also evidence that H_2O_2 activates Ca^{2+} channels in the plasma membrane (Neill *et al.*, 2002) and therefore the chronological order of such transient signalling events is proving difficult to elucidate.

Table 1.4.3: Cellular location of ROI-producing enzymes

Location	Mechanism	Primary ROI	Ref
Chloroplast	Photosynthesis El.T and PSI or II	O_2^-	Takahashi and Asada, 1988
Mitochondria	Respiration El.T	O_2^-	Dat <i>et al.</i> , 2003
Peroxisome	Glycolate oxidase	H_2O_2	Corpas <i>et al.</i> , 2001
	Fatty acid β -oxidation	H_2O_2	
	Xanthine oxidase	O_2^-	
Plasma membrane	NADPH Oxidase	O_2^-	Grant and Loake, 2000
Apoplast	Oxalate oxidase	H_2O_2	Dat <i>et al.</i> , 2003
	Amine oxidase	H_2O_2	Allan and Fluhr, 1997
All of Above	Superoxide dismutase	$O_2^- \rightarrow H_2O_2$	Bowler <i>et al.</i> , 1992
Cell Wall	Peroxidases	H_2O_2	Hammond-Kosack and Jones, 1996

Table describes the subcellular locations of ROI production adapted from Mittler, (2002). El.T, electron transport and PS, photosystem.

Tight control of these powerfully cytotoxic compounds is required to avoid unnecessary damage by ROI signalling and, therefore, plants possess a number of antioxidant mechanisms, both enzymatic and non-enzymatic, to remove ROI from the cell (Mittler, 2002). Plant cells require molecules that scavenge low levels of ROIs used for signalling and also proteins that can detoxify excess ROIs (Mittler, 2002). The H_2O_2 scavenging ascorbate peroxidase and glutathione peroxidase found in most subcellular compartments, together with the peroxisome-specific catalase, are thought to be the most important enzymes for controlling levels of ROIs (Mittler, 2002). In addition, antioxidants such as ascorbic acid and glutathione are fundamental in defence against ROIs and NO causing mutants deficient in their accumulation to be hypersensitive to stress-inducing conditions (Conklin *et al.*, 1996; Creissen *et al.*, 1999). Although high concentrations of H_2O_2 induce cell death and potentiate its own production, lower H_2O_2 concentrations have been shown to activate antioxidant enzymes e.g. glutathione *S*-transferase (GST), via a MAPK signalling cascade at low concentrations (Desikan *et al.*, 1999; Baxter-Burrell *et al.*, 2002). Dependence on concentration for downstream signalling may be responsible for the clear delineation of dead cells from healthy cells characteristic of HR. Many of the signalling pathways regulated by H_2O_2 production have also been shown to require nitric oxide (NO), especially for PCD (Delledonne *et*

al., 2001). There seems to be a delicate equilibrium between ROIs, NO and other intermediates which interact with each other and/or activate other signalling cascades for induction of the appropriate defence responses (Laloi *et al.*, 2004). The literature reviewed in this section has been summarised below in Figure 1.4.3.

1.4.4. Nitric Oxide

Nitric oxide (NO) is a gas that has been well characterized as a mammalian signalling molecule with a range of physiological effects such as immune regulation (Wendehenne *et al.*, 2001). NO production has been reported during plant growth and metabolism, biotic and abiotic stresses and shown to induce defence responses (Delledonne *et al.*, 1998; Beligni and Lamattina, 2000). NO can be produced non-enzymatically from NO_2^- by a reducing agents such as ascorbate under acidic conditions or by carotenoids in the presence of light (Wojtaszek, 2000). In addition, NO can be synthesised enzymatically by nitrate reductase (NR) which is a key enzyme that catalyses the reduction of nitrite to NO from the previous product of nitrogen assimilation also from NR (Dean and Harper, 1988). NO production, akin to ROI production, is biphasic, producing peaks of accumulation at 1-2 hrs and again 3-6 hrs post-inoculation with *P. syringae* (Delledonne *et al.*, 1998; Clarke *et al.*, 2000). In mammals the main source of NO exists as three isoforms of NO synthase in different tissue types, neural (nNOS), endothelial (eNOS) and in macrophages (iNOS) (Bogdan *et al.*, 2000). The activity of nNOS and eNOS has been shown to be regulated by the availability of intracellular free Ca^{2+} (Nathan and Xie, 1994). The source of NO in plant defence responses has been debated for many years, accompanied by determined searches for plant orthologues of mammalian NOS sustained by experimental evidence which suggested that an NOS-like enzyme may exist. For example, the cryptogein-triggered NO burst in tobacco was shown to be sensitive to NOS inhibitors and an NOS-like enzyme was detected in the matrix of most plastids by antibodies raised to the mammalian NOS (del Rio *et al.*, 2002). Recent identification of NOS-like enzymes in tobacco, tomato and *Arabidopsis* reveal that plants, like animals, deploy enzymes with varied NO-producing roles (Chandok *et al.*, 2003; Guo *et al.*, 2003). Also, S-nitrosoglutathione (GSNO), which is central to signal

transduction and host defences, is thought to act as an NO reservoir which releases NO after reaction with Cu^{2+} (Gorren *et al.*, 1996; Mayer and Andrew, 1998; Liu *et al.*, 2004a). In animals, GSNO has been shown to have a pro-apoptotic function depending on the concentration and cell type involved (Messmer *et al.*, 1995). Glutathione-dependent formaldehyde dehydrogenase (GS-FDH) has high affinity for GSNO, regulating GSNO and subsequently NO levels in the cell (Liu *et al.*, 2001).

The cellular concentration of NO in mammals, akin to H_2O_2 , has differential effects on downstream signalling pathways, high concentrations being pro-apoptotic and low concentrations being anti-apoptotic (Bogdan *et al.*, 2000). In mammals, NO protects against apoptosis by inhibiting the subfamily of cysteine proteases, caspases (Kim *et al.*, 1997; Li *et al.*, 1997b). In particular, NO was found to S-nitrosylate the active site of one of the major executing caspases, caspase-3 (Rossig, *et al.*, 1999). In plants, there is accumulating evidence supporting presence of enzymes with caspase-like activity and the inhibition of NO-induced cell death by treatment of *Arabidopsis* with caspase-1 inhibitor (del Pozo and Lam, 1998; Clarke *et al.*, 2000; Chichkova *et al.*, 2004). In addition, NO has been found to strongly induce the expression of the *Arabidopsis* cysteine protease *AtCYS1* consequently blocking PCD. This provides evidence for NO's role as a cell death regulator rather than being directly cytotoxic (Beligni *et al.*, 2002). The pro-apoptotic effect of high concentrations of NO in mammalian macrophages has been well documented (Nathan *et al.*, 1994; Messmer *et al.*, 1996). In macrophages it is apparent that NO triggers cell death by inducing cytochrome C release from the mitochondrial membrane (Brookes *et al.*, 2000) and also reversibly inhibits ATP-generating complexes at the mitochondrial membrane (Drapier *et al.*, 1988). Interestingly, the treatment of carrot cells with SNP led to a reduction in respiration, a decrease in membrane potential and release of cytochrome C, signifying a similar role for NO in plants (Zottini *et al.*, 2002). Like H_2O_2 , NO activates a MAPK that transduces extracellular signals that modulate target gene expression through a signalling cascade, although blocking this MAPK with a specific inhibitor was not found to block NO-induced cell death (Clarke *et al.*, 2000). Additionally, NO was found to increase in cGMP levels that may induce defence gene expression e.g. *PAL1* (Dumer *et al.*, 1998).

However, cGMP treatment alone is not sufficient to induce HR (Wendehenne *et al.*, 2001).

1.4.5. Interplay of ROI and NO

NO and ROIs appear to interact in cells to act in both synergy and antagonism. NO acts synergistically with ROI to potentiate PCD by their reacting together to produce the highly toxic peroxynitrite (ONOO⁻), a known mediator of cell injury in many animal cells e.g. macrophages (Lin *et al.*, 1995). In addition, the treatment of *Arabidopsis* leaves with ONOO⁻ scavenger was found to reduce HR triggered by avirulent *P. syringae*, suggesting that ONOO⁻ is also required for PCD in plants (Alamillo and Garcia-Olmedo, 2001). However, exposure of soybean suspension cells to high concentrations of an ONOO⁻ donor did not induce cell death (Delledonne *et al.*, 2001). An alternative action of NO during plant PCD is its synergistic interaction with H₂O₂. In animals, NO's interaction with H₂O₂ has not been found to be directly cytotoxic thus far, although the hydroxyl radical and singlet oxygen products are both highly reactive and cytotoxic (Delledonne *et al.*, 2003). Other experiments have provided evidence that high levels of NO are not toxic unless accompanied by high concentrations of H₂O₂, suggestive of PCD only being induced by the balanced presence of NO and H₂O₂ (Clarke *et al.*, 2000; Delledonne *et al.*, 2001). Conversely, NO production visualised using an NO stain appeared at approximately the same time as PCD in *Pst* DC3000 and therefore NO may not be required for cell death but could contribute to the HR cell-to-cell signal (Zhang *et al.*, 2003). The literature discussed above is summarised in Figure 1.4.5

The correlation between inducible bursts of reactive oxygen and nitrogen species and the subsequent HR is evident, although their exact cellular functions and consequences of their interactions remain undefined. However, the conservation of many key mechanisms with the animal kingdom stimulates intense investigation in this area, which has been shown to be continuously fruitful.

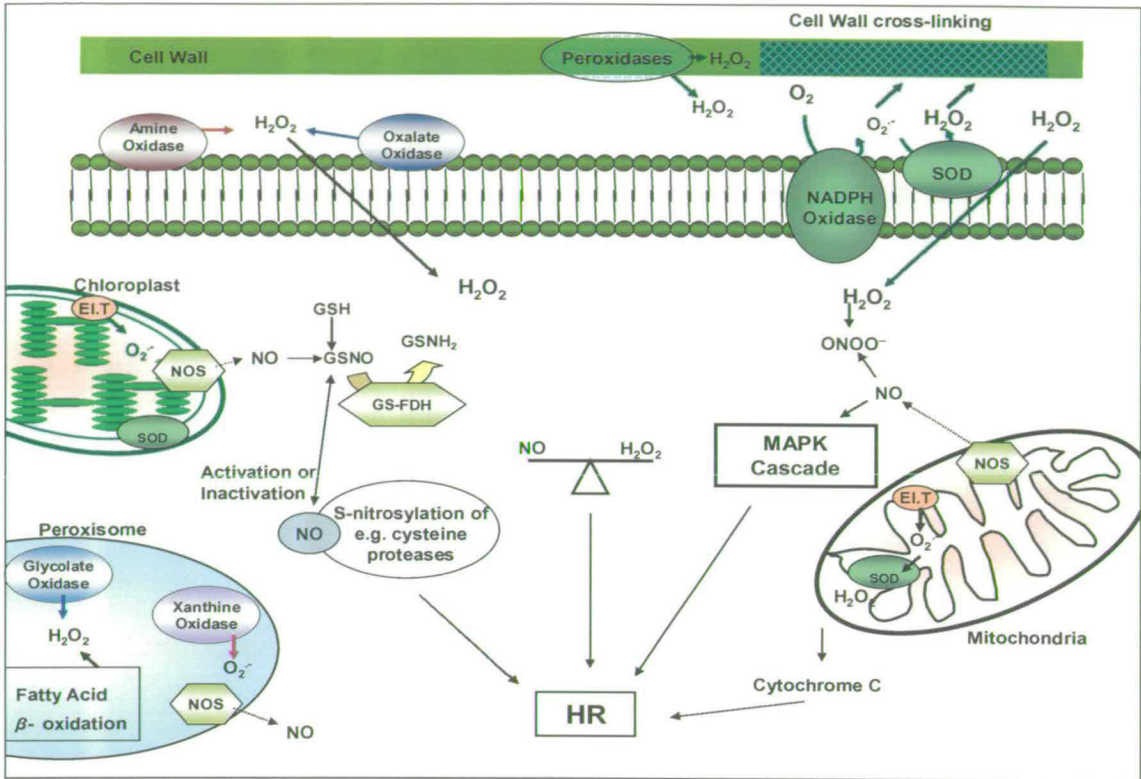


Figure 1.4.5: Synthesis of ROI and NO post-pathogen recognition. Diagrammatical representation of the current literature discussed throughout section 1.4 describing synthesis and interplay of ROI and NO that may induce the HR. ELT, Electron Transport; GS-FDH, Glutathione-dependent formaldehyde dehydrogenase; NOS, Nitric oxide synthase; SOD, Superoxide dismutase.

1.5. Hypersensitive Response

1.5.1. Role of Hypersensitive Response

The HR is almost entirely associated with *R* gene-triggered defence responses and is thought to restrict an avirulent biotrophic pathogen to the site of attempted infection by destroying its nutrient source before it has energy to proliferate (Zhang *et al.*, 2004). The HR is commonly associated with downstream defence gene expression, distinguishing it from developmental and senescence-related PCD (Heath, 2000a). Nonetheless, an *Arabidopsis* defence, no death (*dnd1*) mutant, fails to establish an HR in response to avirulent *P. syringae* but retains downstream defence gene expression and restriction of avirulent pathogen growth (Yu *et al.*, 1998; Clough *et al.*, 2000). This suggests that the HR is a subsidiary form of protection against avirulent pathogens rather

than being sufficient or necessary (Yu *et al.*, 1998). The HR maybe linked to avirulent pathogen recognition because it consists of bursts of cytotoxic signalling molecules that injure both host and pathogen cells causing a necrotic form of cell death. However, the HR has been shown to be a form of PCD which requires the cell to be metabolically and transcriptionally active to proceed and is therefore not a form of necrotic cell death (Greenberg and Yao, 2004). This is supported by the discovery of *Arabidopsis* mutants that possess a vigorous and falsely initiating HR, verifying that HR is genetically controlled (Delaney, 1997). These mutated genes are commonly classified as either lesion stimulating disease response (*lsd*) or accelerated cell death (*acd*) mutants (Weymann *et al.*, 1995; Delaney, 1997). LSD1 has been shown to be required for the activation of CuZn superoxide dismutases in response to SA accumulation (Kliebenstein, *et al.*, 1999). Thus the runaway cell death phenotype in *lsd1* may be manifested by accumulation of $O_2^{\cdot -}$ to a critical threshold concentration, due to a reduction in CuZnSOD activity.

The HR is generally associated with *R* gene-dependent resistance but is also triggered during non-specific resistance (Baker *et al.*, 1997; Heath, 2000b; Dangl and Jones, 2001). HR in pathogen-specific elicitation is not rigorously linked to defence gene expression and typically HR-inducible defence responses can occur in the absence of PCD (Heath, 2000b). *Rx*-dependent resistance in potato, with *Rx* coding for a LZ-NBS-LRR type resistance gene against potato virus X (PVX) is one example of *R* gene-mediated resistance that is not associated with the HR and is referred to as extreme resistance (Shirasu and Schulze-Lefert, 2000). Barley plants, expressing *Mlg*-based resistance toward *B. graminis* f.sp *hordei* represents a second example for *R* gene-mediated resistance not dependent on HR to arrest the spread of the fungus (Görg *et al.*, 1993). Contrary to abundant evidence demonstrating that the HR is a vital component in protection against biotrophic pathogens, recent data suggests that PCD may in fact enhance the ability of necrotrophic pathogens to colonise plant tissue. Growth of the necrotrophic fungal pathogen *Botrytis cinerea* increased when co-inoculated with an avirulent strain of the biotrophic bacterium *P. syringae*. This effect was not observed when the fungus was co-inoculated with *hrp*⁻ strains that are unable to elicit an HR

(Govrin & Levine, 2000). Moreover, *B. cinerea* growth was also suppressed in the HR-deficient *nd1* (Yu *et al.*, 1998), suggesting that cell death may be beneficial to necrotrophs that may molecularly encourage the HR because dead plant tissue is the preferred nutrient source (Govrin & Levine, 2000).

The HR also plays an early warning role for the rest of the plant by releasing signals such as H₂O₂ that prime adjacent cells to respond to pathogen elicitors, inducing an increase in antioxidant gene expression such as *GST1* and also through production of salicylic acid (SA) that activates systemic resistance (SAR) throughout the entire plant (Heath, 2000a,b).

1.5.2. Conservation of Cell Death Regulators

Mechanisms of animal PCD have been studied extensively for many years. However, the first connection between the PCD mechanism and the HR in plants was made relatively recently and only studied in greater detail upon the discovery of *Arabidopsis* lesion mimic mutants (Slusarenko and Longland, 1986; Dietrich *et al.*, 1994; Greenberg, 2005). Experimental evidence from mammalian PCD has provided an indication of the types of processes and proteins expected to be involved in the HR (Dickman *et al.*, 2001). Mitochondria are of particular importance during mammalian PCD (Lam *et al.*, 2001). An alternative oxidase (AOX) that produces ROIs from electron-transfer intermediates has been identified in plant mitochondria and transgenic plant cells lacking this protein have increased susceptibility to mitochondria-dependent and -independent PCD (Robson and Vanlerberghe, 2002). In both systems, DNA laddering, caspase-like proteolytic activity and cytochrome C release from the mitochondria have been described (Richberg *et al.*, 1998). However, HR is additionally associated with autofluorescence of cell walls and cell contents, halt in cytoplasmic streaming, loss of cortical microtubules and the appearance of particles in the vacuole (Heath, 2000a). In both systems, PCD is triggered by a variety of stress-inducing conditions or receptor activation events which induce production of death-promoting stimuli such as ROIs (Jabs, 1999). Whether ROIs are the primary trigger or secondary mediator of PCD, a cell's commitment to apoptosis in both systems has been shown to depend on the extent

of accumulation or inability to scavenge or detoxify ROIs (Levine *et al.*, 1994; Verhaegen *et al.*, 1995; Jabs *et al.*, 1996). Phospholipase D (PLD) derived product, phosphatidic acid (PA) has been shown to activate the $O_2^{\cdot -}$ generating neutrophil NADPH oxidase (Gomez-Cambronero and Keire, 1998). In parallel, depletion of the most prevalent isoform of PLD in *Arabidopsis* resulted in decreased levels of PA and $O_2^{\cdot -}$ in leaf extracts (Sang *et al.*, 2001). In addition, PLD has been identified in rice in response to fungal infection and has also been shown to be activated in tomato and potato (Dyer *et al.*, 1996). Interestingly, phospholipase-like enzymes, EDS1 and PAD4, which are required for TIR-NBS-LRR-related activation of the oxidative burst, may modulate activity of NADPH oxidase (Yun *et al.*, 2003).

In mammalian PCD, activation of “death receptors” such as the CED-4/Apaf-1 family activate pro-apoptotic members of the Bcl-2 protein family which contains both pro- and anti-apoptotic members (Cecconi, 1999). The anti-apoptotic protein, Bcl-2, was demonstrated to inhibit PCD via protection from oxidative stress and from necrotic cell death triggered by glutathione depletion (Kane *et al.*, 1993). Anti-apoptotic members have also been shown to suppress Apaf-1 activity, stabilizing the permeability transition pores in the mitochondrial membrane preventing leakage of the cell death activator cytochrome C into the cytosol (Joza *et al.*, 2002). As a general rule during mammalian apoptosis, cytochrome C leakage stimulates the formation of the mitochondrial “apoptosome” which consists of cytochrome C, APAF1 and the initiator caspase, caspase-9, inducing the cascade that activates downstream effector caspases (Joza *et al.*, 2002) (Figure 1.5.2). Other mammalian initiator caspases (caspase-1, -4 and -5) have been linked to apoptosis although their prime function is the regulation of inflammation (Martinon and Tschopp, 2004). In plants, cytochrome C release from the mitochondria into the cytoplasm has been connected to the HR and used as a marker of plant PCD (Gilroy *et al.*, *under submission*; Greenberg and Yao, 2004). Intriguingly, transgenic tobacco plants expressing the anti-apoptotic human *Bcl-2* and *Bcl-xl*, nematode *CED-9* or baculovirus *Op-IAP* exhibit resistance to several necrotrophic fungal pathogens (Dickman *et al.*, 2001). In addition, Bax, a death-promoting member of the Bcl-2 family analogously triggers cell death in tobacco plants implying that there may be conserved

molecular interactors (Lacomme and Santa Cruz, 1999). Furthermore, *Arabidopsis* and barley homologs of the anti-apoptotic Bax inhibitor-1 (*BI-1*) have been identified and shown not only to impair the HR but also Bax-induced apoptosis in a human cell line (Kawai-Yamanda *et al.*, 2001; Huckelhoven *et al.*, 2003; Bolduc *et al.*, 2003). In *Arabidopsis* and tobacco, BI-1 has been characterised further revealing its suppression of HR induced by endogenous plant signalling molecules, H₂O₂ and SA (Kawai-Yamada *et al.*, 2004).

Bcl-2 family members have been proposed to be the link between cell death receptors such as Apaf-1 and caspases that proteolytically dismantle the cell through the apoptosome (Newton and Strasser, 1998). Interestingly, Apaf-1 shares an NB-ARC domain with NBS-LRR proteins e.g. RPS2 and RPS5 (Shirasu and Schulze-Lefert, 2000). Although the C-terminus of Apaf-1 contains a caspase recruitment domain (CARD) in the region where the NBS-LRR CC or TIR domains are found, a particular CED-4/Apaf-1 family member, CARD4, also possesses a strikingly similar LRR domain in addition to the NB-ARC domain, strengthening the possibility of similar functions (Shirasu and Schulze-Lefert, 2000). Furthermore, a tomato cysteine protease (Rcr3) has been shown to be required for function of the R protein, Cf-2 (Rooney *et al.*, 2005; Krüger *et al.*, 2002). Fifteen caspases (C14 family of cysteine proteases) have been identified in mammals which, upon recruitment by pro-apoptotic Bcl-2 proteins, form a cell death initiation cascade. However, sequence homologues of caspases are not found in plants (Lam *et al.*, 2001). Nevertheless, the *Arabidopsis* genome contains 488 protease genes and so it seems inevitable that one or more could be responsible for the caspase-like activity that has been identified during the HR through biochemical analysis (van der Hoorn and Jones, 2004). In addition, caspase-like cleavage of the virD2 protein has been demonstrated during the HR to TMV in tobacco which could be blocked by a caspase inhibitor (Chichkova *et al.*, 2004). In tomato, cysteine proteases with distant homology to caspases, deemed metacaspases, have been identified and are rapidly up-regulated during necrotrophic fungal infection, although not during chemical-induced PCD (Hoeberichts *et al.*, 2003). However, there are two major classes of plant proteases that have been shown to be involved in the HR, subtilisin-like (S8) and papain-like (C1)

(Beers *et al.*, 2004). For example, the serine protease inhibitor, AEBSF, has been shown to obstruct cell death in response to H₂O₂ (Levine *et al.*, 1994) and cystatin (a cysteine protease inhibitor) impedes the HR in response to H₂O₂ and many avirulent *Pst* strains (D'Silva *et al.*, 1998; Solomon *et al.*, 1999). Furthermore, a cysteine protease, cathepsin B, involved in caspase-independent lysosomal cell death has been shown to be up-regulated early during HR induced by *P. infestans* in potato (Stoka *et al.*, 2001; Avrova *et al.*, 2004). It is also interesting to note that some biotrophic pathogens e.g. *P. infestans* and *P. syringae* have been shown to secrete serine and cysteine protease inhibitors highlighting the importance of proteases in defence and the evolutionary pressure for biotrophic pathogens to evade the HR (Tian *et al.*, 2004; van der Hooft and Jones, 2004).

Inhibiting cysteine proteases also affects downstream expression of specific protease inhibitor proteins that are also regulated by intracellular signals such as SA, ET and jasmonic acid (JA) (Rojo *et al.*, 2004). In addition, mutants with perturbed HR are often unable to induce appropriate downstream defence gene expression. Moreover, the HR suppressing JA- and ET- pathways indicates that other signalling molecules are also intricately involved in defence activation (Solomon *et al.*, 1999). The HR can therefore be summarized as a genetically controlled dismantling of the cell, dependent on an undetermined balance of *R* gene triggered signals, which vary between plant-parasite systems (Lam *et al.*, 1999).

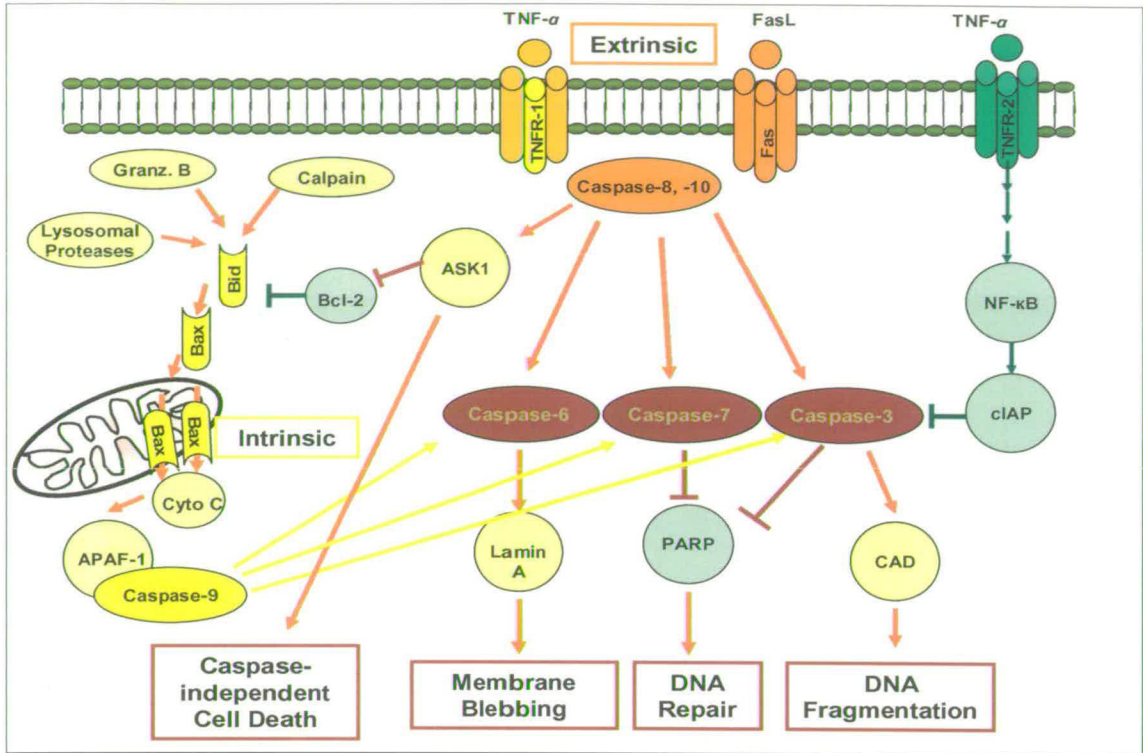


Figure 1.5.2: Basic overview of mammalian caspase cascades in PCD. The extrinsic pathway involves the sensing of ligands by death receptors, that subsequently activates initiator caspases-8 and -10 which then activate caspase-6, caspase-7 and caspase-8. Effector caspases activate or repress cellular target proteins which results in proteolytic dismantling of the cell. An intrinsic cell death pathway involves the Bax-induced release of cytochrome C from mitochondria, which activates APAF-1 which interacts with and activates initiator caspase-9. Caspase-9, like caspase-8 and caspase-10 activates the effector caspases which dismantle the cell. Lysosomal proteases, granzyme B and calpain have also been shown to activate Bax through Bid. Antiapoptotic protein Bcl-2 suppresses the activation of Bax. In addition, a parallel TNF- α -induced antiapoptotic pathway has been identified that involves NF- κ B and is thought to exist in equilibrium with pro-apoptotic pathways. Perception of TNF- α by the cell is likened to a life-death switch that involves a plethora of interacting proteins which can tip the balance. Figure constructed as a summary of relevant literature summarised in 1.5.2 and 4.1.

1.6. Salicylic Acid-dependent Pathway

SA accumulation was originally measured long after the oxidative burst and presumed to be exclusively for induction of defence gene activation that is characteristic of SAR (Dempsey *et al.*, 1999). However, a small transient rise in SA was observed in tobacco plants which overlapped with the second phase of the oxidative burst and occurred prior to the sustained increase in SA (Draper, 1997). Therefore, SA is hypothesised to amplify the oxidative burst, which in turn may drive further SA production in a feed-

back loop that may potentiate the HR in a manner termed the oxidative cell death (OCD) cycle (Draper, 1997; Van Camp *et al.*, 1998). Intriguingly, depletion of SA causes a breakdown of *R* gene-dependent resistance, affecting the ability of the plant to induce an effective HR. Inoculation of *nahG Arabidopsis* (transgenic containing bacterial SA metabolising gene, salicylate hydroxylase, or *nahG*) with an incompatible isolate of *P. parasitica*, leads to the development of severe disease symptoms, whereas wild-type plants are unaffected (Delaney *et al.* 1994). In addition, SA has been shown to inhibit a catalase responsible for the metabolism of H₂O₂, subsequently amplifying ROI-induced responses (Delaney, 1997; Lamb and Dixon, 1997). The treatment of soybean cells with 50 µM SA demonstrated further that SA may regulate accumulation of H₂O₂, induce expression of HR marker genes phenylalanine ammonia-lyase (*PAL*) and glutathione *S*-transferase (*GST*), consequently amplifying HR-related cell death following infection by *P. syringae* pv *glycinae* (Shirasu *et al.*, 1997).

Systemic acquired resistance (SAR) is an inducible defence response that induces the expression of characteristic defence genes (*PR* genes) in the immediate vicinity of the infection site to provide broad spectrum resistance. Concurrently, an unidentified systemic signal is released that establishes, via gene expression, the primed state of defence in uninfected tissues. The importance of SA in this pathway has been shown by studying the block in *PR* gene expression in transgenic *NahG* tobacco plants (Friedrich *et al.*, 1995). However, grafting experiments have shown that SA is not the primary mobile signal exported to uninfected tissues to establish SAR (Vernooij *et al.*, 1994). Dissection of genetic pathways establishing SAR has mainly relied on phenotypic and molecular analysis of *Arabidopsis* mutants (Buell, 1998). In addition to their proposed role in local, *R* gene-mediated resistance, the oxidative burst and cognate redox signalling may also play a pivotal function in the establishment of systemic acquired resistance (SAR). This was highlighted by an elegant series of experiments in transgenic tobacco plants which contained an anti-sense catalase gene (Chamnonpol *et al.*, 1998). Exposure of these plants to high light levels for two days resulted in visible necrosis and induced PR proteins in light shielded local and systemic leaf tissues. In contrast, exposure to high light levels for 4 hours induced PR proteins in local light

shielded but not in systemic tissue, in the absence of necrosis. Thus the ROI-mediated activation of SAR genes could be uncoupled from cell death in local tissues. However, local ROI-mediated cell death was necessary for the accumulation of PR proteins in systemic tissue.

A large number of SA signalling mutants have been isolated and characterised to date (Lorrain *et al.*, 2003). Among the initial mutants isolated, *cpr1* (constitutive pathogen related) plants exhibited a dwarf phenotype, constitutive expression of several SA-inducible defence genes and enhanced disease resistance. However, the gene responsible has not been identified (Bowling *et al.*, 1994).

Downstream of EDS1 and NDR1 (section 1.4.1.1), *SID1* and *SID2* (SA-induction deficient) genes control SA biosynthesis by encoding isochorismate synthase (ICS) that synthesises SA from chorismate (Wildermuth *et al.*, 2001). In *Arabidopsis*, *sid2* mutants could not induce SAR when challenged with an avirulent pathogen (Glazebrook, 2001). The *npr1* (non-expresser of *PR* genes) mutant was identified as a mutant in 1997 because it failed to express genes fused to an SA-sensitive promoter after SA treatment (Cao *et al.*, 1997). Unusually, *NPR1* is constitutively expressed and only increases two-fold upon treatment with SA (Dong, 2004). The *Arabidopsis npr1* mutant can accumulate SA, although it cannot establish SAR and is therefore unable to express *PR* genes and does not inhibit the growth of avirulent or virulent pathogen strains e.g. *P. syringae*. *NPR1* has been shown to localise in the nucleus in response to elevated levels of SA (Glazebrook, 2001) and uses its ankyrin repeats to bind TGA transcription factors that, once activated, induce expression of genes containing GNT35 promoters such as *GST* and *PR-1* (Niggeweg *et al.*, 2000). *NPR1* is therefore presumed to have a role in amplifying *PR* gene expression (Glazebrook, 2001). Mutant alleles of *npr1* (around 12) have been repeatedly isolated from independent loss-of-function *Arabidopsis* screens, indicating that additional genes responding to SA may be embryonic lethal or functionally redundant (Cao *et al.*, 1997). *NPR1* can be repressed indirectly by *SN11* (suppressor of *NPR1* inducible) as homozygous *sn11 npr1-1* double mutants display a nearly wild type phenotype, rescuing SA induction of *PR-1* and resistance to biotrophic

pathogens. This reveals the presence of other SA-inducible, NPR1-independent genes or pathways (Delaney, 2000). On the other hand, WRKY transcription factors, that bind W-boxes, have been shown to repress the SA-inducible gene *PR-1* (Lebel *et al.*, 1998). However, overexpression of *WRKY70* leads to constitutive expression of *PR-1* (Li *et al.*, 2004). In addition, NPR1 has been associated with the inhibition of JA-induced responses as shown by repression of *PDF1.2* transcription in plants over-expressing *WRKY70* (Glazebrook *et al.*, 2003). Conversely, NPR1 has been shown to function downstream of JA and ET in non-pathogenic rhizobacteria-mediated induced systemic resistance (ISR) (van Wees *et al.*, 2000). Therefore, SAR and ISR pathways may activate a parallel set of signalling proteins. However, downstream induction of a variety of SA-dependent transcription factors can act synergistically with, but mostly antagonistically against, the JA- and ET-dependent induction of defence genes (Figure 1.7).

1.7. *Jasmonate and Ethylene*

1.7.1. Jasmonate-dependent Signalling

Jasmonic acid (JA) is a lipid-derived signalling molecule involved in growth inhibition, pollen and seed development, senescence, tuber formation and wound-inducible defences (Doares *et al.*, 1995; Creelman and Mullet, 1997). The wound-inducible biosynthesis of JA has been shown to require lipid oxidation mediated by either ROIs or lipoxygenases (LOX) that catalyze the addition of molecular oxygen to polyunsaturated fatty acids to form volatile, short-chain aldehydes (Martin *et al.*, 1999). Several inducible LOX isoforms have been identified that possess different tissue-specific expression and time course patterns, suggesting varying roles in JA synthesis (Royo *et al.*, 1999). Genes involved in biosynthesis of JA have been identified, e.g. fatty acid desaturase triple mutants (*fad3/fad7/fad8*) that cannot produce linolenic acid (Kunkel and Brooks, 2002). Other genes important for JA signalling have been identified through mutant screens for jasmonate or coronatine insensitivity, *coil* (coronatine insensitive) and *jar1* (jasmonic acid resistant) or for altered JA-regulated gene expression such as jasmonate underexpressing mutants (*jue1*, *jue2* and *jue3*) (Feys *et al.*,

1994; Staswick *et al.*, 2002; Jensen *et al.*, 2002). COI1 is an F-box protein that may inactivate negative regulators of JA-dependent defences or target proteins for ubiquitination (Glazebrook, 2001; Kunhel and Brooks, 2002). Mutations in JA-dependent genes increase susceptibility to necrotrophic pathogens e.g. *Alternaria brassicicola* or *B. cinerea* (Dong, 1998). Additional JA-induced genes, *MPK4* (Mitogen-activated protein kinase 4) and *SSI1-2* (suppressor of SA insensitivity), are shown to negatively regulate the SA-dependent pathways (Petersen *et al.*, 2000; Shah *et al.*, 1997). JA, like SA, also induces a subset of *PR* genes including *PR-3*, *PR-4* and *PDF1.2* (*PR-12*) (Thomma *et al.*, 2001; Kunkel and Brooks, 2002). Comparisons between the potato *Pin2* and the soybean *VspB* genes have revealed a JA-inducible G-box element (TGACG-related) (Rouster *et al.*, 1997). Intriguingly, a JA-regulated terpenoid indole alkaloid (TIA) biosynthesis gene which possesses a GCC-box element in its promoter is also recognised by the ET-related transcription factor, ERF1 (Menke *et al.*, 1999). Induction of particular JA-inducible *PR* genes is associated with a form of broad spectrum, systemic resistance targeted more specifically for protection against insects and necrotrophic pathogens (Royo *et al.*, 1999). Recently, a MYC transcription factor has been found in *Arabidopsis* that, when mutated, conveys insensitivity to JA and decreased expression of JA-responsive genes *AtVSP* and *JRI* (Boter *et al.*, 2004). Interestingly, in addition to inducing expression of JA-responsive genes, this transcription factor may be involved in repressing genes induced by the combination of JA- and ET-dependent signalling (Boter *et al.*, 2004) (Figure 1.7).

1.7.2. Ethylene-dependent Signalling

ET is an important signalling hormone during plant developmental processes, such as seed germination, root nodulation and hair development, flower senescence and fruit ripening (Johnson and Ecker, 1998). In addition, ET signalling has been linked with defence responses to pathogen attack, abiotic stresses including wounding, ozone, chilling and freezing (Thomma *et al.*, 1999). ET production is initiated almost immediately following wounding, JA application or treatment with cell wall oligosaccharides (Dong, 1998). Several ethylene-resistant (*etr11* and *etr2*), ethylene

response sensor (*ers1* and *ers2*) and (ethylene insensitive) *ein1* and *ein2* *Arabidopsis* mutant genes have been identified as ethylene receptors due to their insensitivity to ethylene, linking these genes to ET-dependent signalling (Solano and Ecker, 1998). The first *Arabidopsis* gene cloned was *ETR1* which encodes a receptor associated with the endoplasmic reticulum (Hass *et al.*, 2004). Using *ETR1* as bait in a yeast-2-hybrid assay, the Ser/Thr protein kinase from the RAF MAPKKK family, *CTR1* was identified which also interacts with *ERS1*. In addition, *CTR1* has been shown to act as a negative regulator of ET responses in a cascade including *MPK6*, highlighting a link between these ET receptors and MAPKs (Ouaked *et al.*, 2003). *EIN2* is a membrane-associated protein with sequence similarities to the *Nramp* metal transporters and is thought to act downstream of the *CTR1*-regulated cascade (Dong, 1998). The *ein2* mutant displays increased susceptibility to necrotrophs e.g. *Erwinia carotovora* and decreased disease symptoms to biotrophs e.g. *Xanthomonas campestris* (Dong, 1998). There are two families of ethylene-dependent transcription factors, *EIN3*-like proteins and ethylene response element-binding proteins (EREBPs) (Guo and Ecker, 2004). Interestingly, *EIN3*-like transcription factors bind to a conserved *cis*-regulatory element present in the promoter of EREBP-encoding gene ethylene response factor 1 (*ERF1*) which in turn binds to secondary target genes of ET signalling (Solano and Ecker, 1998). In addition, ET appears to regulate a number of physiological and developmental processes that are also controlled by cytokinin indicating that ET is an important regulator in several pathways (Hass *et al.*, 2004) (Figure 1.7).

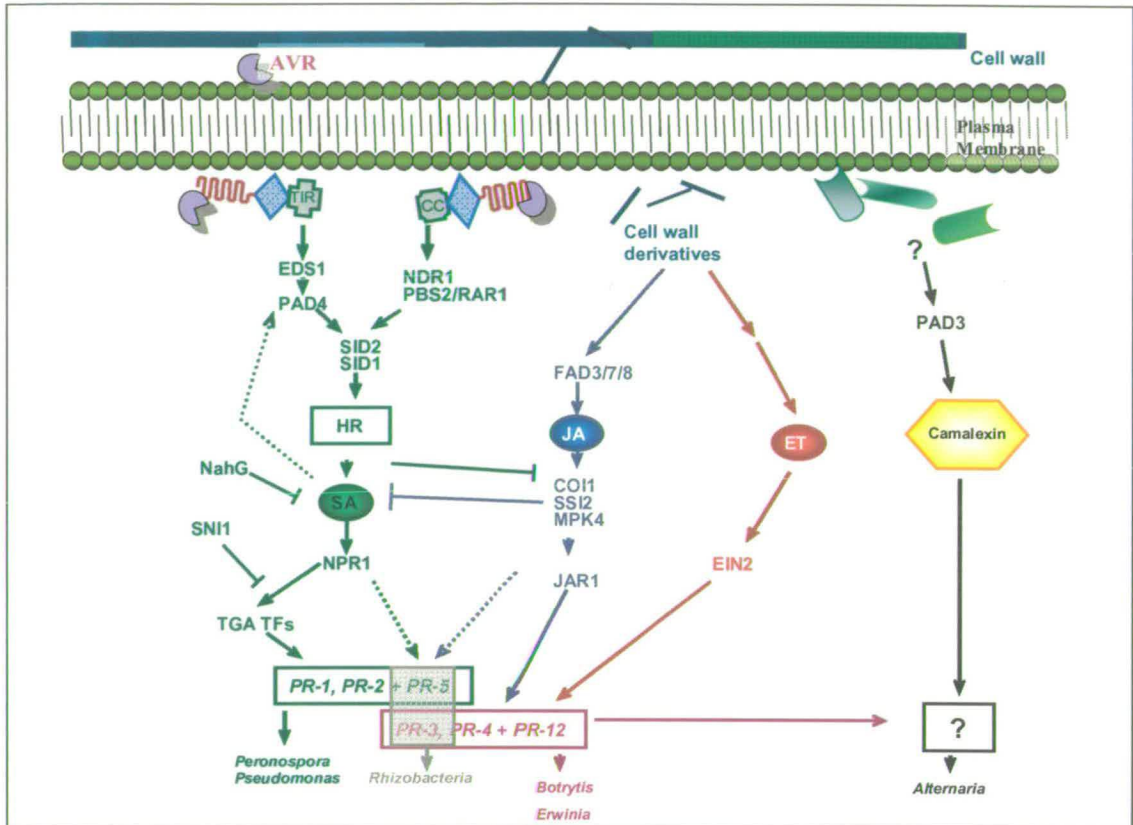


Figure 1.7: SA-, ET- and JA- Dependent Defence Responses. A schematic representation of SA- (green), JA- (blue) and ET- (red) pathways. The defence genes that are consequently activated and examples of pathogens that these genes defend against.

1.8. Signalling-Induced Changes to the Transcriptome

Even though SA-dependent and ET-JA-dependent signalling pathways induce expression of different *PR* genes and confer resistance against distinct pathogens, SA, JA and ET are not completely independent and many studies have found positive and negative interactions between the pathways (Feys and Parker, 2000; Wang *et al.*, 2002; Ton *et al.*, 2002). Microarray analysis of 2375 *A. thaliana* genes after pathogen, SA, Me-JA and ET treatment demonstrated that only a small number are solely regulated by one hormone and most respond to multiple pathways (Schenk *et al.*, 2000) (Figure 1.8). For example, expression and activation of the traditionally JA-associated *PDF1.2* in response to necrotrophic pathogens was shown to require the synergistic interaction between JA and ET but not SA (Feys and Parker, 2000). In addition, inhibition of ET

production has detrimental effects on the JA pathway (Dong, 1998). Microarray analysis has revealed that around half of the genes induced by ET are also induced by JA (Kunkel and Brooks, 2002). Together ET and JA induce defences against the necrotrophic *Botrytis* and *Erwinia* species (Thomma, 2001). Defects in the SA-dependent pathway typically result in enhanced resistance to necrotrophs (Thomma, 2001). Results from microarray analyses have also revealed that SA and ET may function together to induce several defence-related genes (Kunkel and Brooks, 2002). The majority of *PR* genes associated with SAR do not require ET, although it has been reported to enhance inducible *PR-1* expression in *Arabidopsis* (Dong, 1998). The same study also revealed that ET has a negative effect on the basal level of *PR-1* expression by studying *ein2* mutants (Dong, 1998).

The relationship between the SA- and JA-dependent pathways is generally antagonistic. SA appears to have an inhibitory effect on proteinase inhibitor genes that can be partially rescued by application of JA and ET (Dong, 1998). The mutants, *eds4* and *pad4* that cannot accumulate SA are more sensitive to JA. JA has been shown to repress SA signalling and expression of SA-dependent genes in tobacco (Kunkel and Brooks, 2002). *Arabidopsis* plants inoculated with the necrotroph *E. carotovora* resulted in inhibition of SA-dependent *PR* genes (Kunkel and Brooks, 2002). Studies involving the *Arabidopsis* mutants *mpk4* and *ssi2* showed that they possess a constitutively active SAR, constitutively expressing *PR-1*, *PR-2* and *PR-5* in a SA-dependent but NDR1-independent pathway. However, *ssi1* plants also express defensin *PDF1.2*, which is independent of SA and NDR1, but requires JA-ET-signalling (Penninckx *et al.*, 1996). The *edr1* mutant confers enhanced resistance to *P. syringae* in a SA-dependent and JA-ET-independent pathway (Frye *et al.*, 2001). However, pre-treatment of *Arabidopsis* with ET in the *edr1* background resulted in high expression of *PR-1*, which is SA-dependent compared to untreated plants. It is therefore likely that EDR1 is a repressor for SA-inducible defence responses and might be active in a wide range of crops, as EDR1 has been identified in monocots such as rice and barley (Frye *et al.*, 2001). The mutant *coil* expresses SA-inducible responses more rapidly than the wild-type. However, it remains hypersensitive to *P. syringae* (Kunkel and Brooks, 2002).

Concurrent induction of both SA- and JA-dependent pathways has been shown in several lesion mimic mutants e.g. *cpr5*, that exhibit constitutively high expression of both *PR-1* and *PDF1.2* (Glazebrook, 2001).

Microarray analysis identified over 50 genes co-regulated by SA and JA (Kunkel and Brooks, 2002). The mutant *pad3* (phytoalexin deficient) cannot produce camalexin, displays enhanced sensitivity to the necrotroph *A. brassicicola* and retains wild type responses to *P. syringae* (Feys and Parker, 2000). *PAD3* is not regulated by JA, ET or SA, indicating that there may be more signalling pathways to decipher and many more genes to fit into this complex signalling network (Thomma, 2001). It is clear from this information that plants have overlapping mechanisms for inducing defence pathways to suit encountered pathogens and the cross-talk between them may complicate attempts to engineer resistance to the most problematic pathogens.

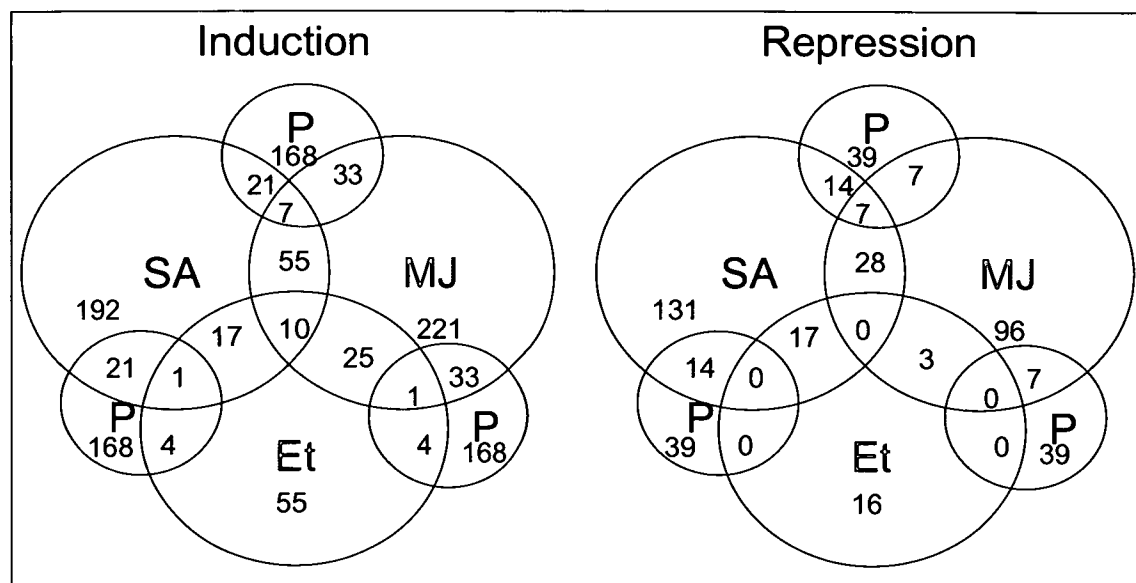


Figure 1.8: Microarray Analysis of *Arabidopsis* Genes Induced by Varying Treatments. Genes expressed and repressed during various treatments that activate the previously described defence pathways (Schenk *et al.*, 2000). The P represents the necrotrophic pathogen *A. brassicicola*. This diagram highlights the extent of overlap between SA, JA (MJ) and ET-responsive genes and the possibility that current representations of these signalling pathways may not be accurate.

1.9. *Potato and Potato Late Blight*

One of the most important crops in the UK is the potato (*Solanum tuberosum* spp *tuberosum* L.) but it is also the fourth most important crop worldwide after rice, wheat and maize (Gebhardt and Valkonen, 2001). It has a genome of around 900 Mb, 9 times larger than the *Arabidopsis* genome (Gebhardt, *personal communication*), consisting of a tetraploid set of 12 chromosomes, making rigorous genetical analyses more difficult than in *Arabidopsis* (Gebhardt and Valkonen, 2001). Most potato varieties are susceptible to one of the most destructive and historically significant pathogens, *Phytophthora infestans*, which caused the infamous potato famines of the mid-19th century (Ballvora *et al.*, 2002). Ireland lost more than 2 million inhabitants to starvation and emigration as a direct consequence of late blight, making *P. infestans* one of the most widely documented plant pathogens (Birch and Whisson, 2001). *Phytophthora* (meaning “plant destroyer”) as its Latin name suggests is an extremely aggressive pathogen that causes devastating necrotic disease symptoms. Little can be done to prevent its spread through certain *Solanaceous* species once it becomes established and it can only be controlled in a preventative manner through weather-dependent fungicide applications throughout the growing season (Birch and Whisson, 2001). In 1999, it was estimated that losses through damage by or controlling *P. infestans* exceeded \$5 billion annually (Duncan, 1999).

Historically, *P. infestans* has been grouped taxonomically with fungi, such as ascomycetes and basidiomycetes, due to its filamentous structure. Nevertheless, metabolic and rRNA analyses have shown that oomycetes such as *P. infestans* are more closely related to diatoms and brown-algae (Pfyffer *et al.*, 1990; Forster and Coffey, 1990; Cooke *et al.*, 2000). It is hypothesised, from isozymic studies of *P. infestans* isolates that a single clonal line migrated from Mexico to Europe in 1845 and through seed potato export spread to Africa and Asia causing global problems. The previous detection of only one mating type of *P. infestans* world-wide (A1) alarmingly changed in the 1980's when the A2 mating type escaped Central Mexico causing increased virulence and genetic variation of blight in nearly all potato-growing regions of the

world (Fry *et al.*, 1993; Goodwin *et al.*, 1994; Sujkowski *et al.*, 1994). The haploid A1 and A2 nuclei can fuse to develop into a thick walled oospore that can survive for many years in the soil and infect any tissues of the next potato crop that come into contact with the soil (Drenth *et al.*, 1995). The most highly effective fungicides used to date are phenylamides, e.g. metalaxyl, which are also applied with another fungicides to combat the increase in currently evolving phenylamide-resistant biotypes (Schwinn and Morton, 1990). Worryingly, 45 % of isolates of *P. infestans* from commercial fields in the Netherlands were found to be metalaxyl-resistant (Fry *et al.*, 1991). This has led to the ban of metalaxyl spraying in some seed producing countries in an attempt to limit the export of resistant biotypes. The combined appearance of this fungicide resistance and the A2 mating type has revived the serious international threat that this rapidly spreading and evolving pathogen poses to modern potato agricultural practices. Studies by Fry *et al.* (1993) and Fry and Goodwin (1997) confirm that *P. infestans* is highly variable and quickly adapts to distinct environments and hosts and the currently high frequency of unique biotypes resembles the genetic diversity that exists where A1 and A2 types coexist in Central Mexico (Fry *et al.*, 1992).

P. infestans has a specific host range that primarily infects foliage and fruits. It spreads predominantly via air-borne or water-splashed asexual sporangia that release motile zoospores in cool (16-21°C), damp, cloudy conditions that rapidly encyst and produce a germ tube. An appressorium develops, from which an infection peg emerges with power to penetrate epidermal cells. After only 3 days, hyphae spread throughout the growing lesion and emerge through stomata, forming sporangiophores that release the next generation of sporangia. Underground tubers may also be infected by sporangia or zoospores dripping off diseased leaves during periods of wet weather (Sato, 1979). *Phytophthora* species can display strong chemotaxis towards and rapidly encyst when in close proximity to its preferred host, highlighting germination as a control point in host specificity dependent on specific chemical or physical signals (Tyler, 2002).

P. infestans has a genome of 250 Mb (more than twice the size of *Arabidopsis*) and the presence of countless biotypes world-wide rules out a quick and efficient genome

sequencing project like other well known pathogens e.g. *Erwinia carotovora* ssp. *atroseptica* (Bell *et al.*, 2004). The development of low-cost, high throughput DNA sequencing has allowed approximately 2000-3000 ESTs from both *P. infestans* (Kamoun *et al.*, 1999) and *P. sojae* (Qutob *et al.*, 2000) to be obtained and funding has been granted for sequencing a further 41,000 *P. sojae* and 14,000 *P. infestans* ESTs. This EST data is stored in the *Phytophthora* Genome Initiative (PGI) database (Waugh *et al.*, 2000; <http://www.ncgr.org/pdi/index.html>). The identification of molecules that trigger or are affected by host recognition shall be facilitated by this EST information and shall also be complemented by the current physical and genetic mapping of the organism (Tyler, 2001).

Breeding race-specific resistance into potato cultivars was considered an effective and environmentally friendly approach to controlling potato blight as sporulation does not occur on potato cultivars that possess race-specific resistance. Some resistant potato cultivars were developed through introgression of single dominant *R* genes to the various *P. infestans* races from wild potato relatives e.g. *S. demissum*, indigenous to Mexico (Ballvora *et al.*, 2002) and were initially highly successful. However, *Phytophthora's* rapidly evolving nature has overcome all eleven *R* genes, foiling the scientists and breeders' efforts (Ballvora *et al.*, 2002). Lipoxygenases are enzymes involved in the biosynthesis of oxylipins that play an important signalling role during wounding and necrotrophic pathogen attack (Gobel *et al.*, 2001). Cultured potato cells treated with a *P. infestans* elicitor accumulated transcripts encoding a linoleate 9-lipoxygenase and the linoleate 13-lipoxygenase (Gobel *et al.*, 2001). Conversely, studying the protein products of these transcripts reveals that 13-lipoxygenase activity and protein levels do not increase in response to elicitor treatment (Gobel *et al.*, 2001). Jasmonic acid (JA), an oxylipin derived from 13-lipoxygen, accumulates in potato leaves after *Pseudomonas syringae* pv. *maculicola* infection. Conversely, JA does not accumulate during *P. infestans* infection (Gebhardt and Valkonen, 2001).

Although *R* genes involved in *P. infestans* recognition have been genetically mapped or cloned, their molecular function and the downstream defence responses they trigger are

poorly understood. This highlights the extent of research required to fully understand this specific recognition event and the Quantitative (field) resistance, which is more stable, does not result in complete immunity, subsequently reducing selection pressure on *P. infestans* to evolve. Consequently, this type of resistance is of increasing interest to plant breeders as an alternative solution to the potato blight problem. Unfortunately, this type of resistance is difficult to engineer as it is conferred by many loci spread throughout the genome and its molecular mechanisms are not well characterised or understood. Nevertheless, through the use of DNA markers, nineteen single dominant *R* genes for resistance to viruses, nematodes and fungi have been situated on the molecular map of potato, fourteen of those are located in five quantitative trait loci (QTL) for resistance. Unfortunately, QTL to late blight, tuber rot caused by bacterium *Erwinia caratovora* spp *atroseptica* and to root cyst nematodes are dispersed on all 12 chromosomes making their combined effect next to impossible to breed by selection (Gebhardt and Valkonen, 2001). Quantitative resistance traits have also been identified in wild potato species and have been mapped to loci of several *R* gene clusters e.g. the QTL, *Grp1*, is located in the *R* gene cluster on potato chromosome V (Gebhardt and Valkonen, 2001).

1.10. Project Aims

A variety of techniques have been developed to isolate genomic and ESTs sequence information from potato and other crop species. Moreover, scientists studying the potato-late blight interaction can also benefit from the sequence information and functional data provided by analyses of the *Arabidopsis* genome. The sequenced model *Arabidopsis* is most amenable for forward genetics-based approaches to gene discovery through large-scale mutagenesis and functional screening.

1.10.1. Screen for gain-of-function susceptible *Arabidopsis* mutants

Arabidopsis thaliana, the model plant, had its entire genome sequenced (120 Mb) by 2000 to supply all scientists with the core sequence data that essentially builds a basic

representative of this kingdom (Arabidopsis Genome Initiative, 2000). The *Arabidopsis* genome sequence has revealed the extent of gene duplication and functional redundancy and therefore an alternative screening method, known as “activation tagging”, has been developed (Weigel, 2000). Activation tagging has been devised to create gain-of-function mutations by taking advantage of the strong activating properties of the cauliflower mosaic virus (CaMV) 35S gene enhancer and the random integration of T-DNA (Weigel *et al.*, 2000). The activation tagging vector (pSKI015), containing four CaMV 35S enhancers and a BAR gene (conveys resistance to the herbicide glufosinate), was used to transform previously engineered *PR-1::Luciferase (LUC) Arabidopsis* (Col-0) plants. T-DNA vector inserts, in the coding region of a gene, cause a loss-of-function phenotype. Alternatively, if this vector inserts into a non-coding region of the genome then it should induce constitutive enhanced expression of immediately adjacent genes from a distance of around 10 Kb (Weigel *et al.*, 2000). Pools of T₁ activation tagged *PR-1::LUC* plants were screened for disease resistance and susceptibility to *P. syringae* pv *tomato (Pst)* DC3000. The main aim was to identify *Pst*-susceptible mutants, caused by the over-expression of novel genes, that may repress SAR, activate the JA- and ET-dependent pathways and may be potential targets of biotrophic pathogens’ virulence factors.

If any novel genes of interest were identified, the role of the genes in plant disease resistance was to be investigated in both *Arabidopsis* and in *Nicotiana* and *Solanum* species. The majority of systems utilised for functional analysis in *Nicotiana* and *Solanum* species relies on obtaining sequence information prior to functional analyses in a reverse genetics approach. The highly accessible and detailed information continuously published from studies into core defence signalling networks in *Arabidopsis* should facilitate the transfer of genetic information that should complement functional analysis of genes in crop species.

1.10.2. Genes involved in *P. infestans*-induced HR in potato

The HR appears to be fundamental to every type of resistance against oomycetes e.g. *Phytophthora infestans* on potato and *P. parasitica* on *Arabidopsis* (Birch and Whisson,

2001). A suppression subtractive hybridisation (SSH) had been performed to create a cDNA library enriched for genes involved in the HR of potato plants against *P. infestans* (Birch *et al.*, 1999). Classes of genes identified are similar to those found in a variety of other cell signalling pathways e.g. transcription factors, protein kinases and cysteine proteases (Birch *et al.*, 1999; Avrova *et al.*, 1999; Solomon *et al.*, 1999). Confirmation of candidate genes' up-regulation during HR and functional characterisation of the most interesting genes was required. Genes confirmed to have a role in HR were carried forward for virus-induced gene silencing (VIGS) experiments (Baulcombe, 1999a; 1999b) that have been previously established in *Nicotiana benthamiana*, a more malleable alternative experimental model (MacFarlane, 1996; Burton *et al.*, 2000; MacFarlane and Popovich, 2000) and an assay was developed to screen for alterations to the HR phenotype following VIGS of candidate genes. However, the HR-related genes of interest were initially identified from potato-*P. infestans* interaction SSHs and, therefore, VIGS in potatoes would be of greater relevance to their resistance role in this pathosystem. Therefore, an important aim of this project was also to develop a VIGS system for cultivated, tetraploid potatoes used for the original SSH libraries.

This project was a collaboration between the *Arabidopsis* community and the crop-based community through which the best resources available to both could be utilised to study aspects of disease resistance. Novel *Arabidopsis* mutants were isolated through an activation tagging approach designed to circumvent lethality and functional redundancy problems associated with classical mutagenesis systems (Chapter 3). Genes identified from *S. tuberosum* were functionally analysed using an established post-transcriptional gene silencing technique in *N. benthamiana* (Chapter 4). Genes of interest from both *Arabidopsis* and *S. tuberosum* were investigated in potato once a gene silencing system had been developed (Chapter 5).

2. Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, UK) or Merck (VWR International Ltd, UK).

2.1. *Growth of Arabidopsis thaliana*

Maximum of 0.4 g seeds were air-sterilised envelopes of Whatmans paper in desiccators containing 100 ml of bleach and 3 ml of hydrochloric acid. Seeds were then spread evenly over wet soil and left at 4 °C for 48 h in dark for stratification. During screening of T₂ generation of Activation tagged lines, pools of approximately 20,000 (0.4 g) seeds were evenly dispersed over each flat of wet, sterilised soil, vermiculite and sand (4 parts: 1 part: 1 part), covered and stratified. After cold stratification, flats and trays of pots were transferred to growth rooms in long day conditions (16 h light and 8 h of dark) at 18 °C and 42 % humidity and remained covered with clear lids until seeds germinated. Seedlings were then sprayed at least twice with selection herbicide Basta (150 µg.ml⁻¹). Seedlings of between 1-2 weeks were transplanted 6 per pot (12 cm x 8 cm), 12 pots per tray and watered by sub-irrigation twice weekly until 4 weeks old when most suitable for inoculation with pathogens. The *Arabidopsis* plants with varying genetic backgrounds used during project are displayed in Table 2.1.

Table 2.1: *Arabidopsis* wild type, transgenic and mutant lines

Ecotype/ mutant	Phenotype	Gene accession	Protein	Reference	Source
Col-0	wild-type			AGI, 2000	NASC
<i>nahG</i>	No SA accumulation	NCBI: X83926	Salicylate hydroxylase	Lawton <i>et al.</i> , 1995	Novartis, USA
<i>ndr1-2</i>	Susceptible to Pst and Pp	At3g20600	membrane associated	Centuary <i>et al.</i> , 1995	Staskawicz, University of California
<i>npr1-1</i>	SAR insensitive	At1g64280	Ankirin Repeats bind TGA transcription factors	Cao <i>et al.</i> , 1995	Dong, Duke University
<i>eds1.2</i> (<i>Ler</i>)	Blocked TIR- NBS-LRR signalling	At3g48090	Lipase-like protein	Parker <i>et al.</i> , 1996	Parker, Max Planck Institute
<i>ein2-1</i>	Ethylene insensitive	At1g47240	membrane associated	Guzman & Ecker 1990	NASC
<i>etr1-1</i>	Ethylene insensitive	At1g66340	NRAMP metal ion transporter	Bleeker <i>et al.</i> , 1988	NASC
<i>coil-1</i>	Jasmonate insensitive	At2g39940	F-box protein	Feys <i>et al.</i> , 1994	Turner, University of East Anglia
<i>cyp1</i>	T-DNA insert	At1g47128	Cysteine Protease & Granulin domain	Alonso <i>et al.</i> , 2003	NASC
<i>cathB</i>	T-DNA insert	At1g02300	Cysteine Protease Cathepsin B	Alonso <i>et al.</i> , 2003	NASC
<i>cathB</i>	T-DNA insert	Ar1g02305	Cysteine Protease Cathepsin B	Alonso <i>et al.</i> , 2003	NASC
<i>cathB</i>	T-DNA insert	At4g01610	Cysteine Protease Cathepsin B	Alonso <i>et al.</i> , 2003	NASC
<i>ads1</i>	pSKI015 tagged	Region F21A14	unknown	Tani <i>et al.</i> , 2004	Loake, Edinburgh University
<i>ads2</i>	pSKI015 tagged	At1g05480 At1g05500	Unknown	Tani <i>et al.</i> , 2004	Loake, Edinburgh University

NASC (Nottingham Arabidopsis Stock Centre)

2.2. *Growth of Solanum tuberosum and Nicotiana species*

Apical 5 cm of young potato stems that contain 4-5 nodes were removed using sterile forceps and scalpel in laminar flow cabinet. Leaves were removed from petioles and then stems were divided into individual pieces each containing a node with an auxiliary bud. Six stem pieces were then planted per 60 ml glass jar containing 20 ml of MS30 medium (4.4 g/l of 1M Murashige and Skoog Medium (Murashige and Skoog, 1962), 30 g/l of Sucrose, 0.7 % Difco Bacto Agar, adjusted to pH 5.8 with NaOH) and covered with a suncap (autoclavable transparent polyethylene film with a built-in 0.02 µm filter,

Sigma, UK) and secured with elastic band. Due to the discontinuation of suncaps (Sigma, UK), potatoes were alternatively grown in Petri dishes with 20 ml MS30 medium, sealed with Nescofilm (Bando Chemical Ind., Japan). Plants were then grown at 22 °C with 16 h photoperiod and light intensity of 110 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Once *in vitro* potato plants were 3-4 weeks old, they were transferred to soil in controlled environment chambers with a 16 h photoperiod (22 °C, light intensity ranging from 400 to 1000 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). *Nicotiana* species were also kept in same chamber with identical conditions. SCRI glasshouse staff provided weekly supplies of *Nicotiana* species which were also maintained in identical chamber conditions.

2.3. Culture of Plant Pathogens

2.3.1. *Pseudomonas syringae* pv *tomato* DC3000

Pseudomonas syringae pv *tomato* (*Pst*) DC3000 (Whalen *et al.*, 1991) with and without *avrB* was grown on petrie dishes containing King's broth (KB) (King *et al.*, 1954) supplemented with 6 mM MgSO_4 , 50 $\text{mg}\cdot\text{l}^{-1}$ rifampicin and an additional 50 $\text{mg}\cdot\text{l}^{-1}$ kanamycin for *Pst*DC3000*avrB* to select for *avrB* carrying plasmid. Liquid cultures were incubated at 30 °C and shaken at 120 rpm overnight. To test for possible contamination of virulent strain (no *avrB*) with an avirulent strain (resistant to kanamycin) during overnight culture, 10 μl of 25 mg/ml kanamycin was added to at least one vial of virulent *Pst*DC3000. Bacterial suspensions were assumed suitable for plant inoculation if the kanamycin containing vial remained clear. The bacteria were pelleted and resuspended in an equal volume of filter sterilized 10 mM MgCl_2 , measuring the optical density at 600 nm and diluted accordingly. An OD_{600} equal to 0.2 is the equivalent of 10^8 colony forming units per ml (CFU/ml).

2.3.2. *Erwinia amylovora* 1430 (Apple Fire Blight)

Erwinia amylovora (Toth *et al.*, 2003) was cultured in liquid King's Broth supplemented with 6 mM MgSO_4 inoculated from colonies plated from glycerol stocks kept at -80 °C.

Cultures were incubated at 30 °C at 120 rpm overnight and optical density read at 600 nm the following morning. Bacterial suspension of 0.002 (10^6 CFU/ml) was inoculated during majority of experiments.

2.3.3. *Phytophthora infestans* simple/complex race

Simple (15.5.1) and complex (99/23) strains of *P. infestans* were maintained by incubating previously infected young potato leaves on damp Kleenex tissue in Q-trays at 15 °C for 6-7 days. For immediate inoculation of aggressive *P. infestans*, leaves were washed in ice-cold SDW and spores counted using a haemocytometer and diluted to 5×10^4 spores per ml. Left in fridge for 30 min to allow trigger release of swimming zoospores and then solution sprayed evenly over potato plants at 4 weeks old. For longer term maintenance of *P. infestans* infected leave pieces were placed directly onto rifampicin (50 mg.l^{-1}) and pimaricin ($250 \mu\text{l.l}^{-1}$ of 2.5 % aqueous suspension) Rye A agar plates (60 g.l^{-1} Rough Rye; 20 g.l^{-1} Sucrose and 10 g.l^{-1} Agar) and thereafter cut 1 cm^2 pieces of infected agar to inoculate fresh plates every 1-2 weeks at 18 °C.

2.4. Disease Resistance Assays

2.4.1. Virulent and Avirulent *P. syringae* DC3000

P. syringae pv *tomato* (*Pst*) DC3000 (Whalen *et al.*, 1991) was grown as described in 2.3.1. Four week old, plants were inoculated by pressure infiltration into the abaxial leaf surface with *Pseudomonas syringae* strains in a 10 mM MgCl_2 solution at optical densities described in Table 2.4.1(Cao *et al.*, 1994).

Table 2.4.1: Range and use of optical densities of *Pseudomonas syringae*

Experiment	OD ₆₀₀ Virulent <i>Pst</i>	OD ₆₀₀ Avirulent <i>Pst</i>
Susceptibility screen of activation tagged Col-0	0.0003	n/a
Colony counts	0.0003/ 0.002	0.02
DAB staining	0.002	0.002
Trypan blue Staining	0.002	0.002

Before large-scale screening of six, 4-week-old susceptible *Arabidopsis* mutants *ndr1*, *eds1* and *NahG* and wild type Col-0, were pressure infiltrated with *vir Pst* DC3000 bacterial suspensions, OD₆₀₀ 0.002, 0.001 and 0.0005 to select a suitable OD which allowed disease in mutants but not in Col-0. Initially, the OD₆₀₀ 0.0005 was inoculated for screening purposes into Basta resistant, activation tagged mutants. The lower OD₆₀₀ 0.0003 was subsequently used reducing percentage of susceptible mutants to 3 % of screened population. Known susceptible mutants and Col-0 plants were inoculated simultaneously with every screening event. Those susceptible plants that developed obvious chlorotic disease symptoms 4-5 days after inoculation were transplanted into individual pots, labelled and later retested with a bacterial solution with an OD₆₀₀ 0.0002 (equivalent of 10⁵ CFU/ml). Plants that develop symptoms in majority of leaves inoculated were kept for seed collection.

For all other experiments, 4 week old, plants were inoculated by pressure infiltration with *P. syringae* strains as described in Table 2.4.1. For viable *Pst* DC3000 recovery colony counting experiments, leaves were harvested 4 days post-inoculation. Whole leaves or leaf segments of equivalent size were ground in 1 ml of 10 mM MgCl₂ using micropestles. Four subsequent serial dilutions of 100 µl sample in 900 µl of 10 mM MgCl₂ were performed then 100 µl of the 10⁻⁴ dilution spread on KB plates containing 50 mg.l⁻¹ rifampicin and incubated at 30 °C for 48 hours.

2.4.2. *E. amylovora* 1430

E. amylovora is a bacterial pathogen of apples which induces a non-host HR in *Nicotiana* and *Solanum* species. *E. amylovora* was grown as mentioned above and resuspended at an OD₆₀₀ = 0.002 (equivalent of 10⁶ CFU/ml) in sterile 10 mM MgCl₂ or 5 mM MES. The suspension was then pressure infiltrated into wild type and TRV::GFP, TRV::Sgt1_{as} and TRV::CathB_{as} infected *N. benthamiana* (Cao *et al.*, 1994). Cathepsin B inhibitors were diluted from 100 mM stocks to 1 mM with 10⁶ CFU/ml suspension in 5 mM MES (Cathepsin B, S and L inhibitor, Z-FGNHO-Bz; Cathepsin B inhibitor I, Z-FA.fmk; Cathepsin B inhibitor II, Ac-LVK-CHO and Cathepsin B Inhibitor IV, CA-074

Me) (Calbiochem[®], Merck Biosciences, UK). These solutions were also pressure infiltrated into wild type *N. benthamiana* as above.

2.4.3. *P. infestans* simple/complex races

Assessment of foliage blight symptoms was performed according to scale published in Cruickshank *et al.* (1982). Briefly, scoring was as follows: 1= >90 % necrotic tissue, 2= 81–90 % necrotic, 3= 71–80 % necrotic, 4= 61–70 % necrotic, 5= 41–60 % necrotic, 6= 26–40 % necrotic, 7= 11–25 % necrotic and 8= <10% necrotic. Majority of scoring was performed by Ruth Solomon-Blackburn.

2.5. *Cloning of and Infection with Viral Vectors*

2.5.1. Tobacco Rattle Virus (TRV) Constructs

Initial TRV constructs pPNC96 (RNA1) and pPNC97 (RNA2) with and without the 2B nematode transmission protein were created and supplied by Dr S MacFarlane (Liu *et al.*, 2002; MacFarlane and Popovich, 2000; MacFarlane *et al.*, 1996) as RNA transcripts for potato *Pds* silencing experiment. The construct pPNC97 contained either tomato *Pds* (X59948) or transgenic GFP gene fragments. These constructs were transcribed using T7 RNA polymerase as described in MacFarlane *et al.*, 1996) mixed with RNA1 transcripts and inoculated on to *N. benthamiana* leaves using aluoxite as a slight abrasive. Around 2 µl of sap from infected *N. benthamiana* plants was used to inoculate 2 week old *N. benthamiana*, *N. tabacum* cv Samsun, *N. tabacum* cv Xanthi, *Solanum tuberosum* cultivars Spey, Stirling and Wilja, 10 plants per construct.

Subsequent silencing experiments were also performed utilising TRV vector (Liu *et al.*, 2002) using cloning sites *AvrII* and *KpnI* (see Table 2.5.2 for sequences and primers). Primers were designed using Sequence Navigator and Oligo software for Apple Mac. Fragments were cloned from *N. benthamiana* cDNA using Hi-Fidelity Taq Polymerase (Promega Ltd, UK) and cloned into pGemT-easy (Promega Ltd, UK). Plasmid preparation of bacterial DNA was performed using QIAprep plasmid DNA purification

kit (Qiagen Ltd, UK). Positive pGemT Easy clones were checked by sequencing using BigDye sequencing kit (Applied Biosystems Ltd, UK) and used for subsequent cloning into TRV construct as used in Valentine *et al.*, (2004). The restriction enzymes predominantly used were from NEB (New England Biolabs, USA) and Qiagen gel extraction kits (Qiagen Ltd, UK) used for purification of plasmid digestion products. Sepharose 6B (Sigma) was utilised for desalting products (Lehmann, 1970) from intermediate cloning steps which required changes of buffer. Transcription reactions of linearised plasmids were performed using Ambion mMessage mMachine T7 kit (Ambion Inc, Austin, USA) according to manufacturer's instructions using wherever possible baked apparatus. Transcripts (2 µl) were inoculated on to first two true leaves of 2-week-old *Nicotiana* species by lightly sprinkling abrasive powder, aluoxite, over leaves and stroking leaves gently twice. Plants were washed with cold water and left for 2-3 weeks (conditions mentioned in section 2.2) for silencing to develop.

Table 2.5.1: Sequences and primers for TRV cloning

Construct	NCBI No.	Primer name	Primer sequence (5'-3')	Size (bp)
TRV.SGT1as	AF516181	NbSGT1For	TTTGGTACCTTCGCCGACCGTG	607
		NbSGT1Rev	TATTCCTAGGGCAGGTGTATCTC	
TRV.CathBas	AF359422	NbCathBFor	TTGGGTACCTAAGCGCCTTCTTG	376
		NbCathBRev	TTTCCATGGGTAAGGATCACACTCTC	
TRV.CYP1as	AJ245924/ AF172856	NbCathKFor	TTTTGGTACCGTCAAGCCACCTAC	246
		NbCathKRev	TTTTCCTAGGCGCTTCATTGCCTTC	
TRV.ADR1as	BG596098/ A1776307	NbAdr1For	TTTTGGTACCGATTCTTGATGATG	994
		NbAdr1Rev	TTCCTAGGGTTGGTTAAATGACTG	

GGTACC = AvrII restriction site; CCTAGG = KpnI restriction site

2.5.2. Potato Virus X Constructs

Cloning of *PDS* and *GFP* into PVX as described in Faivre-Rampert *et al.* (2004). Briefly, cDNA was cloned into PVX vector (pGR106, Jones *et al.*, 1999; Lu *et al.*, 2003) obtained from David Baulcombe (Sainsbury Laboratory, Norwich, UK) using *AscI* and *NotI* restriction sites. Primers were designed using Sequence Navigator and Oligo software for Apple Mac. Gene fragments were amplified from *S. tuberosum* cDNA using Hi-Fidelity Taq Polymerase (Promega Ltd, UK) and initially cloned into pGemT-easy (Promega Ltd, UK), positive clones sequenced as in section 2.5.1. Plasmid DNA preparations as in section 2.5.1. *Agrobacterium tumefaciens* strain LB4404, carrying the

helper plasmid pSoup (Hellens *et al.*, 2000) was transformed with PVX constructs. Agroinfiltration of *N. benthamiana* and *Solanum* species with PVX vector was performed as previously described (Lu *et al.*, 2003).

Table 2.5.2: Sequences and primers for pGR106 cloning

Construct	NCBI No.	Primer	Primer sequence (5'-3')	Size
PVX.GFP	U62637	GFPe3up	TTTAGGGCGGCCATGGCTAGCAAAGGAG	723 bp
		GFPe3low	TATTGCGGCCGCTTATTGTAGAG	
PVX.PDSas	AY48445	PDSPup	TTTTGCGGCCGCTTTGCCACTCCAG	415 bp
		PDSPlow	TTTTGGCGCGCCTTGGAGTTTTGAC	
PVX.hpPDSN bSt	AY48445/ I23876	HPNbStup	AAAGGCGGCCACATGTAAGGAATATTACAAC	94 bp (47 bp loop)
		HPNbStlow	AAAAGGATCCACCAATTCCAACATAGAC	
PVX.SGT1as	BG599125	PocSGT1For	TTTGCGGCCGCTCGCCGACCGTG	584 bp
		PocSGT1Rev	TTTTGGCGCGCCGAGGTGTTATCTTG	
PVX.CathBas	BG590588	PoCathBFor	TTTTGCGGCCGCTAAGCGCCTTCTTG	376 bp
		PoCathBRev	TGCGCGCCGTAAGGATCACACTCTTC	
PVX.CYP1as	AJ245924	PoCathK_Gr For	TTTTGCGGCCGCGTCAAGCCACCTAC	248 bp
		PoCathK_3R ev	TTTTGGCGCGCCCGCTTCATTGCCTTC	
PVX.ADR1as	BG596098	PoADR1CoF or	TTTTGCGGCCGCTATGAGATATCGGT	391 bp
		PoADR13'Re v	TTTTGGCGCGCCGTTGGTTAAATGACTG	

GGCGGCC = *AscI* restriction site; GCGGCCG = *NotI* restriction site

2.5.3. Tobacco Mosaic Viral (TMV) Constructs

TMV VirD2::GFP fusion construct that displayed a hypersensitive response induced- and caspase-like cleavage and the negative control plasmid GFP::VirD2 as described in Chichkova *et al.*, (2004) were received from Michael Taliansky. Linearised plasmids were transcribed using Ambion mMessage mMachine T7 kit (Ambion Inc., USA) according to manufacturer's instructions using wherever possible baked apparatus. Previously prepared TMV strain U1 virion coat protein 10 mg.ml⁻¹ (8.75 µl) was mixed with 51.25 µl autoclaved assembly buffer (26.8 mM Na₂HPO₄ and 19.5 mM NaH₂PO₄, pH7) and incubated for 6 hours at 25 °C. Freshly prepared TMV transcripts (5 µl) were rapidly added to reassembling TMV coat protein without chilling on ice and incubated at 25 °C overnight. Transcript and coat protein reassemblies were immediately inoculated onto first two true leaves of 2-week-old *Nicotiana* species by lightly sprinkling abrasive powder, aluoxite, over leaves and stroking leaves gently twice. Plants were then washed

with cold water and incubated at either 33 °C (optimal) or 24 °C. Inoculated leaves were examined for nuclear localised GFP in viral growth foci 2 days post-inoculation.

2.6. Treatments using Exogenous Signalling Molecules

2.6.1. 1-Aminocyclopropane-carboxylic acid (ACC)

ACC (1-aminocyclopropane-carboxylic acid) plates were made using MS agar media (4.5 g.l⁻¹ Murashi and Skoog, 1 % Sucrose 4 g.l⁻¹ Agar and pH adjusted to 5.8 with 1 M Sodium hydroxide) cooled to 60 °C. Concentrations, conditions and examination 7 days later for triple response phenotype and root length measured as described in (Guzmain and Ecker, 1990). Wild type Col-0 and *ein2.1* were used as controls.

2.6.2. Methyl jasmonate (MeJA)

Methyl jasmonate (MeJA) plates were made using MS media described above with a range of concentrations (0 µM - 150 µM) and examined for percent germination similar to that displayed by MeJA insensitive mutants and root length. Wild type *Arabidopsis* Col-0, *coi-1* and/or *jar1* were used as controls although had problems with the seed.

2.6.3. Salicylic acid (SA) and analogue benzothiadiazole (BTH)

The SA analogue Benzothiadiazole (BTH, Bion[®], Norvartis, Frimley, UK) was used as a systemic acquired resistance inducer as a 1.2 mM solution made with SDW sprayed over 3-4 week old plants usually twice at least 4 days before inoculation with *Pst*.

2.7. Histochemical Analyses and Microscopy

2.7.1. Trypan Blue Staining

Trypan blue staining procedure was employed to identify dead plant cells in challenged leaves as used in Yun *et al.* (2003). Leaves were submerged in 5 ml of lactophenol trypan blue (1 part water with 1 mg.ml⁻¹ trypan blue; 1 part Lactic acid; 1 part Phenol; 1

part Glycerol) and boiled for 2-5 minutes. Solution was removed and leaves kept in destaining solution of 2.5 g.ml⁻¹ Chloral Hydrate for 48 hours. Leaves were mounted on glass slides with 60 % glycerol, covered with a coverslip and secured with nail varnish. Slides examined under a Nikon Eclipse E600 microscope with x 10 and x 20 objectives.

2.7.2. DAB Staining

Hydrogen peroxide production was visualised using 3, 3-diaminobenzidine (DAB) as described by Thordal-Christensen *et al.* (1997). 1 g.l⁻¹ of DAB was dissolved in boiling SDW. Leaves were submerged in 10 ml of cooled DAB solution and left overnight. Leaves were transferred to 10 ml of 96 % ethanol and boiled for 10 min. Leaves were rinsed twice in sterile distilled water and mounted on glass slides with 60 % glycerol under a coverslip and secured with nail varnish. Slides were examined under a Nikon Eclipse E600 microscope with x 10 and x 20 objectives.

2.7.3. GFP Imaging with Scanning Laser Confocal Microscope

Transgenic *Nicotiana tabacum*, CB9, carrying TMV movement protein were inoculated with 2 µl of TMV construct and coat protein reassemblies as mentioned in section 2.5.3. Two days post-inoculation, primary leaves were examined through a Leica SP2 scanning laser confocal microscope (Leica Microsystems Ltd, UK) for GFP localisation in the nuclei of upper epidermal cells using excitation wavelength of 488 nm and collecting emission wavelengths of 505-520 nm employing an Argon/ HeNe laser. Viral growth foci displaying nuclear GFP localisation were infiltrated with either 10 mM MgCl₂, *E. amylovora* suspension of 10⁶ cells per ml with or without 1 mM Cathepsin B inhibitor II, Ac-LVK-CHO (Calbiochem[®], UK) reconstituted in SDW. Hypersensitive response-related cleavage of nuclear localisation signal from fusion protein, VirD2::GFP, in upper epidermal cells was investigated in upper epidermal cells at 4 and 24 hours post-inoculation.

2.8. PCR-based methods

2.8.1. General PCR method

For general PCR amplification applications Taq Polymerase (Promega Ltd, UK) was used. Hi-Fidelity Taq Polymerase (Promega Ltd, UK) was used for amplification of fragments intended for cloning into silencing vectors.

2.8.2. Thermal Asymmetric Interlaced (TAIL) PCR

TAIL PCR was performed with both *ads1* and *ads2* homozygous mutant DNA extracted using Qiagen DNeasy[®] Plant mini Kit (Qiagen Ltd, UK) using protocol and PCR cycle settings as described in Liu *et al.* (1995). Primers used listed in Table 2.8.2 below.

Table 2.8.2: Primers used for TAIL-PCR of *ads1* and *ads2*

Primer Name	Sequence (5'-3')
AD 1	NTCGASTWTSGWGTTT
AD 2	NGTCGASWGANAWGAA
AD 3	WGTGNAGWANCANAGA
AD 4	TGWGNAGSANCASAGA
AD 5	AGWGNAGWANCAWAGG
AD 6	STTGNTASTNCTNTGC
Right Border 21	ACGACACTCTCGTCTACTCCAAG
Right Border 53	AACAAACGGTAATATCGGGAAAC
Right Border 205	TAAAGGAAAGGCTATCGTTCAAG

AD=Arbitrary degenerate; N=any nt; S=[G/C]; W=[A/T]

2.8.3. Genotyping of NASC and ABRC T-DNA Knockout mutant

Seeds were obtained through the TAIR website (see section 2.15) through the functional genomics link which lists all T-DNA insert mutants available for any particular loci. The SIGnAL website (see Table 2.15 for address) was used to order suitable T-DNA insert mutants which arrive as progeny of a heterozygous parent and therefore T-DNA primer express website was used for design of gene specific primers based on gene loci and SALK description. Primers designed are shown in table 2.8.3. Plant DNA was extracted using cetyltrimethylammonium bromide (CTAB) extraction buffer (100 mM Tris HCl, pH 8; 1.4 M NaCl; 20 mM EDTA; 2 % CTAB and 0.2 % 2-Mercaptoethanol)

from at least 10 plants. DNA extraction as described in Doyle and Doyle (1990). Gene specific primers were used together with left border specific primer LBB1 in one PCR tube. The larger band represented the wild type allele whereas a shorter band around 400 bp represented the mutant allele. Seed was collected from plants homozygous for the insert.

Table 2.8.3: Gene specific primers designed for T-DNA mutant genotyping

Gene	Loci	Description	Code	Primer name	Primer sequence (5'-3')
Left Border	T-DNA	pROK2	N/A	LBB1	GCGTGGACCGCTTGCTGCAACT
CathB	At1g02300	SALK_049118 (A1)	N549118	CathBGenFor	AGAACCGCTTGGTCACATTTC
				CathBGenRev	TGTGCAAAGTCCTGAAGAACAAA
CathB	At1g02305	SALK_138261	N638261	CathBbGenFor	GAGGAACGAACGAATGTGGCA
				CathBbGenRev	TTTTCAGCTGCAATACCTGAGA
CathB	At4g01610	SALK_019630	N519630	CathBcGenFor	GGTTTTGGTTTAGGGACATTGTGG
				CathBcGenRev	GCCTCTGTTCCATTGATTTGCC
CYP1	At1g47128	SALK_090550	N590550	CathKGenFor	AGAATGGCGTTGCGGGTTTAC
				CathKGenRev	CGCCAATTACAAACGAAAGGAGA

2.8.4. Quantitative Real-Time Reverse Transcriptase PCR

Total RNA was extracted from frozen leaves using the Qiagen RNeasy Plant Mini kit (Qiagen, Crawley, UK), following the manufacturer's instructions and quantified spectrophotometrically (Uvikon, Kontron Instruments, UK) using 1 A₂₆₀ of ssRNA = 40 µg.ml⁻¹ H₂O and also the ratio of A₂₆₀/A₂₈₀ ≥ 2.0; the RNA was then stored at -80 °C until required. DNaseI treatment (Ambion Inc, USA) and first strand cDNA synthesised from 3 µg of RNA using Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen, UK) were as previously described (Lacomme *et al.*, 2003). For SYBR real-time RT-PCR (QuantiTect SYBR GreenPCR kit, Qiagen Ltd, UK) experiments, primer pairs were designed outside the region of cDNA targeted for silencing using the Primer Express software supplied with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Ltd, UK) following the manufacturer's guidelines (see Table 2.8.4 for Taqman primers). The GenBank accession numbers for *N. benthamiana* PDS and ubiquitin cDNA are as previously mentioned (Lacomme *et al.*, 2003). Primer concentrations giving the lowest threshold cycle (cT) value were selected for further

analysis. Detection of real-time RT-PCR products, calculations and statistical analysis were performed as previously described (Lacomme *et al.*, 2003).

Table 2.8.4: Primers used for Quantitative Real-Time RT-PCR

Plant	Gene	Primer Name	Sequence (5'-3')
<i>Solanum tuberosum</i>	StUBI	StUBIfwd	ACACCATTGATAATGTCAAGGCTAAG
		StUBIrev	GCCATCCTCCAATTGCTTTC
	StPDS	StPDSfwd	CCAAGACCAGAGCTAGACAATACAGT
		StPDSrev	CCACCCAAACCTGCA
<i>Nicotiana benthamiana</i>	NbPDS	NbPDStaqFw	TTCTTCAGGAGAAAACATGGTTCAA
		NbPDStaqRv	TCCACAATCGGCATGCAA
	NbCathB	NtCathBtaq2_For	CGAGCAAGGAGAGGACTATTGG
		NtCathBtaq2_Rev	GCTGAAGGCAATCCTGCAA
	Le_25S	Tom25S_For	CACGGACCAAGGAGTCTGACAT
		Tom25S_Rev	TCCCACCAATCAGCTTCCTTAC

2.8.5. Reverse Transcriptase PCR

First strand cDNA obtained as in section 2.8.4. For RT-PCR of TRV RNA2 coat protein primers were previously designed by Dr S MacFarlane (see Table 2.8.5). For RT-PCR analysis of silencing, primers that anneal outside the region of the cDNA cloned into the virus vectors were designed (see Table 2.8.5). In cases where semi-quantification of RT-PCR was not as important, Qiagen one step RT-PCR kit (Qiagen Ltd, UK) was used with primers previously used for *Arabidopsis* T-DNA insert genotyping (see section 2.8.3).

Table 2.8.5: Primers used for RT-PCR

Species	Gene	Primer Name	Sequence (5'-3')	Expected Product (cDNA)
Tobacco Rattle Virus	Coat Protein	157	CGCGGTAGAACGTACTTAT	1200 bp
		208	GTGTCGTCAAGCCACTTCC	
<i>Solanum tuberosum</i>	StUBI	RTPoUBIfor	GCA GTTGGAGGACGGAC	266 bp
		RTPoUBIrev	GGCCATCTTCCA ACTGTTTC	
	StPDS	RTPoPDSfor	CTCGAGGTCGTCTTCTTTGG	263 bp
		RTPoPDSrev	GTTTAGTTGGCGTGGAGAA	
<i>Arabidopsis ads2 pSKI015 Insert Region</i>	At3g05480	5480 For	CCTCTGATGCTGCAAGTGAA	308 bp
		5480 Rev	TGCATCAAAACTGGAGCTTG	
	At3g05490	5490 For	ATTCACCGAGAAGGTTTTGG	168 bp
		5490 Rev	TCCTGTGCATCCAGAGAAGA	
	At3g05500	5500 For	TCTTCAAACGAATCGCCTTT	347 bp
		5500 Rev	GACGGTCAAGCTCAGTCACA	
	At3g05510	5510 For	GGCTTTTTATCGCAGACGAG	303 bp
		5510 Rev	AGCGTCCACCTCAAATTACG	
	At3g05520	5520 For	GGTTTTGAGCGATGAGGAAG	360 bp
		5520 Rev	GGACCCTGGACCCTCATTAT	
	At3g05530	5530 For	TTGTACCCTCATTCCGATTTG	163 bp
		5530 Rev	AGAATAGGTCTATATACCATGAACCAA	

2.9. BAC Library Construction

Bacterial artificial chromosome (BAC) library construction was performed using high-molecular-weight (HMW) DNA extracted from homozygous *ads1 Arabidopsis* mutant based on technique used by Chalhoub *et al.*, (2004) and described in Hein *et al.* (2005). Changes from the published protocol are as follows. The pIndigoBAC5 vector (Caltech UK Ltd, UK) came pre-digested with *Hind*III and was not purified on CsCl gradient. Nuclei extraction and preparation of HMW DNA was as described (Peterson *et al.*, 2000) and partially digested using *Hind*III range between 0.5–80 units, incubated on ice for 1 hr to allow enzyme diffusion into plug pieces followed by 20 min at 37 °C. Enzyme was heat denatured and plugs purified using 1 % standard agarose/ 0.25 x TBE gel using CHEF–Mapper XA pulsed field gel apparatus (Bio-Rad Laboratories, USA) as described in Hein *et al.* (2005). Standard 1 % agarose/ 0.25 x TBE gel was used for size fractionation and gel extraction of partially digested DNA population (50-250 Kb). Electro-elution, ligation, transformation and antibiotic selection as described in Hein *et al.*, 2005). Genetix Qbot was utilised to pick 18,432 individual clones into 364 well plates (Genetix Ltd, UK) with Genetix robot, Qstack, used to fill plates with 60 µl of LB (supplemented with 25 µg.ml⁻¹ chloramphenicol) per well generating the BAC library.

Table 2.9.1: pSKI015 specific probes used to screen BAC library

Probe	Description	Size	Primer name	Primer sequence (5'-3')
35S Enhancer	pSKI015	339 bp	For	GATCCCAACATGGTGGAGCACG
			Rev	TAGATATCACATCAA TCCACTTGC
BAR	pSKI015	610 bp	For	GAAGTCCAGCTGCCAGAAAC
			Rev	CACCAAATCGACTCTAGCCA

Once positive clones were identified, plasmids were prepared using a method from Dr Glenn Bryan: 10 ml of liquid culture was grown overnight in LB supplemented with 12.5 $\mu\text{g}\cdot\text{ml}^{-1}$ chloramphenicol. The 4 ml of the liquid culture was pelleted in 2 in 2 ml eppendorf by centrifugation in an Eppendorf 5415 centrifuge (Eppendorf, UK) at full speed for 5 min. The pellet was resuspended in 200 μl of chilled Solution 1 (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.9 % Glucose) and placed on ice. 400 μl of freshly prepared Solution II (1 % SDS, 0.2 M NaOH) was added to each sample and mixed thoroughly by inversion and left on ice for 4 min. Next, 300 μl of Solution III (3 M KOAc, 11.5 % Glacial Acetic Acid) was added, mixed thoroughly by inversion and left on ice for 1 hr after which the BAC preps were centrifuged at full speed for 10 min. The supernatant was transferred to a fresh tube and mixed with an equal volume of cold isopropanol, mixed by inversion and spun immediately for 10 min at full speed. The pellet was then washed twice in 70 % ethanol, air dried and resuspended in 50 μl of SDW. The 7.5 μl of each positive BAC DNA was then digested with 1 unit of *Not*I to check insert size. These were separated on a 1 % Agarose/ 0.25 x TBE CHEF gel, Southern blotted using standard Sambrook method and probed with 35S enhanced probe mentioned in Table 2.9.2. The BACs were then end sequenced with the pIndigoBAC-5 sequencing primers shown in Table 2.9.2 using the ICAPB sequencing service (Kings' Buildings, Edinburgh University, UK) using $\frac{1}{2}$ reaction size of ABI-Prism[®] 7000 Sequencing Kit (Applied Biosystems Ltd, USA).

Table 2.9.2: pIndigoBAC-5 Sequencing primers (Cambio, Cambridge)

Primer name	Primer sequence (5'-3')	Position
pIndigoBAC-5 Forward sequencing primer	GGATGTGCTGCAAGGCGATTAAGTTGG	230-256 bp
pIndigoBAC-5 Reverse sequencing primer	CTCGTATGTTGTGTGGAATTGTGAGC	311-330 bp

2.10. Southern Blot Analysis

2.10.1. DNA extraction

DNA extraction for Southern Blot analysis using plant DNA was performed using a Qiagen DNeasy[®] Plant mini Kit (Qiagen Ltd, UK).

2.10.2. DNA Probe Synthesis

Probes were prepared by amplification of appropriate sequences using PCR and directly purified using a kit (Promega, UK) (Table 2.9.1 and Table 2.2). Sequences for the PCR primers and templates used for each probe are reported. Probes were labelled with α -³²P-dCTP (Amersham, UK) by random priming using the Prime-a-Gene[®] labelling system (Promega, UK).

2.10.3. Southern Blot

E. coli containing the pIndigoBAC5 vector containing *Hind*III partially digested *ads1* DNA (Caltech UK Ltd, UK) were spotted onto nylon membrane (Genetix, UK) using the Qbot according to the supplier's instructions (see 2.9). Probes were labelled with α -³²P-dCTP (Amersham, UK) by random priming using the Prime-a-Gene[®] labelling system (Promega, UK). Southern blotting, hybridisation and washing performed as described in (Sambrook *et al.* 1989).

2.11. Northern Blot Analysis

2.11.1. RNA Extraction

RNA was extracted from the frozen bulked leaf samples using the Qiagen RNeasy plant mini kit and procedure followed as advised by the manufacturer's instructions (Qiagen Ltd, Crawley, West Sussex, UK). The RNA was quantified as mentioned in 2.9.4. RNA samples were DNased using the Ambion (Ambion Inc, USA) DNA-free protocol as in section 2.9.4.

2.11.2. Northern Hybridization

RNA filters were prepared by separating 15 μg of denatured total RNA by gel electrophoresis following the Ambion NorthernMax protocol (Ambion Northern Max system, Ambion Inc, USA) with modifications. Northern gel was prepared using 10x denaturing gel buffer (Northern Max System, Ambion Inc, USA) Samples were run using 1x MOPS gel running buffer (10x MOPS buffer: 200 mM 3 (N-morpholino) propanesuphonic acid (MOPS); 80 mM Sodium Acetate trihydrate; 10 mM EDTA in 0.1 % DEPC SDW). RNA samples (5 μg) were mixed with 3 volumes of formaldehyde load dye (Ambion Inc., USA). The RNA was then denatured by incubation at 65 °C for 15 min before loading onto gel and run at a low voltage overnight. The gel was then viewed and photographed using a transilluminator (Uvitec, Thistle Scientific, UK). Blotting was performed as in Sambrook *et al.* (1989) though using Ambion 1x transfer buffer (Ambion Inc., USA). The membrane was washed in 1 x running buffer, examined and photographed using the transilluminator (Thistle Scientific, UK). The membrane was crosslinked on the maximum setting using the spectrolinker XL-1500 UV crosslinker (Spectronics Corporation, USA). For membrane pre-hybridisation, the ULTRAhyb pre-hybridisation solution (Ambion Inc., USA) was pre-heated and used according manufacturer's instructions. Probe was synthesised as in section 2.10.3 except using components of the Strip-EZ DNA kit (Ambion Inc): 2.5 μl 10x decamer solution, 5.0 μl 5x buffer -dATP/-dCTP, 2.5 μl 10x dCTP, 5.0 μl [α -³²P]dATP (3000 Ci.mmol⁻¹, 10 mCi ml⁻¹), 1.0 μl exonuclease-free Klenow. Probes used for Northern blot analysis are shown in Table 2.11.2. Subsequent hybridisation and washing performed as in Sambrook *et al.* (1989). X-ray film (X-OMAT LS-1, Kodak, USA) was used in cassettes with intensifying screens for an appropriate length of time at -80 °C depending upon the final signal strength.

2.11.3. Removal of strippable DNA probe from the membrane

The membrane was stripped using the protocol and solutions provided with the Strip-EZ kit (Ambion Inc, USA) and the membrane sealed in a polythene bag and stored at -20 °C until required.

Table 2.11.2: Primers used to synthesize Northern probes

Species	Gene	NCBI No.	Primer Name	Sequence (5'-3')
<i>Solanum tuberosum</i>	CYPI	AJ245924	pCATHK	GCAGCTCACAGCTCAACTCTC
			pCATHK	CAAAGGCGTAGTCCATAAGACC
	ADRI	BG596098 & BG591972	pADR_1	ACAAACCTTTCCATGCACGAG
			pADR_4	CCCTTAACCAGTAGACCATT
	SGTI	BG599125	pSGT_1	TGAGCCCTAAGAACCCTGAA
			pSGT_3	TAGGGCTAAAGAGGCGTTCA
	SNFI	BI433152	pSNF_1	TGGATTGTTATTCCCCTGGA
			pSNF_2	GAGTTTGAGACGCCCATTC

2.12. Western Blot Analysis

Protein extraction and western-blot analysis were as previously described (Lacomme and Santa Cruz, 1999) with the exception of extraction buffer which consisted of an equal mix of 50 mM Potassium phosphate monobasic (KH_2PO_4) and Potassium phosphate dibasic (K_2HPO_4). Buffer was adjusted to pH 6.8 by adding monobasic or dibasic solution as required. For PARP-1 western 160 ng or 80 ng of bovine PARP was incubated with either Bovine Cathepsin B (Sigma-Aldrich Company Ltd, UK) or crude protein extracts with or without 1 mM Cathepsin B Inhibitor I, Ac-LVK-CHO (Calbiochem[®], Merck Biosciences, UK) diluted from 100 mM stock dissolved in SDW, adapted from D'Silva *et al.*, (1998). Pre-cast NuPAGE[®] Novex 4-12 % Bis-Tris gels (Invitrogen, UK). Mark12[™] unstained Protein Marker was used and visualised using Ponceau's stain (Sambrook *et al.*, 1998). Membranes were probed with rabbit polyclonal antiserum, raised against the PVX CP as previously described (Santa Cruz *et al.*, 1996) or polyclonal anti-PARP-1 as previously described in D'Silva *et al.*, (1998). Secondary anti-rabbit antibody (IgG) utilised was raised in goat and fused to alkaline phosphatase. One Tetrazolium tablet was dissolved in 10 ml of SDW, added to membrane and shielded from light for 0 - 4 hr to visualise protein.

2.13. Cathepsin B Activity

Frozen material was finely ground using liquid Nitrogen and baked pestle and mortar. 300 μ l of extraction buffer (50 mM Monobasic, 50 mM dibasic Potassium phosphate, pH6.8 and 1 mM DTT) was added and tissue homogenised. Protein samples were incubated on ice for 15 min before being spun at max speed for 15 min to pellet cell debris. Supernatant was transferred to fresh Eppendorf tube (Eppendorf Ltd, UK) and kept on ice or frozen at -70 °C. Assays performed using clear 96 well microtitre plates (Fisher Scientific, UK) and DIAS plate reader (Dynatech Laboratories, UK).

2.13.1. Bradford Assay

Bradford reagent (Bio-Rad, UK), was diluted 1:5 with SDW, aliquoted 199 μ l to each well of 96 well microtitre plate and 1 μ l of protein extract was subsequently added and gently mixed by pipetting. Reaction left for 30 min at room temperature and optical density measured at 595 nm. Protein concentration was calculated using formula derived from a Bovine serum albumin dilution series ranging from 0 – 6 μ g ($y = 0.2186x + 0.3304$).

2.13.2. Cathepsin B Substrate I - Colorimetric Assay

Cathepsin B colorimetric substrate (Calbiochem[®], Merck Biosciences, UK) was diluted to 1 mM in SDW. Assay buffer was prepared with SDW as recommended by Calbiochem[®] (46.3g/L Sodium acetate; 4 mM Disodium EDTA; 60 mM Acetic Acid and 8 mM DTT). Assay buffer and colorimetric substrate were mixed together as equal volumes then mixed well with 1.8 x volume of SDW. 140 μ l of buffer/substrate solution was aliquoted into each well of 96 well microtitre plate and 10 μ l of protein extract added, mixed and incubated at room temperature for 30 min. The optical density was read at 405 nm using DIAS plate reader. Protein activity was divided by total protein (mg/ml) in crude extracts calculated using Bradford Assay, reading optical density at 595 nm. Assay was repeated using Cathepsin B Inhibitors diluted to 1 mM from 100 mM stocks kept at -80 °C (Cathepsin B, S and L inhibitor, Z-FGNHO-Bz; Cathepsin B

inhibitor I, Z-FA.fmk; Cathepsin B inhibitor II, Ac-LVK-CHO and Cathepsin B Inhibitor IV, CA-074 Me) (Calbiochem[®], Merck Biosciences, UK).

2.14. Quantification of Anthocyanin

Leaf samples (100 mg) from six individual plants per line were collected. The tissue samples were ground in 300 µl of acidified methanol (1 % HCl v/v) and incubated for 2 hr at 30 °C. Following centrifugation (10,000 x g for 10 min) the supernatant was transferred to a new tube. Two volumes of chloroform were then added and the samples vortexed before centrifugation (10,000 x g for 5 min). The supernatant (methanol phase) was carefully collected and transferred to a new tube. The chloroform step was repeated and the supernatant collected and added to 600 µl of water and vortexed. This fraction was read spectrophotometrically at 550 nm for anthocyanin.

2.15. Homology searches and sequence analyses

The bioinformatics tools from web-based databases presented in Table 2.15 were used for sequence searches and analysis. Homology searches in *Arabidopsis* were carried out using BLAST at the TAIR website. Searches which included sequences from *Nicotiana*, *Lycopersicon* and *Solanum* species were performed using the nucleotide and protein databases at the NCBI website. Alignments were performed using both Multialign website and the bl2seq (at NCBI website).

Table 2.15: Web-sites used for sequence search and analysis

Name	Function	Web address
BLAST	Homology search in <i>Arabidopsis</i>	http://www.arabidopsis.org/Blast/
	Homology search between species	http://www.ncbi.nlm.nih.gov/blast/
Multialign	Multiple alignment of nt/aa sequences	http://prodes.toulouse.inra.fr/multialin.html
bl2seq	Alignment of 2 nt/aa sequences	http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html
<i>At</i> R genes	<i>Arabidopsis</i> resistance Genes Database	http://niblrrs.ucdavis.edu/At_RGenes/
The Arabidopsis Information Resource	Arabidopsis genome annotation and functional genomics resources	http://www.arabidopsis.org/Index.jsp
National Center for Biotechnology Information (NCBI)	Cross-database search engine for nucleotide sequences, protein sequences and publications from which they originate	http://www.ncbi.nlm.nih.gov/Entrez/index.html
Salk Institute Genomic Analysis Laboratory (SIGnAL)	Sequence-Indexed Library of Insertion Mutations in the Arabidopsis Genome	http://signal.salk.edu/about.html
The European Cultivated Potato Database	A resource for potato breeders, scientists and farmers	http://www.europotato.org

3. Isolation and Gene Identification of Susceptible Activation Tagged *Arabidopsis* Mutants

3.1. *The Model Plant: Arabidopsis*

The flowering weed, *Arabidopsis*, was first used as a model organism by Luiback, in 1943, because of its small size, short life cycle (6 weeks) and large seed production (Meinke *et al.*, 1998; Dennis and Surridge, 2000). However, research using *Arabidopsis* for genetic analysis of disease resistance pathways was only established in the late 1990's (Buell, 1998). The *Arabidopsis* Genome Initiative (AGI) was formed in 1996 to co-ordinate complete sequencing of the genome to provide plant scientists with a genetic blueprint for a simple model plant. The genome sequence was completed in 2000 and revealed that functional redundancy may be extremely common because around 70 % of genes have been duplicated (Walbot, 2000).

3.2. *Development of Gain-of-Function Screens*

Classical mutant screens have been used extensively as a means of dissecting genetic pathways and have traditionally utilised loss-of-function mutants generated by transposable elements, neutron particle bombardment, T-DNA tagging and ethyl methane sulfonate (EMS) mutagenesis. EMS and irradiation efficiently generate large numbers of mutations that effectively saturate the genome. However, identifying the mutated gene by map-based cloning can be arduous. On the other hand, T-DNA tagging and transposable elements provide a molecular anchor from which to determine the surrounding DNA sequence (Tani *et al.*, 2004). In addition, loss-of-function mutagenesis rarely identifies functionally redundant or essential genes. Due to the high degree of gene duplication in the *Arabidopsis* genome and therefore other plant genomes (Bevan *et al.*, 1998), a number of methods have been designed to promote identification of these redundant or essential gene families (Tani *et al.*, 2004). By identifying regions highly conserved (> 90 % identity) between all genes in a family, and cloning them into a silencing vector, the function of the gene family as a whole can be investigated. In

addition, virus-induced gene silencing in adult plants may circumvent potential lethality of given loss-of-function phenotypes at earlier developmental stages. However, this approach requires prior knowledge of the candidate gene and gene family to be investigated. Enhancing the expression of one gene family member is another method of investigating functionally redundant genes and has been previously shown to generate recognizable phenotypes e.g. MyoD in fibroblasts (Davis *et al.*, 1987). Therefore a method of generating enhanced expression of random genes was required to identify gain-of-function mutants and concomitantly novel genes. A method known as “activation tagging” has subsequently been developed that takes advantage of the strong activating properties of the cauliflower mosaic virus (CaMV) 35S gene enhancer (Weigel *et al.*, 2000). The pSKI015 T-DNA vector has been manipulated to contain four CaMV 35S enhancers and the *BAR* gene, a selectable marker which conveys resistance to the herbicide ammonium glufosinate (commonly known as Basta) (Figure 3.2, A). Insertion of this vector into the genome may cause a loss-of-function phenotype (Figure 3.2, B). However, the activation of genes adjacent to pSKI015 can occur at distances from 380 bp to 3.6 kb (Weigel *et al.*, 2000) (Figure 3.2, B). An advantage of using the tagged pSKI015 vector is that many protocols have been developed to isolate adjacent genomic sequence. One PCR-based technique, known as thermal asymmetric interlaced (TAIL) PCR, uses nested primers specific for the 35S enhancers with degenerate primers which may anneal in the adjacent genomic DNA (Liu *et al.*, 1995). In addition, a plasmid rescue procedure can also be performed to digest the T-DNA vector out of the genome with some adjacent genomic DNA (Ichikawa *et al.*, 2003). There are a few drawbacks with T-DNA-based technologies, including: non-random vector insertion preventing complete genome saturation; and the presence of insulator sequences in the genome which may protect some genes from activation (Weigel *et al.*, 2000; Jeong *et al.*, 2002). This activation gain-of-function strategy has been utilised successfully in *Arabidopsis* and is now being applied to other plant species, particularly the second fully sequenced plant and model monocot, rice (Jeong *et al.*, 2002).

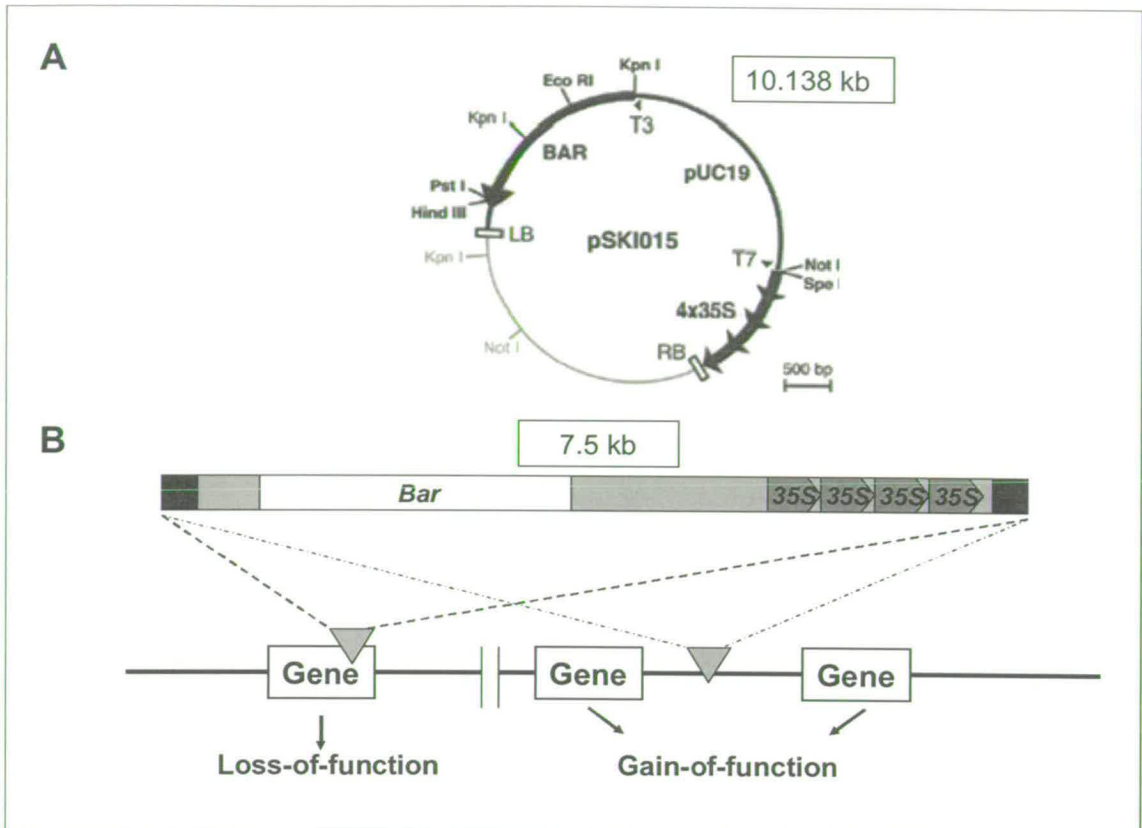


Figure 3.2: Activation tagging vector pSKI015 and two possible insertion events. **A**, Map of pSKI015 vector (Accession no, AF187951), including *BAR* gene for herbicide resistance and 4 x 35S CaMV enhancers (adapted from Weigel *et al.*, 2000). **B**, Insertion of vector into a gene may result in a loss-of-function phenotype and insertion adjacent to genes may result in a bi-directional activation of one or more genes resulting in a gain-of-function phenotype.

3.3. Activation Tagging for Negative Regulators of Disease Resistance

Classical mutagenesis screens for loss-of-function mutants in race-specific disease resistance pathways have predominantly identified mutant alleles of the cognate *R* gene e.g. *RPM1* (Bisgrove *et al.*, 1994) and only a few additional genes. Similarly, loss-of-function screens undertaken in a number of laboratories for insensitivity to SA have only uncovered *npr1/nim/sai1/eds16* (Cao *et al.*, 1997; Ryals *et al.*, 1997; Shah *et al.*, 1997). Therefore, many defence signalling components may be encoded by either redundant or essential genes. Hence, activation represents an attractive research strategy. In this

context, activation tagging has been successfully utilised to identify defence genes such as the cathepsin D-like *CDRI* and the *R* gene-like *ADRI* (Xia *et al.*, 2004; Suzuki *et al.*, 2004; Grant *et al.*, 2003). Nearly all screens of *Arabidopsis* have focused on loss-of-function or gain-of-function mutations in genes required for disease resistance induced by avirulent, non-host or necrotrophic pathogens (Eckardt, 2002). Virulent pathogens grow aggressively *in planta*, which can lead to recognition of infection, inducing SA-dependent defence responses that are often too late to prevent disease (Crute and Pink *et al.*, 1996). However, few screens have been developed to identify genes that could be involved in response to virulent pathogens and therefore many basal defence responses. Most *R* genes bred into commercial crops have not been durable in the field (Kearney and Staskawicz, 1990; Li *et al.*, 2001). Therefore identifying genes involved in basal resistance could provide alternative targets for breeding durable resistance in crop species (Stuiver and Custers, 2001; Wichmann and Bergelson *et al.*, 2004).

Despite the possible applications of these types of genes, screens identifying over-expressed suppressors of basal disease resistance have not been published thus far. A few papers describing enhanced disease susceptibility (*eds*) mutants caused by loss-of-gene function defining positive regulators of basal resistance have been described (Parker *et al.*, 1996; Rogers and Ausubel, 1997; Volko *et al.*, 1998; Reuber *et al.*, 1998). Therefore, it was of interest to develop a screen for activated disease susceptibility. This strategy was designed to uncover novel genes encoding negative regulators of SA-dependent disease resistance.

3.4. Screen for Mutants Susceptible to Virulent Pst DC3000

Previously, Col-0 *Arabidopsis* plants were transformed with the activation tagging vector pSKI015 generating a T₀ population which were left to self-pollinate giving rise to approximately 20 million T₁ seeds (Tani *et al.*, 2004) supported by Akadix Inc. (CA, USA). The T₁ transformed lines are heterozygous for the pSKI015 insert and lines that survived repeated spraying with the commercially available herbicide Basta must contain

at least one T-DNA insertion. Basta resistant lines were transferred from large flats to pots and left until plants of all genotypes were a suitable size for pathogen inoculation. Plants were then pressure infiltrated with *Pst* DC3000 in a bacterial suspension of $OD_{600} = 0.0003$ (10^5 CFU/ml). This inoculum does not induce disease symptoms in Col-0 plants but does induce chlorosis at 4-5 days post-inoculation (dpi) in the susceptible mutants *nahG* and *eds1* (Lawton *et al.*, 1995; Parker *et al.*, 1996). In order to check the aggressiveness of the bacteria used for every screening event, six Col-0, *nahG* and *eds1 Arabidopsis* plants were pressure infiltrated with the inoculum used for screening. Putative susceptible candidates were selected if symptoms were comparable to inoculated *eds1* and *nahG* plants. Putative susceptible candidates were then labelled and left to set seed. The lines which did not show any increased susceptibility were left to flower and seed was collected as a pool per tray (70 plants) for future screens.

In addition, plants displaying abnormal morphological phenotypes were also kept for seed collection. Frequently, ectopic over-expression of ordinarily inducible defence pathways produce plants of reduced stature (Frye and Innes, 1998; Maleck *et al.*, 2002; Pilloff *et al.*, 2002). Overall, more than half of the T₁ seed, corresponding to about 1.8 million seeds and 6,000 individual T₁ tagged plants (transformation rate of approximately 0.3 %) were screened for disease susceptibility. Twelve individual candidate lines that conveyed disease susceptibility were identified for further analysis.

3.4.1. Heritability of the Activation Tagging Vector

The following generation (T₂) of all twelve susceptible mutants were analysed for the inheritance of the pSKI015 vector by Basta spraying. As progeny of a heterozygous mutant with a single pSKI015 insertion, Basta treatment should result in a segregation ratio of 3:1 for Basta resistance to sensitivity. Identifying the gene responsible for the disease susceptibility phenotype in a line with more than one insert would require generations of back-crossing with Col-0 to identify the insertion that links with the disease susceptibility phenotype. Table 3.4.1 reveals the segregation ratio of mutant candidates firstly inoculated with virulent *Pst* DC3000 and then sprayed with Basta.

The putative susceptible candidate line L had a segregation for Basta resistance of 55/75 = 73 % (3:1) and 53/75 = 71 % (2.5:1) for disease susceptibility. N displayed 52/73 = 71 % (3:1) segregation for Basta resistance and 47/73 = 64 % (2:1) for disease susceptibility making them the most suitable lines for further investigation. Hereafter, susceptible candidate L will be referred to as activated disease susceptibility 1 (*ads1*) and N as activated disease susceptibility 2 (*ads2*). The ratios obtained from susceptibility screening and Basta resistance did not fit the perfect Mendelian ratio and could possibly be due to biased selection of seedlings at time of transplant, sensitivity of mutants to transplant procedure or inadequate *Pst* DC3000 inoculation of all plants, especially smaller homozygous mutants.

Table 3.4.1: Initial segregation analysis on the T₂ progeny of susceptible candidates for disease susceptibility to *Pst* DC3000 and Basta resistance.

Mutant	Total	Susceptible to <i>Pst</i> DC3000	Resistant to <i>Pst</i> DC3000	Ratio	Basta sensitive	Basta resistant	Ratio
A	70	27	43	1:2	6	64	1:11
B	70	26	44	1:2	26	44	1:2
C	70	12	58	1:5	70	0	1:0
G	70	10	60	1:6	14	56	1:4
J	63	16	47	1:3	0	63	0:1
L	75	53	22	2.5:1	20	55	1:3
M	72	17	55	1:3	72	0	1:0
N	73	47	26	2:1	21	52	1:3
O	47	23	24	1:1	21	26	1:1
P	48	17	31	1:2	36	12	3:1
Q	48	23	25	1:1	32	16	2:1
R	48	17	31	1:2	6	42	1:7

In total, approximately 75 % (226/297) of the T₂ progeny of *ads1* retained the susceptible phenotype and Basta resistance. The sum of *ads2* plants that retained the susceptibility phenotype and Basta resistance was approximately 73 % (166/228). All plants exhibiting a wild-type phenotype, about 25 % of *ads1* (71/297) and 27 % (62/228) of *ads2* were BASTA susceptible and did not show chlorotic symptoms post-inoculation with *Pst* DC3000 at 4 dpi. These results indicate that a single dominant mutation was responsible for each of these mutant disease susceptibility phenotypes.

3.5. Characterisation of *Pst* DC3000 Susceptibility in *ads* Mutants

3.5.1. Disease susceptibility to 10^5 CFU/ml inoculum of virulent *Pst* Dc3000

Following determination of disease susceptibility to virulent *Pst* DC3000 co-segregating with Basta resistance, confirmation and quantification of the extent of susceptibility to virulent *Pst* was required. A bacterial suspension of 10^5 virulent *Pst* DC3000 was pressure infiltrated into *Arabidopsis* Col-0, *nahG*, *eds1* and *ads* mutants. Bacterial colony counts were performed 4 dpi to provide an indication of whether *ads* mutants were more or less susceptible to virulent *Pst* DC3000. Unfortunately, dwarfed homozygous *ads2* mutants were too small to inoculate adequately by pressure infiltration and therefore bacterial growth was evaluated from both heterozygous *ads1* and *ads2* plants. Due to time constraints, dipping or spraying of *Pst* DC3000 over *Arabidopsis* was not optimised or quantified. In addition, these mutants were originally identified during the susceptible screen as heterozygous mutants in the T₁ and should therefore still possess a dominant susceptible phenotype. The resulting viable colony counts displayed in Figure 3.5.1 revealed that heterozygous *ads1* and *ads2* allowed 2-log greater virulent *Pst* growth but almost 1-log less than the well characterised transgenic plant *nahG* and the homozygous recessive mutant *eds1*. This experiment was repeated twice with similar results.

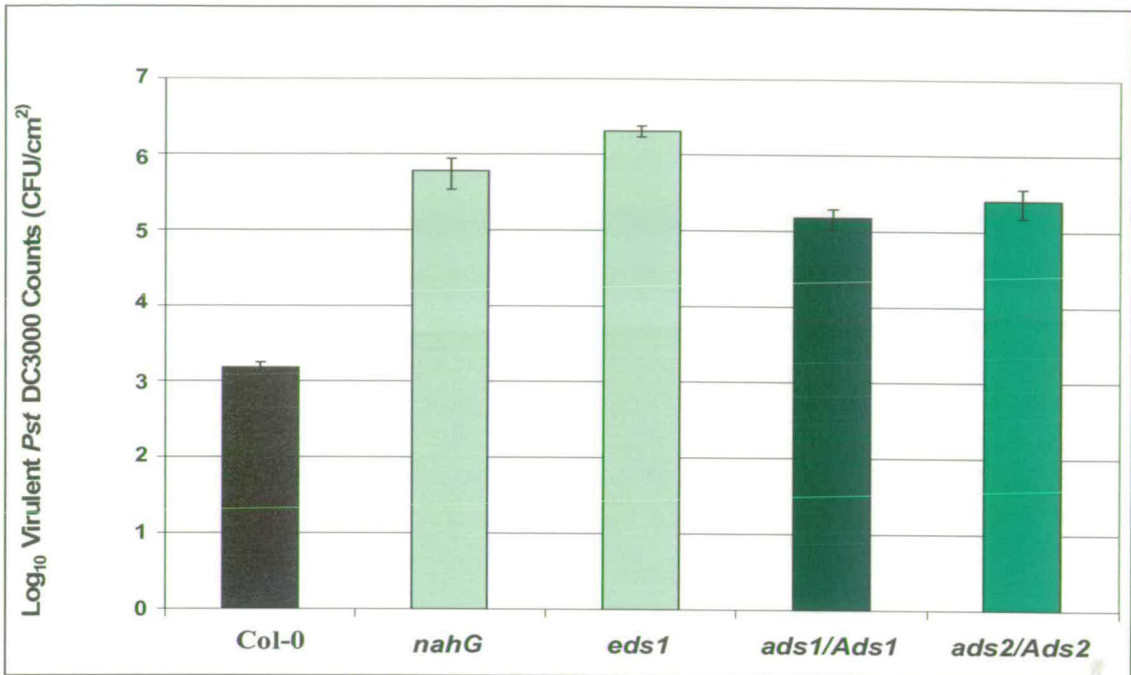


Figure 3.5.1: Growth of low OD suspension of *Pst* DC3000 in Col-0 and associated mutants. Comparison of colony forming units (CFU) recovered from wild-type (Col-0) and susceptible mutants 4 dpi with a suspension of 10^5 CFU/ml of *Pst* DC3000 ($OD_{600} = 0.0003$). Error bars represent standard error from 8 plants in one biological replicate (SE).

3.5.2. Response of *ads* mutants to 10^6 CFU/ml inoculum of *Pst* DC3000

The bacterial suspension of 10^5 CFU/ml used for screening T_1 activation tagged population for susceptible mutants and inoculated to produce Figure 3.5.1 (shown above) does not produce disease symptoms in Col-0. Following this, it was examined whether the difference in resistance to *Pst* DC3000 in *ads* mutants and Col-0 would differ when using a greater inoculum strength of 10^6 CFU/ml ($OD_{600}=0.002$) which does causes disease symptoms in Col-0 (Figure 3.5.2). The susceptible control mutant *nahG* was only 10-fold more susceptible than Col-0 using a suspension of 10^6 CFU/ml *Pst* DC3000. The *ads1* heterozygous mutant was 5-fold more susceptible to *Pst* whereas *ads1* homozygous mutant was as susceptible to *Pst* DC3000 as *nahG*. In addition, *ads2* was almost as susceptible to *Pst* DC3000 as *nahG* and *ads1*. Viable colony counts were performed twice with similar results.

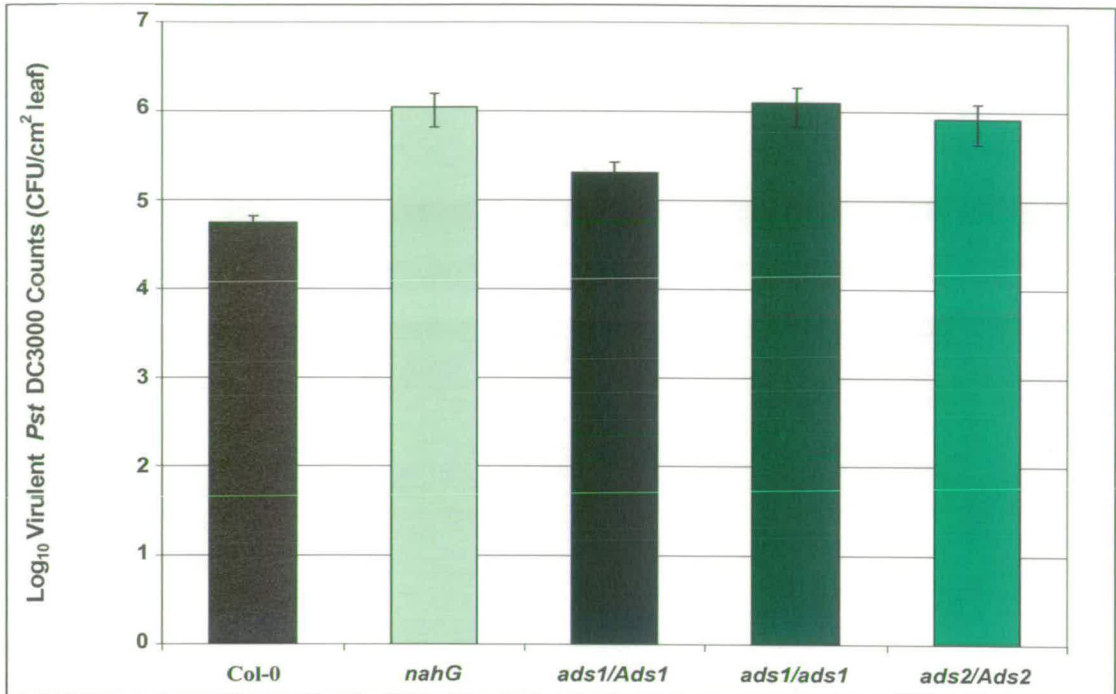


Figure 3.5.2: Growth of high OD suspension of *Pst* DC3000 in Col-0 and associated mutants. Graph displays viable colony counts of *Pst* DC3000 from plants 4 dpi with 10^6 CFU/ml, error bars represent SE from six individual plants in one biological replicate. Error bars represent SE of one biological replicate with five plants per genotype.

3.5.3. Response of *ads* mutants to avirulent *Pst* DC3000 (*avrB*)

Although these mutants are susceptible to *Pst* DC3000, it was of interest to investigate whether these lines were also susceptible to *Pst* DC3000 (*avrB*). Therefore, the recovery of bacteria from inoculated leaves was performed and counted from *ads* mutants using 10^6 CFU/ml suspension of *Pst* (*avrB*). Figure 3.5.3 reveals that the *nahG* mutant is around 1.5-logs more susceptible to *Pst* DC3000 (*avrB*). Similarly, *ads1* heterozygous mutant was shown to be almost 2-logs more susceptible and displayed extensive chlorosis. On the other hand, *ads2* appeared to be as susceptible as Col-0 with the same inoculum although displayed a stronger HR. Therefore, *ads1* is as susceptible to both virulent *Pst* DC3000 and avirulent *Pst* DC3000 (*avrB*) in a similar fashion to *nahG* plants. Interestingly, *ads2* appears to be only perturbed in basal defence responses and reacts like Col-0 in response to avirulent *Pst* DC3000 (*avrB*). This experiment was repeated twice with similar results.

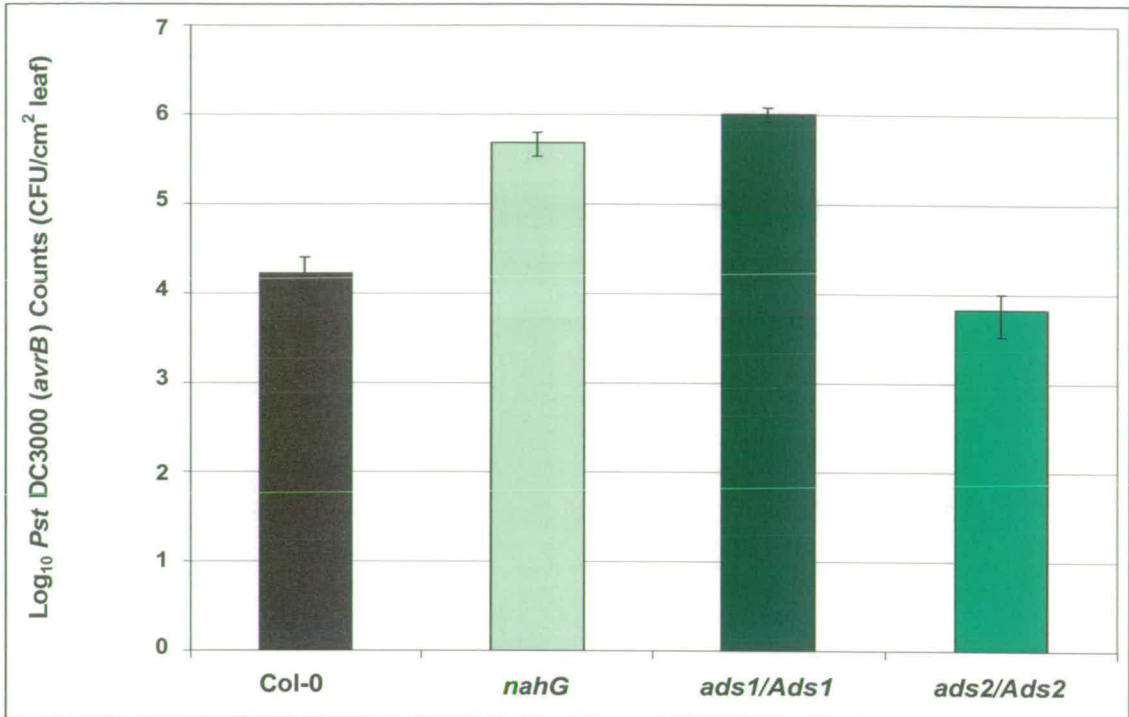


Figure 3.5.3: Growth of *Pst* DC3000 (*avrB*) in *ads* mutants and associated lines. Graph displays the recovery of viable avirulent *Pst* DC3000 (*avrB*) from plants 9 dpi with suspension of 10⁶ CFU/ml. Error bars represent SE from one biological replicate with six individual plants per line.

3.5.4. Hydrogen peroxide accumulation and cell death in *ads* mutants

As discussed in Chapter 1, hydrogen peroxide (H₂O₂) is a signalling molecule involved in both inducing the HR and, to a lesser extent, in basal resistance (del Rio *et al.*, 2002). Since both *ads* mutants respond differently to *Pst* DC3000 (*avrB*) shown by examining resistance, it was proposed that early defence signalling such as H₂O₂ may be perturbed in these mutants. The stain, 3, 3-diaminobenzidine (DAB) is oxidised to produce an insoluble brown substrate by peroxidase which simultaneously reduces hydrogen peroxide (Thordal-Christensen *et al.*, 1997). Therefore, DAB was used to examine H₂O₂ production in *ads* mutants (Figure 3.5.4a). As expected, Col-0 does not accumulate H₂O₂ before inoculation, a small amount after inoculation with virulent *Pst* DC3000 4 hpi and to a greater extent 4 hpi with avirulent *Pst* DC3000. Transgenic *nahG* plants which has both perturbed basal and HR defences did not accumulate H₂O₂ after any treatments tested. Interestingly, *ads1/ADS1* displayed significant accumulation of H₂O₂ in untreated plants, and massive accumulation after inoculation with both virulent *Pst*

DC3000 and avirulent *Pst* DC3000 (*avrB*). The *ads2/ADS2* mutant displayed more acute H₂O₂ accumulation at the site of inoculation in response to both *Pst* DC3000 (*avrB*) and, to a lesser extent, with virulent *Pst* DC3000.

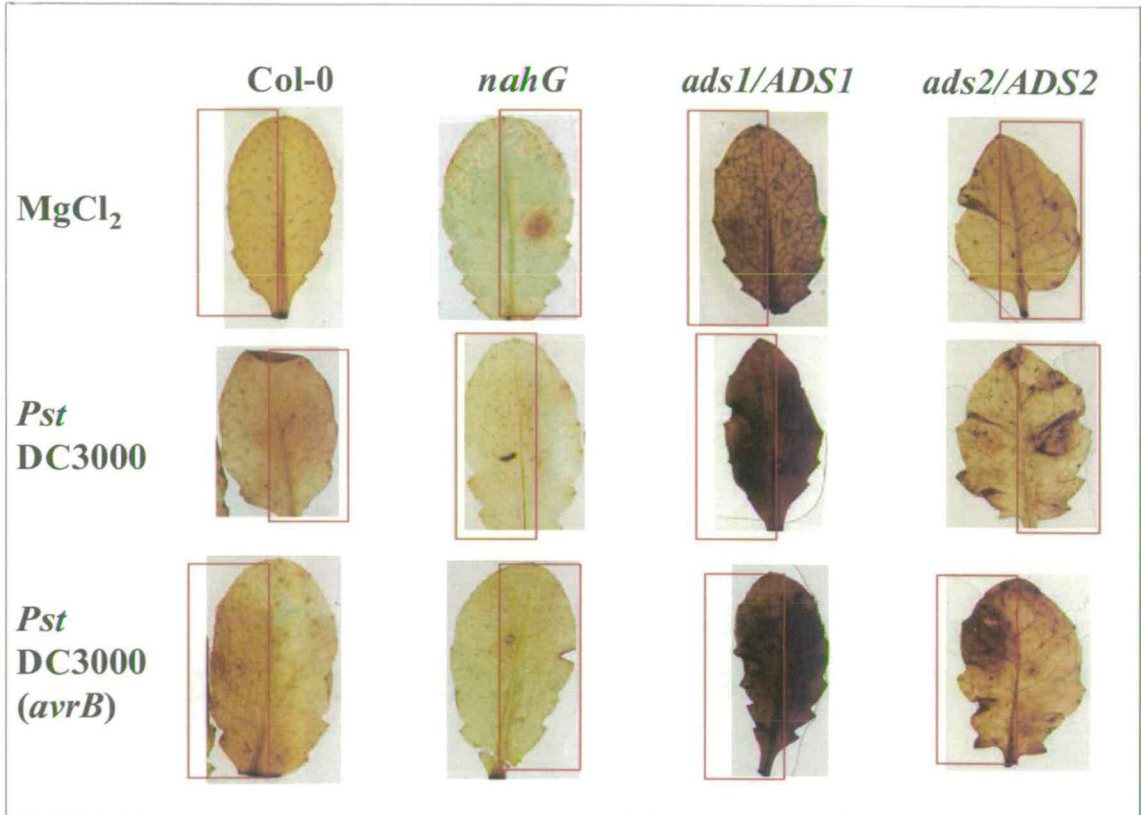


Figure 3.5.4a: Hydrogen peroxide accumulation in whole leaves of *ads* mutants. Leaves DAB stained 4 hpi after half leaves (red box) were infiltrated with buffer or with bacterial suspension of 10⁵ CFU/ml of *Pst* DC3000 or *Pst* DC3000 (*avrB*). Three biological replicates produced similar results.

Stained leaves were also examined under the microscope to observe the H₂O₂ accumulation at the cellular level (Figure 3.5.4b, A). Examination of DAB stained leaves at the cellular level revealed that *ads1* mutant plants accumulate H₂O₂ extensively and uniformly throughout the leaf tissue, and production increases further in response to virulent and avirulent *Pst* DC3000. In Col-0 plants, H₂O₂ accumulation is more localised to sites of pathogen growth. In addition, *ads2* plants display patchy H₂O₂ accumulation in response to *Pst* DC3000 (*avrB*). In addition to DAB staining, trypan blue staining was performed to visualise cell death in leaves at 12 hpi. Trypan blue is a stain that can distinguish viable cells from non-viable cells, but cannot distinguish

between cells killed by disease or by the HR. Live, healthy cells appear round without absorbing the blue dye, whereas dead cells appear blue and, in later stages asymmetrical (Yun *et al.*, 2003). After inoculation Col-0 responds to *Pst* DC3000 (*avrB*) with an extensive HR by 12 hpi and with absent or limited cell death after virulent *Pst* DC3000 infection (Figure 3.5.4b, B). The *nahG* mutant displays limited cell death in response to both virulent and avirulent *Pst*. The *ads1/ADS1* mutant shows more cell death than *nahG* in response to virulent *Pst* DC3000 and *Pst* DC3000 (*avrB*). The mutant *ads2/ADS2*, displayed similar responses to *Pst* DC3000 and *Pst* DC3000 (*avrB*) as *nahG*. In addition, *ads2/ADS2* displayed less cell death in the form of asymmetric cells as Col-0 in response to *Pst* DC3000 (*avrB*).

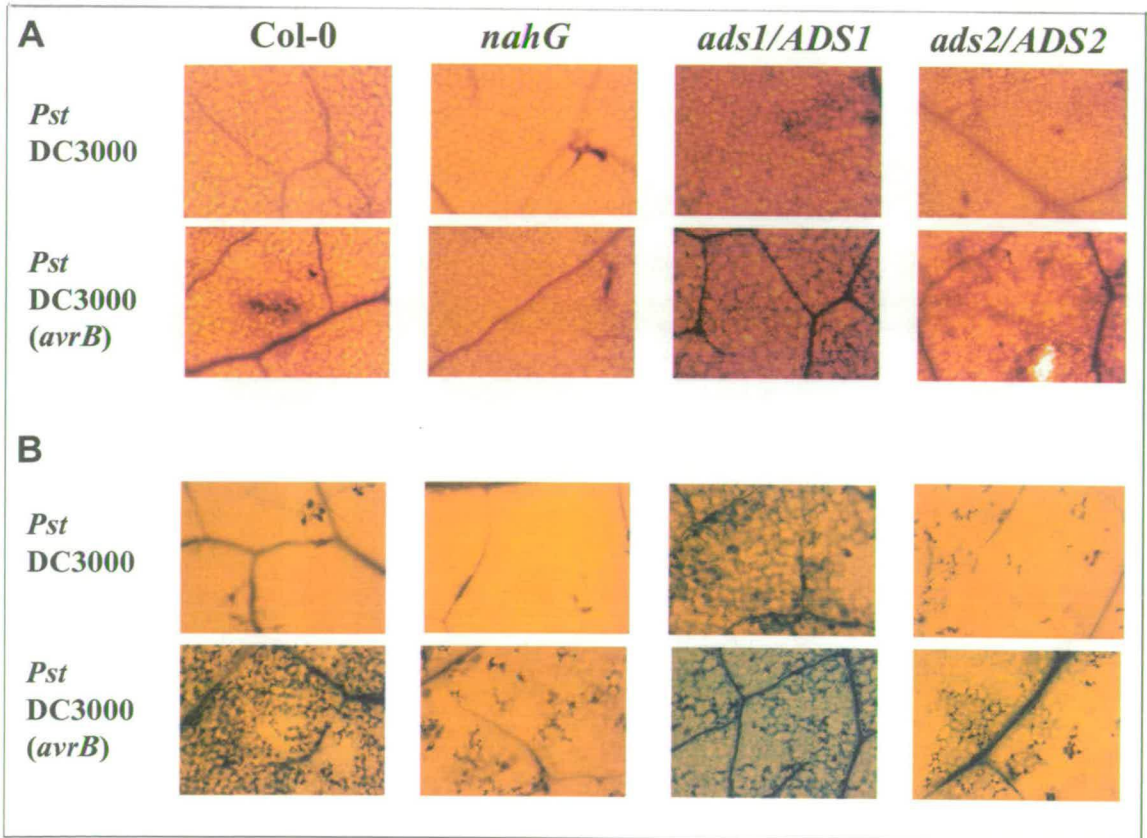


Figure 3.5.4b: Microscopic images of hydrogen peroxide accumulation and cell death. A, DAB staining for H₂O₂ at 4 hpi with either virulent or avirulent *Pst* DC3000. B, Trypan blue staining for dead cells at 12 hpi with either virulent or avirulent *Pst* DC3000. Five leaves per time point were stained for each genotype and pathogen challenge. Experiment was repeated three times with similar results.

3.5.5. Anthocyanin accumulation in *ads2* mutant

Closer inspection of the *ads2* homozygous mutant revealed that anthocyanin was strongly accumulating in most leaves by 21 days old. Anthocyanins are antioxidant compounds produced by the stress-inducible flavanoid biosynthesis pathway that include the enzymes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), which also produces antimicrobial phytoalexins (VanEtten *et al.*, 1994; Abraham *et al.*, 1999). Light-induced production of anthocyanin has been shown to decrease after fungal pathogen challenge which induces an increase in phytoalexins production (Lo and Nicholson, 1998). There has also been a report that anthocyanins have a strong inhibitory effect on nitric oxide production in macrophages (Wang and Mazza, 2002). Anthocyanin was extracted from 100 mg of leaf tissue from six individual Col-0 and *ads2* homozygous and heterozygous mutants using acidified methanol, purified using chloroform and absorbance of the methanol phase was read at 537 nm and 650 nm. The difference between these two readings differentiates between anythocyanin and other flavanols. Figure 3.5.5 shows that both the untreated heterozygous and homozygous *ads2* mutants accumulated more anthocyanin than Col-0 at the same age. The clear visible phenotype associated with anthocyanin accumulation in homozygous *ads2* can be observed in the photographs also shown in Figure 3.5.5.

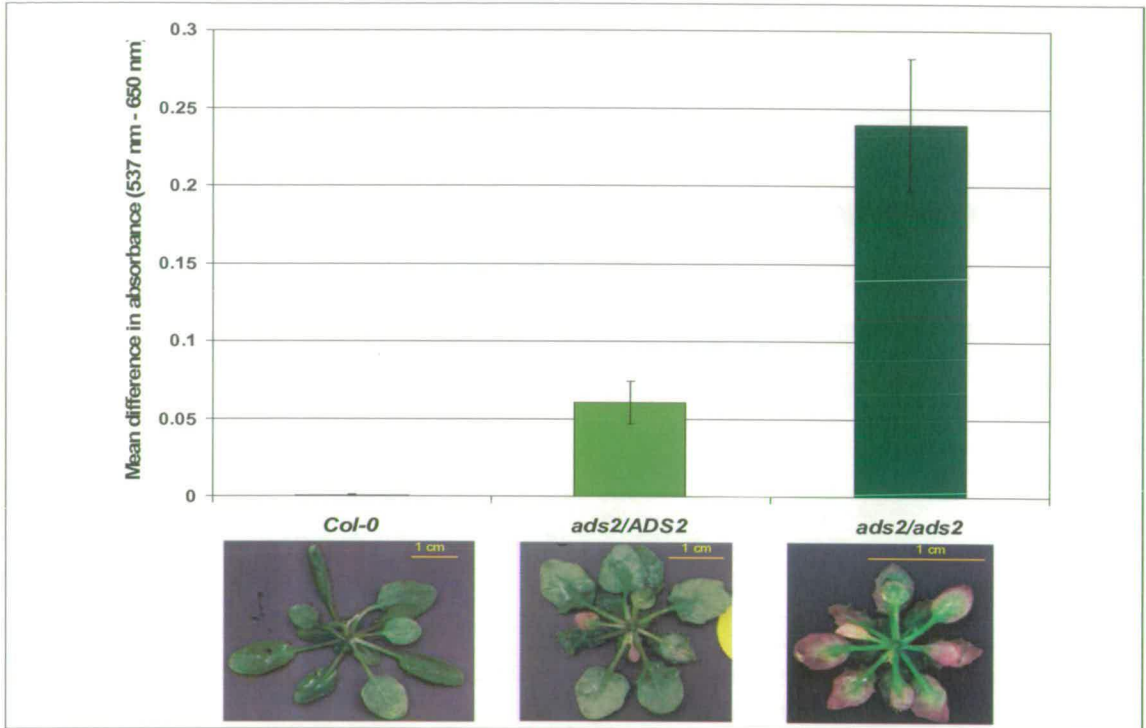


Figure 3.5.5: Anthocyanin accumulation in *ads2* mutant lines and Col-0. Anthocyanin was extracted from six individual plants of each genotype, one plant photographed below. Values in graph represent the mean of all six plants from two biological replicates. Error bars represent SE.

3.5.6. Morphological phenotypes of *ads1* and *ads2* candidates

Both *ads1* and *ads2* developed unusual morphological phenotypes that segregated with the Basta resistance phenotype, susceptibility to virulent *Pst* and therefore with the activation tag (Figures 3.5.6a and 3.5.6b). The co-dominant mutant, *ads1* displayed a loss-of-apical dominance phenotype which resulted in both heterozygous and homozygous mutants commencing flowering approximately two weeks before Col-0 in long day conditions (Figure 3.5.6a). The leaves and siliques of the heterozygous *ads1* mutants were smaller than wild type and exhibited a dwarfed stature with perturbed leaf architecture. In addition, florescences grew to a maximum height of 6 cm compared with Col-0 florescences (~20 cm). Homozygous *ads1* mutants exhibited a more severe dwarfed stature, normal leaf shape but smaller leaf size and similar leaf architecture as heterozygous plants. In addition, homozygous *ads1* mutants produced little or no seed, as normal silique development did not commence after flowering from 4 cm tall.

Interestingly, the majority of older leaves of both homozygous and heterozygous mutants had senesced by six weeks.

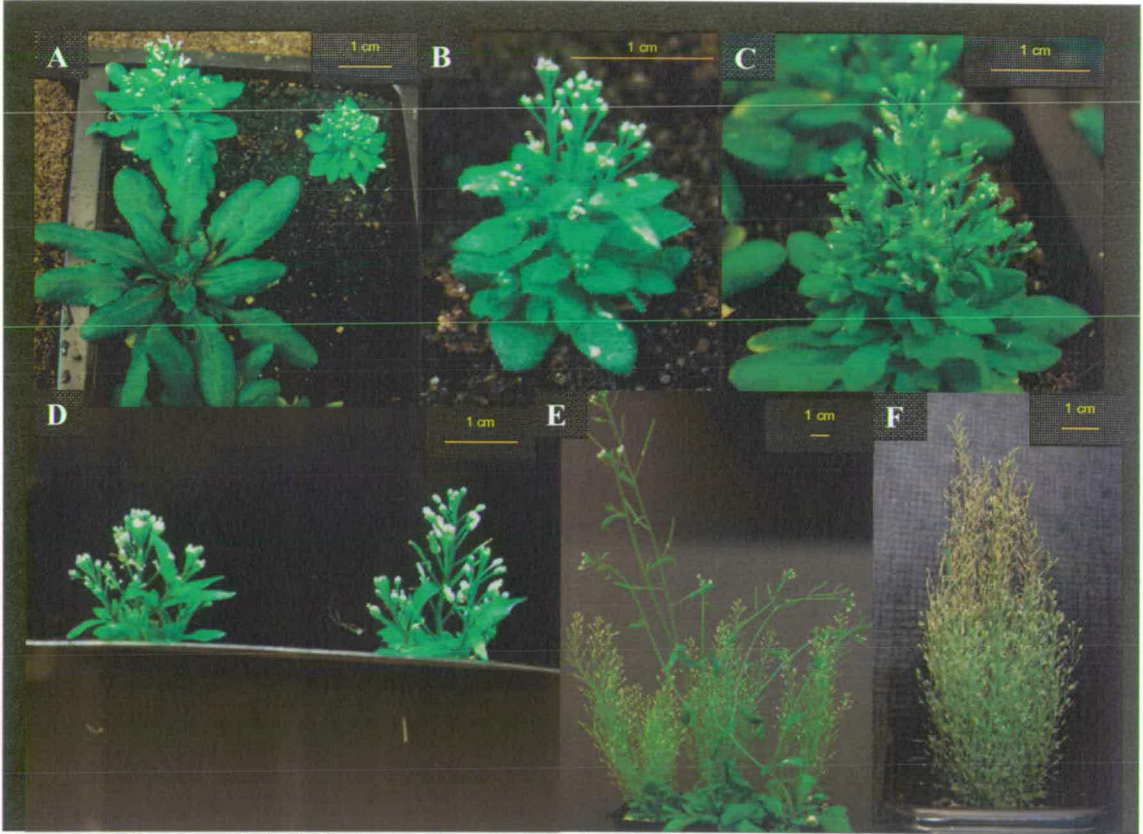


Figure 3.5.6a: Morphological phenotypes of *ads1*. **A**, *ads1* heterozygous mutant (top left), homozygous mutant (top right) and Col-0 at 4 weeks old. **B**, homozygous *ads1* mutant at 4 weeks. **C**, heterozygous of *ads1* mutant at 4 weeks. **D**, side view of 4 week old homozygous *ads1* mutants. **E**, *ads1* mutants and Col-0 of same age in background for comparison at 6 weeks. **F** is the original T₁ mutant identified from susceptibility screen.

The co-dominant *ads2* mutants also displayed a distinctive morphology. Heterozygous, *ads2* mutant leaves possessed an undulating surface and short petioles although their size was only slightly smaller than Col-0 (3.5.6b). The heterozygous *ads2* mutant was fertile although the siliques were smaller than Col-0. The homozygous *ads2* mutant was also dwarfed in comparison with Col-0 (Figure 3.5.6b, A), possessed very small and narrow leaves that displayed a perceptible accumulation of anthocyanin from four weeks (Figure 3.5.6b, D). In addition, the homozygous *ads2* mutant was infertile as flowers failed to open and senesced early (Figure 3.5.6b, E).

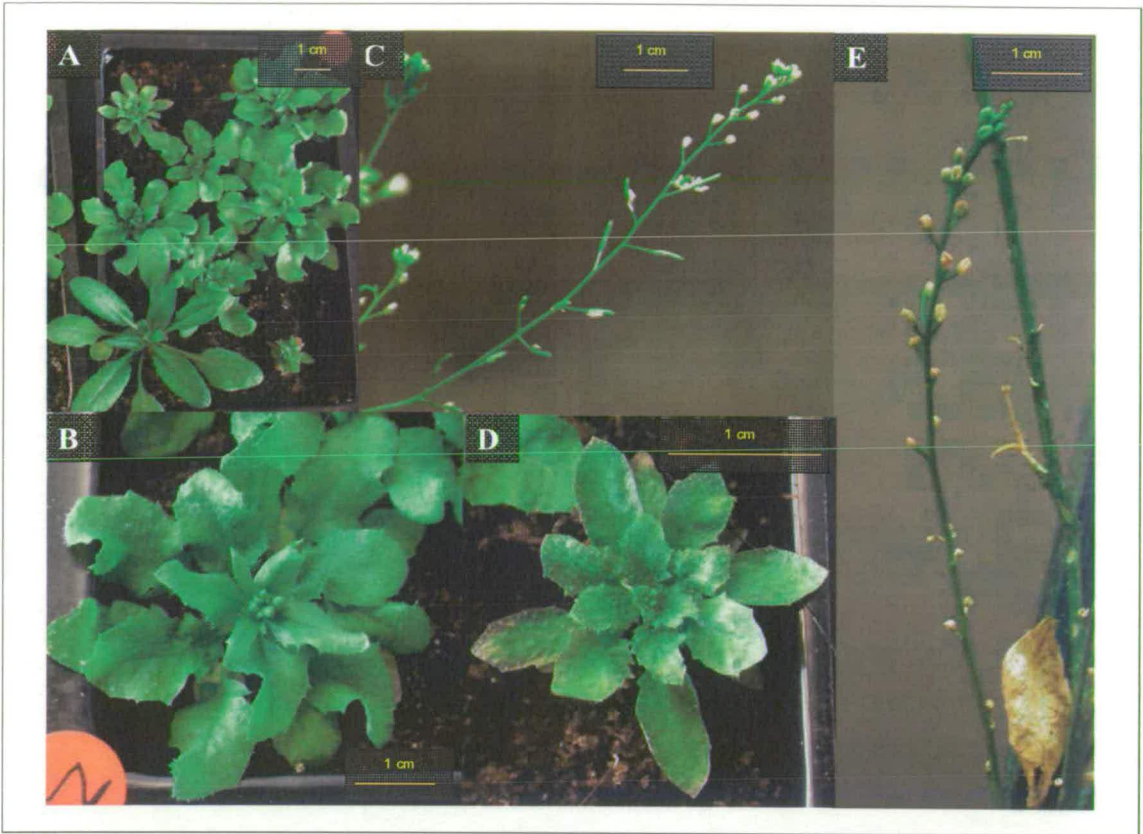


Figure 3.5.6b: Morphological phenotypes of *ads2*. **A**, *ads2* mutant T2 progeny at 4 weeks old. **B**, close up of heterozygous *ads2* mutant at 4 weeks. **C**, close up of heterozygous *ads2* mutant florescence at 4 weeks. **D**, close up of homozygous *ads2* mutant at 4 weeks. **E**, close up of homozygous *ads2* mutant florescence which shows no silique development as compared with heterozygous at same stage (**C**).

3.6. Defence Signalling Pathways in *ads* Mutants

SA, ET and JA have each been demonstrated to have a role in regulating basal resistance responses induced against different pathogens (Ton *et al.*, 2002). The SA-dependent pathway is associated characteristically with defence mechanisms induced against avirulent and virulent biotrophic pathogens, whereas ET- and JA- dependent pathways predominantly regulate defences against necrotrophic pathogens (Bent *et al.*, 1992, Lund *et al.*, 1998; Greenberg *et al.*, 2000). However, the *Arabidopsis* mutant, named constitutive expression of *Vsp1* (*cevl*), possesses enhanced resistance to a range of biotrophic powdery mildew fungi and constitutively active ET- and JA-dependent

pathways (Ellis and Turner, 2001). Evidence suggests that these parallel defence pathways can be mutually antagonistic but not mutually exclusive.

3.6.1. SA analogue, Benzothiadiazole (BTH) treatment of *ads* mutants

The *ads* mutants could possibly be susceptible to virulent *Pst* DC3000 due to a dysfunctional SA-dependent pathway, perturbed up-stream or down-stream of SA signalling. BTH spraying of the mutant *nahG*, which has a blocked SA-dependent pathway can rescue the pathway and induces SAR (Lawton *et al.*, 1996). The response to *Pst* DC3000 of both *ads* mutants after BTH treatment was investigated to determine if these mutants could be rescued by application of exogenous SA. Both *ads* mutants plus Col-0, *nahG* and *eds1* plants were sprayed twice with BTH, 2 and 1 week before inoculation of a 10^5 CFU/ml suspension of *Pst* DC3000. Figure 3.6.1 reveals that BTH treatment resulted in a 20-fold decrease in bacterial growth in Col-0 plants. BTH treatment of *nahG* resulted in restoration of resistance to virulent *Pst* DC3000 to a level observed in untreated Col-0 plants. EDS1 is required for SA-dependent signalling through TIR-NBS-LRR-mediated resistance (Falk *et al.*, 1999). BTH treatment of *eds1* plants partially restored its resistance to *Pst* DC3000 by 20-fold. Nevertheless, the *eds1* mutant is still one log more susceptible to virulent *Pst* DC3000 than untreated Col-0 plants. In addition, both BTH treated *ads* mutants exhibited a reduction of *Pst* DC3000 growth of 2.5 logs compared to untreated plants in Figure 3.5.1. BTH appears to have partially restored basal resistance in both *ads* mutants to a level greater than untreated Col-0 plants but not to the extent of BTH-treated Col-0 plants. This suggests that both *ads* mutants are perturbed in signalling events upstream of SA accumulation and can be partially rescued by BTH treatment.

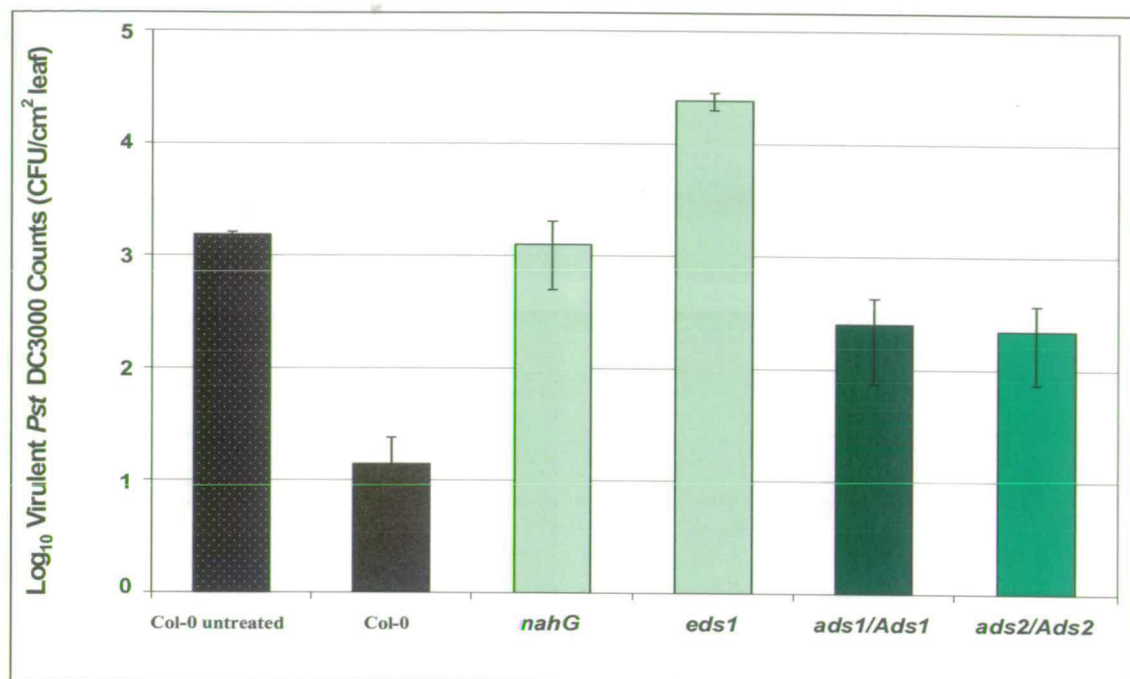


Figure 3.6.1: Growth of *Pst* DC3000 in untreated Col-0 plants and *ads* mutants and associated lines after BTH treatment. Comparison of colony forming units (CFU) recovered from Col-0 and susceptible mutants 4 dpi with a 10^5 CFU/ml suspension of *Pst* DC3000. Untreated Col-0 included from Figure 3.5.1 as both figures derived from same experiments using same inoculum. Error bars represent SE from one biological replicate with six individual plants per line. Experiment repeated twice with similar results.

3.6.2. Response of *ads* seedlings to 1-aminocyclopropane-carboxylic acid (ACC)

Arabidopsis seedlings grown in the dark and in the presence of ET should develop radial swelling of the hypocotyls and inhibition of root and hypocotyls growth, known as the “triple-response phenotype”. Screening for ET insensitive mutants has uncovered mutants that either do not develop the triple-response in the dark and presence of ET (e.g. *ein2*) or display a constitutive triple-response phenotype in the absence of ET (e.g. *eto1*) (Guzman and Ecker, 1990). Interestingly, the *ein2* mutant also displays increased susceptibility to necrotrophs and decreased disease symptoms to biotrophs (Dong, 1998). In contrast, mutants perturbed in the SA-dependent pathway typically result in enhanced resistance to necrotrophs (Thomma, 2001). As the SA pathway in *ads* mutants could be partially restored upon application with BTH, it may be possible that increased biotrophic susceptibility may be caused by activity in the concomitant ET- and JA-dependent pathway (Solano and Ecker, 1998).

In order to investigate the response to ET in *ads* mutants, sensitivity to the ET donor, ACC was examined. Surface sterilised seeds were sown on 5 plates for each concentration of ACC in a range between (0-50 μM). The effect of each ACC concentration on root length was measured at 7 days (Figure 3.6.2). In the absence of ACC, *ads1* and *ads2* responded to the dark conditions like Col-0, indicating that *ads* mutants do not over-produce or are insensitive to ET as *ein2* (which is both insensitive to ET and thus overproduces ET) (Guzman and Ecker, 1990). However, *eto* mutants have been shown to overproduce ET but are not insensitive to it and therefore ET measurements in *ads* mutants would be required to support this experiment (Chae *et al.*, 2003).

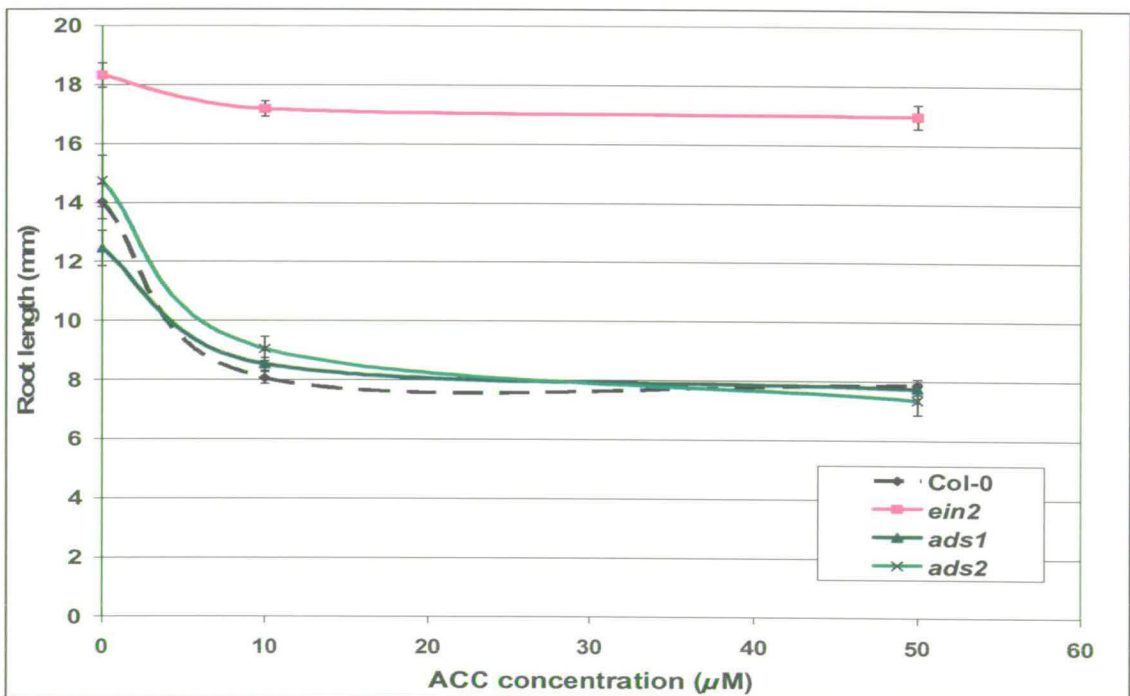


Figure 3.6.2: Root length in response to increasing concentrations of the ethylene donor, ACC. Surface sterilised *ein2.1*, Col-0 and the progeny of heterozygous *ads* mutants were sown on MS plates containing 0, 10 and 50 μM ACC. Root length measured at 5 days. Error bars represent SE from two biological replicates including 30 seeds per genotype.

3.6.3. Response of *ads* seedlings to methyl jasmonate (Me-JA)

Constitutive increased susceptibility in *ads* mutants may be caused by suppression of SA-dependent defences due to activation of the JA-dependent defence-pathways.

Jasmonate-induced genes such as *MPK4* and *SSII* have been shown to suppress SA-dependent pathways (Petersen *et al.*, 2000). COI1 is an F-box protein that may negatively regulate the JA-dependent pathway and the recessive *Arabidopsis* loss-of-function mutant is insensitive to Me-JA and more susceptible to necrotrophic pathogens (Feys *et al.*, 1994; Dong, 1998). It has also been shown that the mutants *eds4* and *pad4*, which cannot accumulate SA, are more sensitive to jasmonate (Dong, 1998). To investigate the response of the *ads* mutants to Me-JA, surface sterilised seeds were subsequently sown on MS agar plates containing a range of Me-JA concentrations (0-150 μM). The effect of each Me-JA concentration on root growth was measured at 7 days (Figure 3.6.3.) Results revealed that *ads1* and *ads2* responded to increasing Me-JA concentrations in a similar manner as Col-0 seedlings. However, the root length of *ads2* seedlings at 150 μM Me-JA was 20 % of untreated seedlings while Col-0 and *ads1* plants were 30 % root length of untreated seedlings. This experiment provides an indication that there may be a difference in sensitivity to Me-JA between Col-0 and *ads2* seedlings.

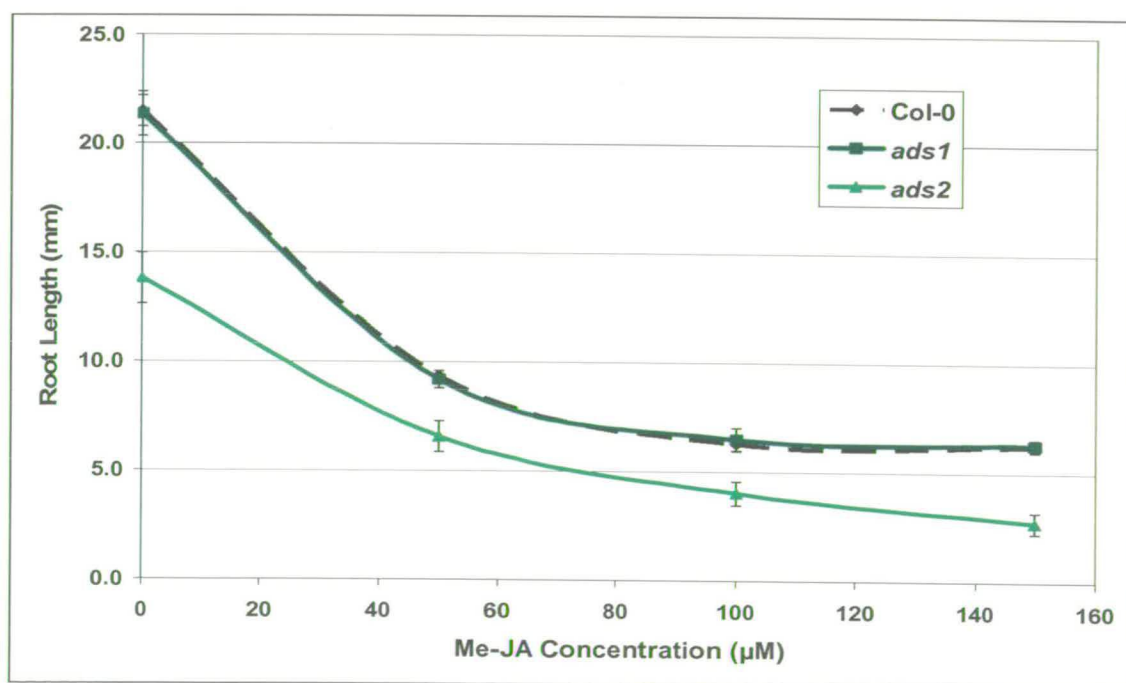


Figure 3.6.3: Root length of *ads* mutants and Col-0 in response to increasing concentrations of Me-JA. Surface sterilised Col-0 and the progeny of heterozygous *ads* mutants were sown on MS plates containing 0, 50, 100 and 150 μM of Me-JA. Root length was measured at 7 days. Error bars represent SE of one biological replicate involving 40 seedlings per genotype.

3.7. *Inserted pSKI015 Location in ads1 Mutant*

3.7.1. Southern blot analysis of *ads1* genomic DNA

The co-dominant phenotype of *ads1* co-segregated with Basta resistance, with a ratio of 3:1, indicative of a single copy pSKI015 insertion. However, this was confirmed by Southern blot analysis by probing restriction enzyme digested *ads1* DNA with a 370 bp PCR product, corresponding to the 35S enhancer element at the right border of pSKI015 (Figure 3.7.1). Southern blotting revealed that only one specific band resulted from DNA digestion with the restriction enzymes utilised, thus confirming the presence of only one T-DNA insertion. Probing of the same filter with a 700 bp PCR product corresponding to the *BAR* gene was employed and confirmed the presence of a single and intact T-DNA insertion (data not shown).

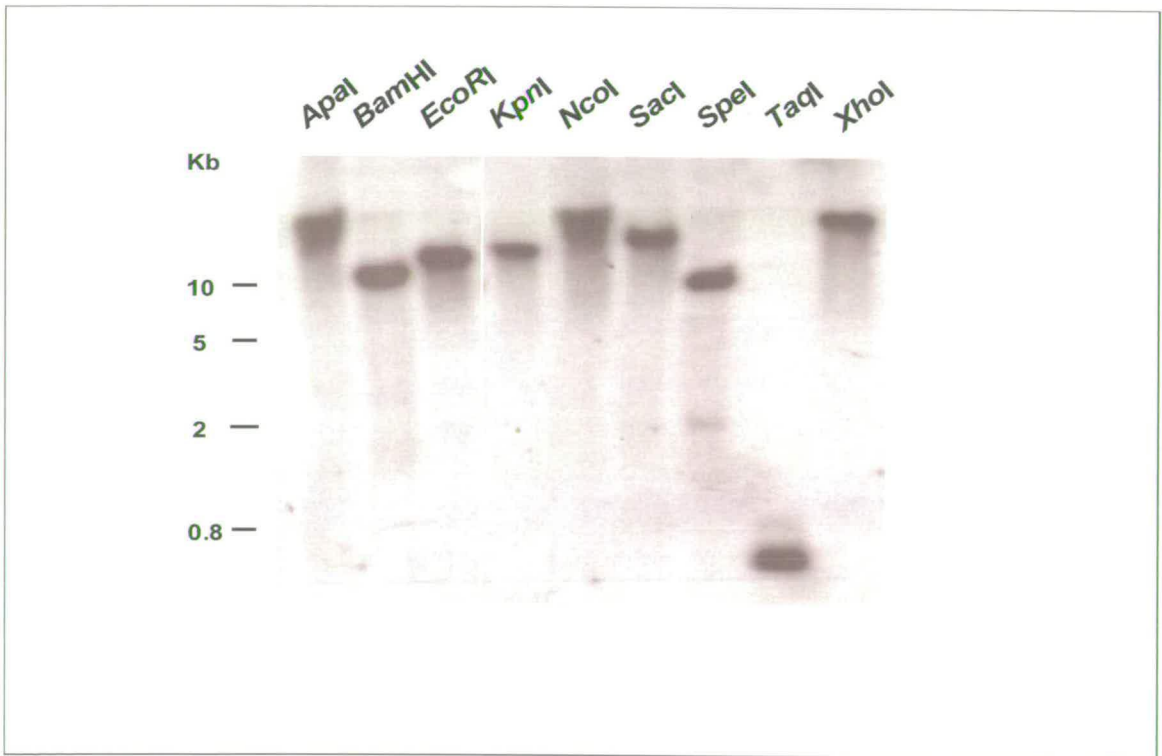


Figure 3.7.1: Southern blot of digested *ads1* genomic DNA with 35S enhancer probe. Blot demonstrates that a single copy of the pSKI015 is present in *ads1*.

Subsequently, a TAIL-PCR procedure was employed to identify the genomic region in which the pSKI015 was located (Liu et al., 1995). TAIL-PCR utilises six possible

nested PCR primers specific for the 35S enhancer at the right border of pSKI015 or six primers specific for the left border in combination with six different degenerate primers which could anneal to adjacent genomic DNA (Liu *et al.*, 1995). However, this method failed to produce PCR products. Subsequently, an inverse PCR procedure was employed but also failed (Yuanxin *et al.*, 2003). In addition, a plasmid rescue method, repeated with the enzymes *EcoRI*, *HindIII*, *KpnI*, *SpeI* and *TaqI* also failed to uncover the genome location of the activation tag (Ichikawa *et al.*, 2003). All these experiments were performed by Andrea Chini.

3.7.2. Screen of BAC library for clones possessing pSKI015

The nature of the genomic DNA surrounding the pSKI015 insert prevented attempts to identify the tag's location utilising immediately adjacent sequences and therefore a vector that could clone larger genomic fragments was required. A bacterial artificial chromosome (BAC) genomic library was assumed to be the best method that should be utilised for identifying of the location of the activation tag in *ads1*. High-molecular-weight (HMW) DNA was extracted from the nuclei isolated from homozygous *ads1* mutants using a protocol adapted from Chalhoub *et al.*, (2004) by Ingo Hein (Hein *et al.*, 2005). The HMW DNA was partially digested, cloned into pIndigoBAC5 vector to generate a BAC library of 18, 432 transformed colonies. This was estimated to be an 8-fold coverage of the *Arabidopsis* genome. The library was arrayed onto several nylon membranes and then probed with the 35S enhancer sequence and the *BAR* gene specific probe, both used earlier in 3.7.1. The filter probed with the 35S enhancer sequence is shown in Figure 3.7.2. Only clones that were successfully identified by both probes were picked for further analysis, which resulted in 36 positive BAC clones.

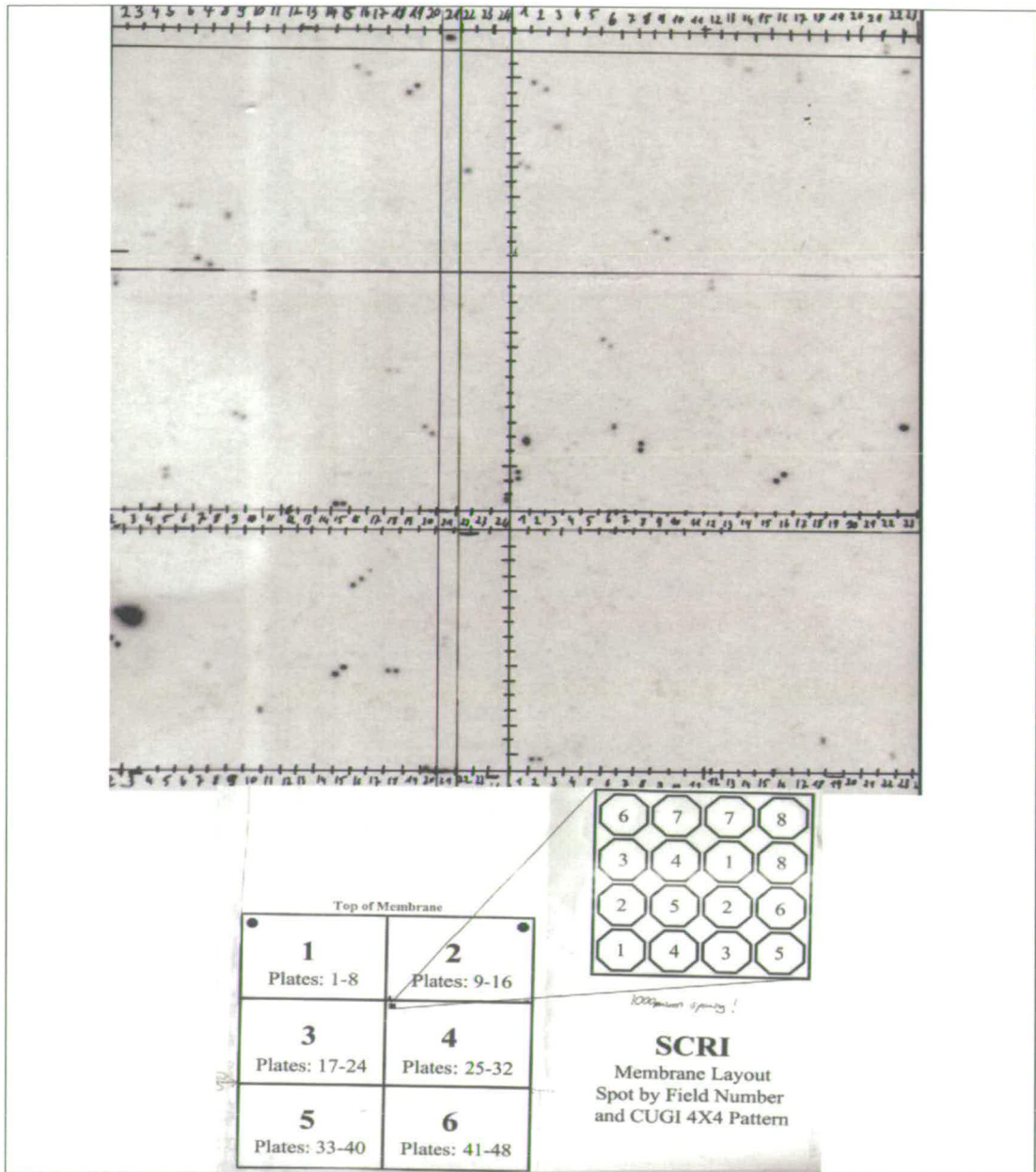


Figure 3.7.2: Screen of BAC library for clones possessing pSKI015 using 35S probe. Scanned image of the 35S enhancer probed BAC filter revealing 36 positive BAC clones. Below the filter is a diagrammatical representation of the spotting pattern used to generate the filters.

3.7.3. Sizing and screening of pSKI015 positive BAC clones

The resulting 36 positive clones were then picked from the library and grown in LB liquid media supplemented with chloramphenicol overnight. The BAC DNA was then extracted as described in 2.9, digested with *Hind*III and run on a contour-clamped

homogenous electric field (CHEF) gel to analyse the size of the inserts (Figure 3.7.3, A). The gel was then used for Southern blot analysis, again employing the 35S enhancer probe to reconfirm whether the pSKI015 insert was present (Figure 3.7.3, B). Ten BAC clones with the smallest inserts (Table 3.7.4), were selected for further sequence analysis.

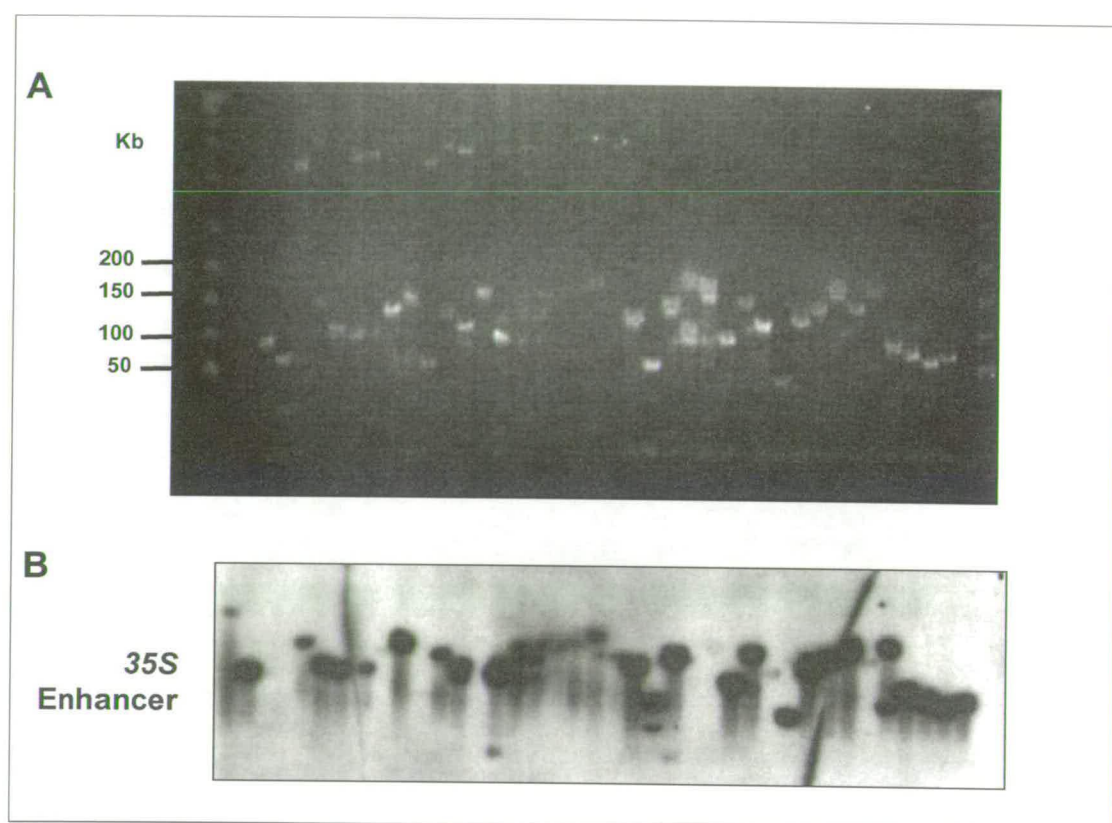


Figure 3.7.3: Sizing of BAC inserts and Southern to confirm presence of pSKI015. A, BAC clones, digested with *Hind*III and run on a CHEF gel for insert sizing. B, Southern blot of gel with 35S enhancer probe to re-confirm presence of pSKI015. 8/36 of BAC clones were shown to be false positives.

3.7.4. BAC-end Sequencing

Ten BACs were therefore grown in liquid culture, DNA extracted and sequenced using primers ordered from Cambio (Cambridge, UK). The forward primer successfully produced BAC-end sequences for six of the ten BACs selected (Table 3.7.4). BlastN analysis of the BAC-end sequences with the AGI BAC clone database hit multiple regions on two annotation unit, F21A14 and, with slightly less homology, F23H14. This could be explained by the centromeric or telomeric genome locations of the BACs

F21A14 and F23H14 (Table 3.7.4 and Figure 3.7.4). Therefore, one likely position of pSKI015 insertion is 77 kb either upstream or downstream of the identified regions on F21A14 annotation unit on chromosome 3 (Figure 3.7.4, A). The second possible position of the pSKI015 insert was identified on F23H14 which is at the very start of chromosome 2. This region has also been shown to consist of repetitive sequences and many retrotransposons.

Table 3.7.4: BlastN of *adsI* BACs with AGI BAC database

BAC	Size of <i>adsI</i> BAC	BlastN BAC hit	AGI Position on BAC	E Value
7J22	90 kb	F21A14	71759-72033	e-124
			61350-61624	e-122
			71688-71884	3 e-89
			61279-61475	3 e-89
		F23H14	8750-8910	6 e-66
8M8	95 kb	F21A14	71850-72189	e-177
			61441-61780	e-177
			71753-71926	1 e-80
		F23H14	8750-8968	e-100
32K6	130 kb	F21A14	71850-72189	e-180
			61441-61780	e-180
			71753-71926	1 e-80
		F23H14	8750-8968	e-100
			9095-9208	1 e-42
32L1	150 kb	F21A14	71837-72302	0
			61428-61893	0
		F23H14	8750-8968	1 e-95
42P2	65 kb	F21A14	71850-72255	e-174
			61441-61846	e-174
		F23H14	8750-8968	9 e-090
40M10	75 kb	F21A14	71758-71269	0
			61349-60870	0
		F23H14	8825-8339	0

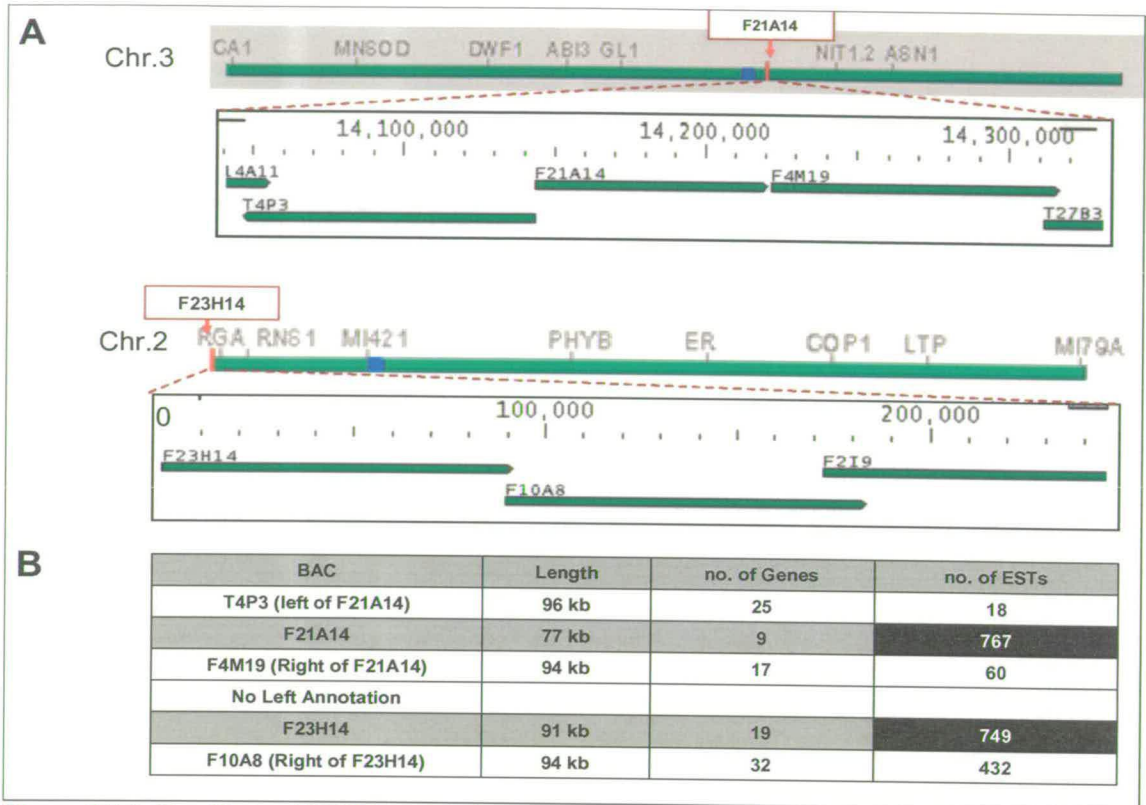


Figure 3.7.4: Candidate pSKI015 containing genome regions in *ads1* mutant. A, The position of AGI BAC, F21A14, in the *Arabidopsis* genome is adjacent to the centromeric region on chr.3. The position of AGI BAC, F23H14 is close to the 5' telomere of chr. 2. Chromosome and BAC images are from the TAIR website. B, The ESTs annotated to BACs F21A14 and F23H14 and immediately adjacent BACs.

3.7.5. Sub-cloning of BAC 40M10

In order to be more confident about the location of the activation tag, the BAC 40M10, which provided the best quality BAC-end sequencing results and, as one of the smallest positive BACs, was chosen for sub-cloning. The annotation units of F21A14 and F21H14 were inserted into the restriction map analysis programme at the TAIR website. Restriction maps generated for all 6 base-pair cutters revealed that the sequence was indeed repetitive and the majority of restriction enzymes either completely digested or cut rarely, generating fragments larger than 10 kb. The restriction enzyme, *Pst*I, was predicted to cut this region with the most regularity. Even so, at least two fragments of around 20 kb would be generated making this sequence difficult to subclone into smaller fragments using standard cloning vectors. The BAC 40M10 was therefore subjected to physical shearing by nebulisation which breaks DNA into regular fragments,

independent from the nature of the sequence. The nebulised DNA was then ligated in standard pGEM-T easy vector (Promega, UK) and transformed into *E. coli* by electroporation. Approximately 5000 white colonies were picked for a sub-clone library which was spotted onto a nylon filter as in Figure 3.7.2. The filter was initially probed with the 35S probe used previously which gave only one positive that contained just pSKI015 DNA. The library was then probed with a (300 bp) left border probe which identified 10 positive sub-clones. These sub-clones were sequenced using M13 forward and reverse primers and arranged into contigs (0.7-2 kb) using Sequencher software (all performed with Ingo Hein). BlastN analysis of the AGI BAC database revealed matches of higher identity and length of homology with F21A14 than F23H14, distinguishing between the two regions (Table 3.7.5). Figure 3.7.5 reveals the location of the contigs on AGI BAC F21A14.

Table 3.7.5: BlastN of positive 40M10 sub-clone contigs against AGI BAC database

Contig	Length (bp)	BAC hit	E Value	% Identity
1	1765	F21A14	0.0	99
		F23H14	1e-87	97
2	2096	F21A14	0.0	99
		F23H14	0.0	99
3	1896	F21A14	0.0	98
		F23H14	0.0	97
4	1874	F21A14	0.0	98
		F23H14	0.0	97

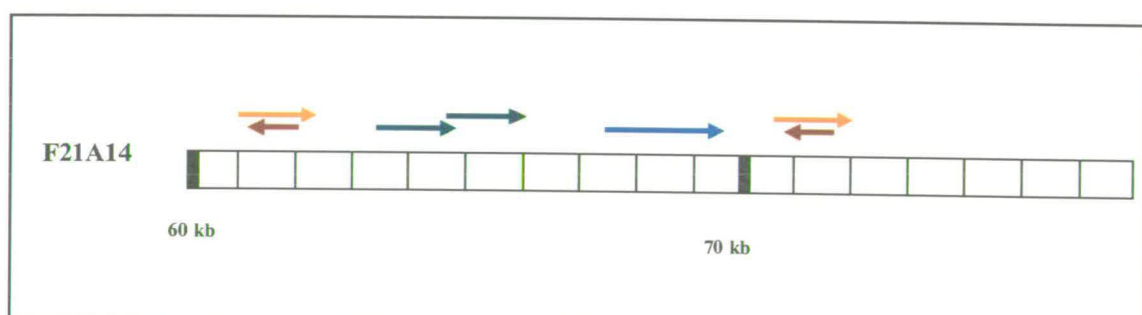


Figure 3.7.5: Regions of homology of 4 contigs with AGI BAC F21A14. Different coloured arrows represent each contig (described in Table 3.7.5) compiled from ten 40M10 sub-clones positive for the left border of pSKI015.

Annotation unit F21A14 contains only 9 genes, described as either pseudogenes or genes encoded by transposable elements in the first 40 kb of the BAC (Table 3.7.6). The pSKI015 vector is most probably located in the 60-77 kb region of F21A14 in which there are presently no genes annotated (Table 3.7.6). However, in 2003 there was an 18S rRNA which has more recently been removed from the TAIR annotation of F21A14 and the NCBI website declares that this BAC is still being completely sequenced. It is therefore possible that the pSKI015 insertion responsible for the *ads1* phenotype has permitted expression of micro-RNA (miRNA) sequences or enhancer functions in regions of the chromosome usually suppressed by heterochromatin.

Table 3.7.6: Genes present on annotation unit F21A14

BAC F21A14 (77 kb)		
Gene	Co-ordinates (bp)	Description
At3g34299	1007 - 2635	Mutator-like transposase family
At3g35707	4716 - 6198	pseudogene, hypothetical protein
At3g36411	9543 - 10091	gypsy-like retrotransposon family
At3g37820	14275 - 19163	gypsy-like retrotransposon family (Athila)
At3g38525	20282 - 22300	pseudogene, hypothetical protein
At3g39230	28754 - 31564	gypsy-like retrotransposon family
At3g39935	35123 - 36594	gypsy-like retrotransposon family (Athila) and isocitrate dehydrogenase (NADP+) activity
At3g40640	38200 - 38673	pseudogene, similar to putative AP endonuclease/reverse transcriptase
At3g41345	39484 - 40120	CACTA-like transposase family (Ptta/En/Spm)
At3g41763	65636-67443	18S rRNA (present in 2003)

3.8. *Insert Location and Gene Identification in ads2 Mutant*

3.8.1. TAIL-PCR of homozygous *ads2* genomic DNA

The first method used to identify the position of the pSKI015 insert in *ads2* was the TAIL-PCR procedure utilising three of the six possible nested PCR primers specific for the 35S enhancer elements in combination with the six different degenerate primers described (Liu *et al.*, 1995). The 35S enhancer primers used in combination with degenerate primer 4 (AD 4) produced a PCR product which reduced by the appropriate size after each of the three rounds of PCR with one of the nested 35S enhancer primers (Figure 3.8.1).

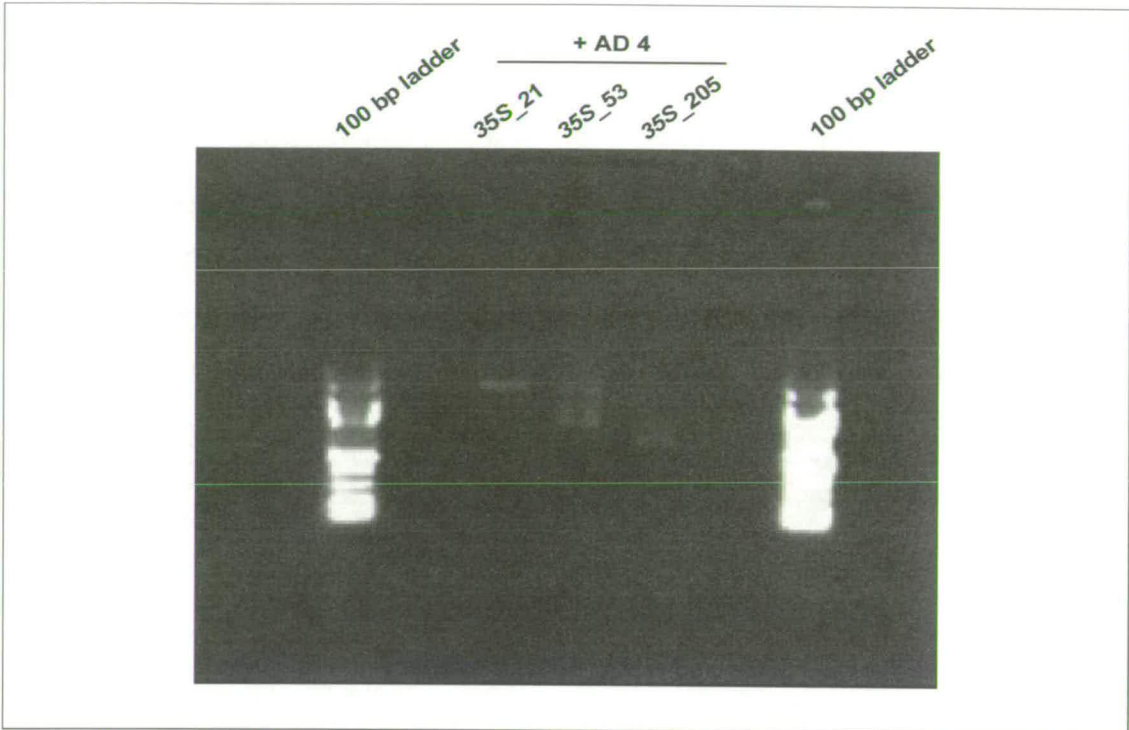


Figure 3.8.1: TAIL-PCR derived fragments of the 3 specific 35S enhancers and degenerate primer, AD4. PCR product of 35S_21 (furthest from the right border) and AD 4 was around 1 kb. The secondary PCR using the product of 35S_21 and AD 4 as a template produced several bands. The tertiary PCR reaction using the template of the secondary PCR reaction with 35S_205 and AD 4 produced a product of around 700 bp.

3.8.2. Sequencing of TAIL-PCR products and BlastN results

The PCR products of 35S_21 and 35S_205 with AD 4 were purified by gel purification and sequenced using the enhancer specific primers that amplified them. These sequences, shown in Figure 3.8.2., A, were found to be homologous to each other (Figure 3.8.2., B). These sequences were then analysed using the TAIR BlastN programme and found to match a member of the rubber elongation factor family (At3g05500) with no other significant matches (Figure 3.8.2., C). This provides evidence for the genomic region in which the pSKI015 has inserted.

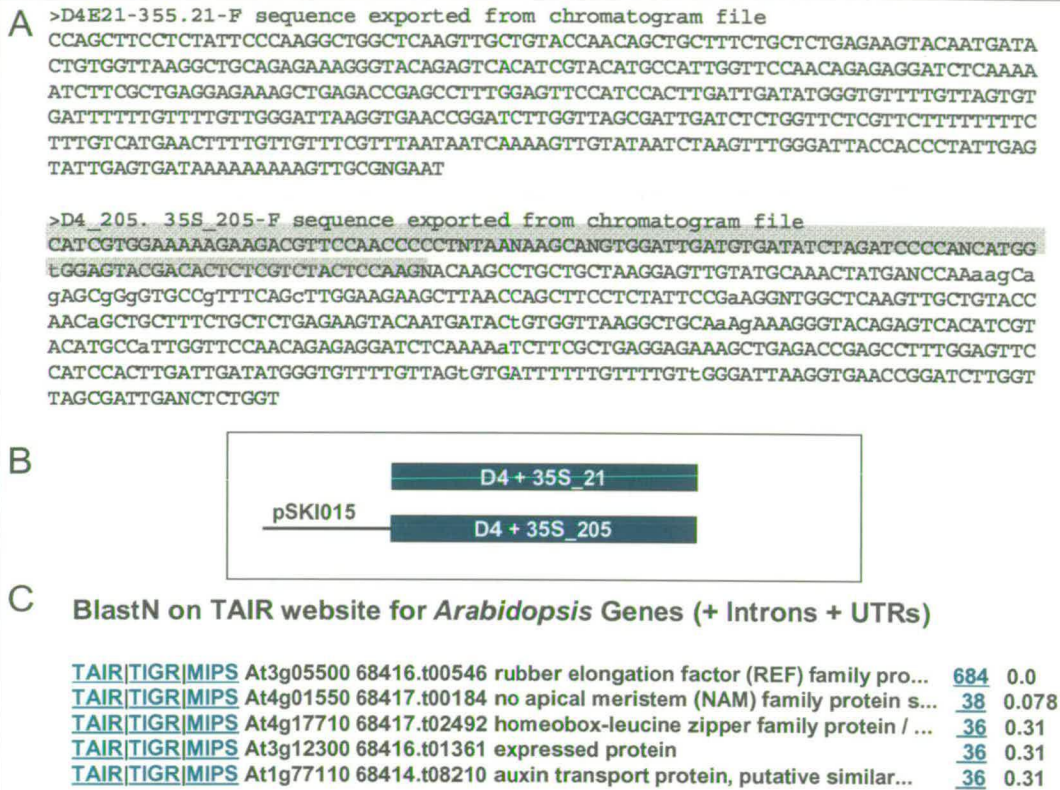


Figure 3.8.2: Sequence of TAIL-PCR products and BlastN results. **A**, sequences derived from TAIL-PCR products amplified with the relevant 35S enhancer specific primer. The grey shading of D4_205 PCR product matches the pSKI015 right border. **B**, pairwise alignment of both PCR products show 100 % homology for each other. **C**, TAIR BlastN analysis revealed that sequences were exact matches to a member of the rubber elongation factor family (At3g05500) with no other significant homologous matches, therefore providing a location for the pSKI015 vector.

3.8.3. Analysis of genomic region surrounding insertion site

Pairwise alignments of the products of TAIL-PCR with the genomic sequence of At3g05500 revealed that the insertion site is in the middle of the last exon, at position 1080 out of 1262 bp of the open reading frame (ORF) (Figure 3.8.3., A). The majority of over-expressed genes have been found to be located within 3.5 kb, both upstream and downstream of the activation tagging vector pSKI015 (Weigel *et al.*, 2000). However, one example of an over-expressed gene located more than 10 kb from the insertion site has been reported (Weigel *et al.*, 2000). Therefore, genes located up to 10 kb upstream or downstream of At3g05500 were examined further (Figure 3.8.3., B). Six genes were identified, in the same orientation (At3g05480-At3g05530) and all have potential for producing a disease resistance-related phenotype (Table 3.8.3).

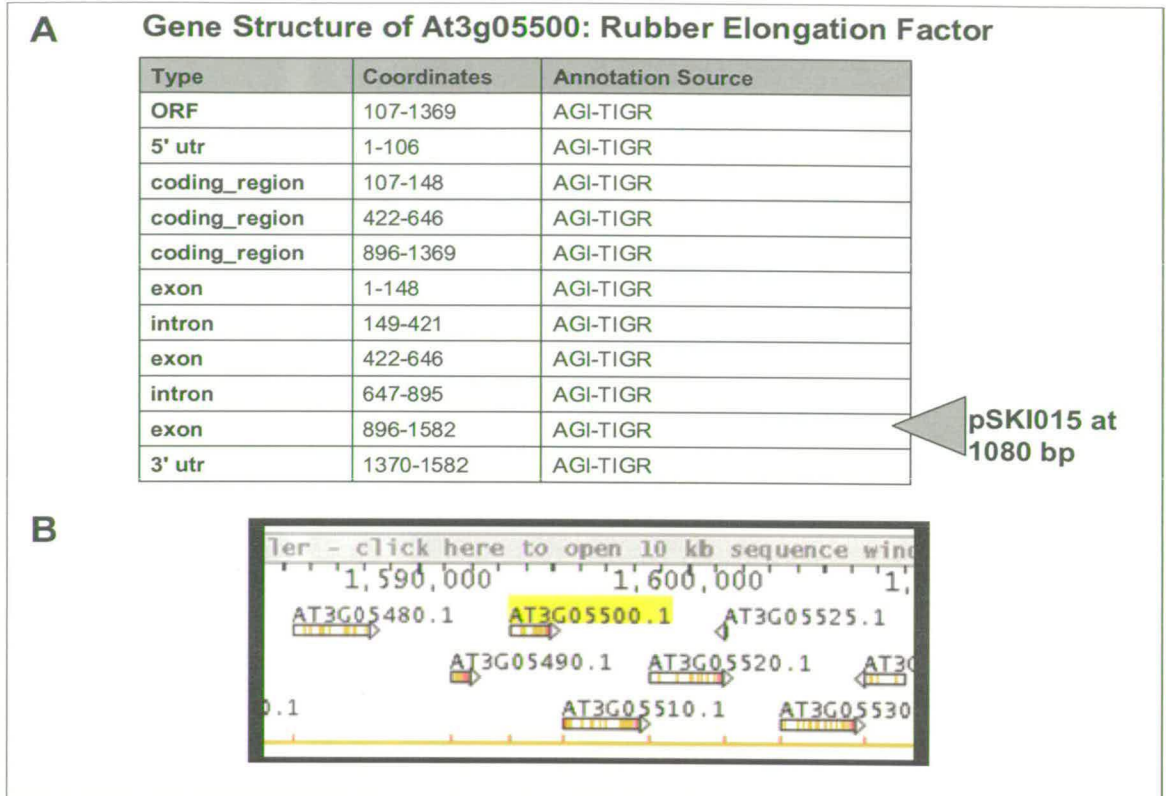


Figure 3.8.3: Gene annotation of At3g05500 and 20 kb of the surrounding genome. **A**, gene annotation of At3g05500 and the position of insert at 1080 bp. **B**, 20 kb region surrounding At3g05500 which contains five other annotated genes all in the same orientation, At3g05480, At3g05490, At3g05510, At3g05520 and At3g05530. At3g05525, is unlikely to be overexpressed as it is in reverse orientation and encodes tRNA-Glu (anticodon: TTC). Image of gene annotation from the TAIR website.

Table 3.8.3: Description of protein domains encoded by genes surrounding pSKI015 insertion site in *ads2*

Loci	Encoded Protein Homology	Embl-EBI InterPro	Possible Related Function	Reference
At3g05480	Rad9	Rad9: IPR007268	Rad9 cleaved by Caspase-3	Lee <i>et al.</i> , 2003
	N-myristoyltransferase	NMT: IPR000903	Addition of myristic acid to proteins of diverse function e.g. Pto kinase	Qi <i>et al.</i> , 2000
At3g05490	Tobacco Rapid Alkalinization Factor (RALF), RALK-like 22	none	Alkalinization of apoplast during HR	Pearce <i>et al.</i> , 2001; Olsen <i>et al.</i> , 2002
At3g05500	Rubber Elongation Factor (REF) Domain	REF: IPR008802	Stress-related proteins induced by abiotic stress	Kim <i>et al.</i> , 2003
At3g05510	Phospholipid/Glycerol acyltransferase	Acyltransferase: IPR002123	Stress induced phospholipid signalling	Munnik <i>et al.</i> , 2000
	1-acyl-sn-glycerol-3-phosphate acyltransferase	AGP acyltrn: IPR004552		
	Tafazzin	Tafazzin: IPR000872		
At3g05520	F-actin Capping Protein, alpha subunit	F-actin-cap-A: IPR002189	Involved in actin cytoskeleton function in resistance in <i>Arabidopsis</i>	Yun <i>et al.</i> , 2003
At3g05530	ClpX, ATPase regulatory subunit	ClpX: IPR004487	Calmodulin-binding proteins are differentially regulated in response to internal signals that in turn regulates transcription of other genes	Glickman <i>et al.</i> , 1998; Fu <i>et al.</i> , 1999; Peng <i>et al.</i> , 2002; Du and Poovaiah, 2004
	AAA-protein subdomain	AAA_sub: IPR003960		
	AAA ATPase, central region	AAA_ATPase_cent: IPR003959		
	26S proteasome subunit P45	26S_p45: IPR005937		
	Peptidase M41, FtsH	Pept_M41_FtsH: IPR005936	Other domains associate with the proteasomes that control protein ubiquitination	
	Sigma-54 factor, interaction region	Sig54_interact: IPR002078		
Holliday junction DNA helicase RuvB	RuvB: IPR004605			

3.8.4. Analysis of gene expression in pSKI015 insertion region

RT-PCR was employed to investigate the expression of the six genes in close proximity to the activation tag insertion site. RNA was extracted from Col-0 and *ads2* homozygous plants at 4 weeks old and quantified. Subsequently, cDNA was synthesised from 3 μ g RNA from each sample using an oligodT primer. Therefore, gene-specific primers were designed on the coding sequence of all six genes to be used for RT-PCR and northern blot analysis. RT-PCR was performed using primers designed to amplify a 100 bp product from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a control of cDNA amount in each sample. For each sample, 10 μ l were loaded on to a gel to reveal if any obvious differences could be observed in the

expression of any of the genes in this region. Figure 3.8.4 reveals that three genes may have altered expression caused by the presence of the pSKI015 vector. At3g05480 and At3g05500 both appear to be overexpressed when compared with the Col-0 sample. In contrast, At3g05520 appears to be down regulated in *ads2*. There appears to be genomic DNA contamination in *ads2* cDNA amplified with At3g05510 and At3g05520 producing an amplification product larger than that amplified from Col-0 or *ads2* cDNA. Therefore At3g05480 and At3g05500 may be the genes activated by pSKI015 in this region.

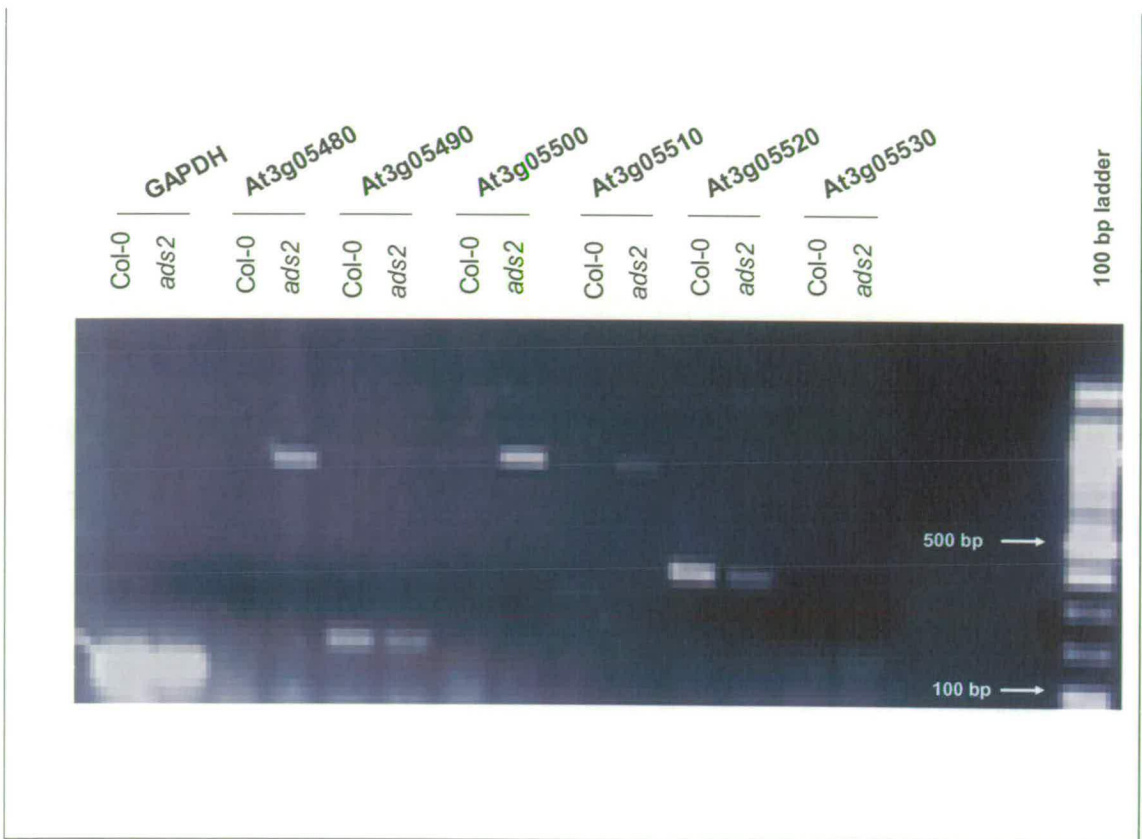


Figure 3.8.4: RT-PCR of six genes in region surrounding pSKI015 insertion site. First two wells contain control amplification using GAPDH primers to examine amount of cDNA synthesised from each RNA sample. Then the genes as they appear in the genome first with Col-0 derived cDNA and then homozygous *ads2* cDNA, At3g05480, At3g05490, At3g05500, At3g05510, At3g05520 and At3g05530. RT-PCR was repeated three times with the same RNA giving similar results.

3.9. Discussion

Activation tagging is a technique used to enhance the expression of random genes in *Arabidopsis* that consequently generate gain-of-function mutants. Screening of the T₁ progeny of activation tagged *Arabidopsis* was performed to isolate novel genes that regulate resistance to virulent *Pst* DC3000 and to circumvent the identification of alleles from previously characterised loci. Two of the twelve *ads* mutants identified in the susceptibility screen were co-dominant for susceptibility to *Pst* DC3000 and morphological phenotypes and segregation of pSKI015 vector tested through Basta resistance. The near 3:1 ratio of Basta resistance and *Pst* provided an indication that a single gene has been disrupted. The segregation ratios calculated in Table 3.4.1 for N and L could be explained by biased seedling selection during transplanting. In addition, a small number of homozygous N seedlings would die shortly after transplanting and could have possibly disrupted the ratio of plants inoculation with *Pst*. The observed ratio for *Pst* susceptibility appeared further from the expected 75 % than Basta resistance and could be explained by the difficulty in adequately inoculating all homozygous plants, especially homozygous mutants, in comparison with Basta spraying. The dominance of the gene affected in these mutants is suggested by the susceptibility phenotype identified in the T₁ generation of activation tagged lines. Unfortunately, the homozygous *ads1* and *ads2* mutants were infertile and therefore analysis with a completely homozygous line was not possible. However, both mutants were identified as possessing a susceptibility phenotype to *Pst* DC3000 in the heterozygous T₁ generation and therefore analysis was possible using the heterozygous plants.

The co-dominant *ads1* mutant displayed an extreme loss-of-apical dominance, a dwarfed stature, premature leaf senescence and in the homozygous mutant reduced fertility (Figure 3.5.6a). The observed loss-of-apical dominance phenotype is similar to that commonly observed in *Arabidopsis* mutants with reduced gibberellin levels (Dill and Sun, 2001; Wen and Chang, 2002; Schomburg *et al.*, 2003). The gene or genes perturbed by the activation tag must be affecting development of leaves, florescences and siliques.

To quantify the extent of susceptibility, inoculation of two suspensions of virulent *Pst*DC3000 (10^5 and 10^6 CFU/ml) revealed that *ads1* was more susceptible than Col-0 by 10-fold and comparable to the level of susceptibility exhibited by *nahG* plants (Figure 3.5.1 and Figure 3.5.2). In addition, *ads1* was also shown to be as susceptible to avirulent *Pst* DC3000 (*avrB*) as *nahG* plants (Figure 3.5.3). This implies that *ads1* is perturbed in a gene involved in both basal and *R* gene mediated resistance to *Pst* DC3000 although testing with pathogens containing other AVR proteins would be required to confirm this hypothesis. Intriguingly, DAB staining revealed that the *ads1* mutant accumulated H_2O_2 to highly elevated levels in comparison with Col-0 and *nahG*. This was surprising as H_2O_2 is thought to be a signalling molecule for inducing defences such as the HR and SAR and could be directly toxic to cell-invading pathogens. In fact, transgenic crop plants have been generated that accumulate H_2O_2 resulting in increased resistance to biotrophic pathogens (Wu *et al.*, 1995). The rapidity of ROI production triggered by avirulent pathogens to induce downstream pathways is independent of transcription and regulated by secondary messengers such as Ca^{2+} and H^+ and by proteins such as kinases and G-proteins (Yang *et al.*, 1997; Cessna and Low, 2001). In addition, a tightly regulated balance of NO and H_2O_2 production has been shown to be required for HR (Clarke *et al.*, 2000). The *ads1* mutant may therefore be deficient in one of these other early signalling molecules essential for inducing downstream defence responses. Alternatively, downstream responses may be suppressed by the activation of a negative regulator. Basal disease resistance has been shown to require an active SA-dependent pathway in many plant-pathogen interactions (Dong, 2004). Nevertheless, H_2O_2 production and induction of defence responses to virulent and avirulent *Pst* DC3000, in the *ads1* mutant appears to be uncoupled. Unfortunately, expression analysis of key marker genes of HR and SAR were not examined and therefore it cannot be determined whether downstream defence responses are being induced or not. The extent of cell death was examined using trypan blue staining. This revealed that at 12 hpi, cell death was occurring in *ads1* leaves inoculated with virulent *Pst* DC3000. Trypan blue staining can not differentiate between PCD and necrotic cell death; however

the biotrophic phase of virulent *Pst* DC3000 is between 1-3 days and therefore the dead cells are not likely to be the result of severe disease symptoms.

The susceptibility of *ads1* to *Pst* DC3000 may also be caused by a dysfunctional SA-dependent pathway. Therefore, BTH, a SA analogue, was sprayed onto *ads1* and associated *Arabidopsis* lines to investigate whether exogenous application of SA could rescue resistance against *Pst* DC3000. Figure 3.6.1 reveals that BTH spraying could induce a level of resistance to virulent *Pst* comparable with untreated Col-0. However, *ads1* allowed almost one and a half logs more bacterial growth than BTH treated Col-0. This suggests that *ads1* can respond to exogenously applied SA and that the SA pathway in *ads1* is functional downstream of SA production. The SA-dependent pathway may therefore be perturbed between H₂O₂ production and SA synthesis. Perhaps the pathway is perturbed by insensitivity to H₂O₂, by absence of another essential signalling molecules or by activation of negative regulators of the SA-independent signalling cascades downstream of H₂O₂ production e.g. MAPK kinase (Petersen *et al.*, 2000). Some repressor proteins of the SA-pathway are activated by the wounding and necrotrophic defence pathways regulated by ET and JA (Thomma, 2001). It is therefore possible that the *ads1* plants possess active ET- or JA-dependent pathways which subsequently suppress defence responses against biotrophic pathogens. Previously characterised mutants with constitutive ET-dependent pathways have been shown to be insensitive to ethylene and show unaffected root and hypocotyl growth e.g. *ein2* (Guzman and Ecker, 1990). To investigate whether *ads1* responds to ET differently to Col-0 plants, seedlings were grown on ACC plates (exogenous ethylene donor) in a method adapted from Guzman and Ecker (1990). Figure 3.6.2 reveals that *ads1* responds to ACC as Col-0, indicating that it is neither more nor less sensitive to ET. It is probable that even if a completely homozygous *ads1* mutant line was used for this experiment that it would not be revealed as being as dramatically insensitive to ACC as the *ein2* mutant (Guzman and Ecker, 1990). Me-JA has been shown to inhibit root growth and stimulate anthocyanin production of Col-0 plants but not the insensitive mutant, *coil* (Feys *et al.*, 1990). Therefore the effect of Me-JA on the root growth of *ads1* was examined. Figure 3.6.3 reveals that *ads1* was no more or less sensitive to Me-

JA than Col-0. Expression analysis of marker genes *GST1*, *PRI* and *PDF1.2* would be required to establish the active state of the HR, SA-dependent and the ET- and Me-JA-dependent pathways. Consequently, it is more likely that *ads1* is insensitive to H₂O₂ or perturbed in the production of another signal essential for inducing basal and *R* gene-mediated disease resistance.

Identifying the region of pSKI015 insertion was initially attempted by employing commonly utilized techniques, such as TAIL-PCR and plasmid rescue. Repeated attempts to identify the insertion region in these ways failed and so a genomic scale library was deemed necessary. Therefore, a BAC library of homozygous *ads1* genomic DNA, partially digested to fragments of 50-250 kb, was constructed. Southern blot analysis of the BAC library with both a *35S* enhancer probe and a *BAR* gene-specific probe identified around 36 positive BAC clones (Figure 3.7.2). All 36 positive clones were sized by digestion with *Hind*III and another Southern blot was performed with the *35S* enhancer probe to confirm the presence of the pSKI015 vector (Figure 3.7.3). BAC-end sequencing revealed a sequence which is repeated many times in the *Arabidopsis* genome, including the AGI BAC, F21A14 and F23H14 (Table 3.7.4). All six BAC end sequences matched F21A14 with higher significance than F23H14, suggesting that F21A14 may be the more probable region contained in these BACs. Subcloning and further sequencing supported BAC-end sequencing by matching F21A14 with longer sequences and higher identity (Table 3.7.5). The sequences from subcloning BAC 40M10 all aligned with the 3' end of F21A14 (60-77 kb) (Figure 3.7.5). However, there are no genes annotated on this BAC from 40 kb to 77 kb. This suggests that there may be a protein or regulatory element present in this region which has not been investigated or annotated yet. It still remains to be tested whether all the BACs identified from *ads1* using *35S* and *BAR* probes contain this region. Activation tagging has facilitated the identification of a novel mutant. It is therefore possible that the pSKI015 insertion responsible for the *ads1* phenotype has permitted expression of micro-RNA (miRNA) sequences or enhancer functions in regions of the chromosome usually suppressed by heterochromatin. Isolating the factor responsible for the susceptible phenotype may require expression analysis and genetic reconstitution experiments.

The co-dominant *ads2* mutant displayed an undulating leaf surface and in the homozygous mutant, observable anthocyanin accumulation, a dwarfed stature and failure to develop flowers into siliques (Figure 3.5.6b). Interestingly, increased anthocyanin production as found in *ads2* (Figure 3.5.5) has been associated with Me-JA treated plants (Feys *et al.*, 1994). Anthocyanins have antioxidant activity and have been shown to have an inhibitory effect on NO production in macrophages (Kong *et al.*, 2003). It is therefore possible that the high concentration of anthocyanins present scavenges free ROI in the leaves preventing defence gene induction. In addition, Lo and Nicholson (1998) have shown that anthocyanin production can be decreased by pathogen-induced production of antimicrobial phytoalexins. It may also be possible that the anthocyanin production in *ads2* prevents phytoalexins production, subsequently affecting basal resistance to *Pst* DC3000. To confirm the susceptibility of *ads2* to virulent *Pst* DC3000, inoculation of two suspensions of virulent *Pst* (10^5 and 10^6 CFU/ml) revealed that *ads2* was more susceptible than Col-0 by at least 1-log and to a comparable level exhibited by *nahG* plants (Figure 3.5.1 and Figure 3.5.2). On the other hand, *ads2* was shown to be as resistant to *Pst* DC3000 (*avrB*) as Col-0 (Figure 3.5.3). This suggests that the presence of the antioxidant, anthocyanin is not preventing the HR response required for resistance to *Pst* DC3000 (*avrB*) and therefore the gene or genes perturbed in the *ads2* mutant are only necessary for basal resistance responses. DAB staining revealed that the *ads2* mutant only accumulated H_2O_2 in response to *Pst* DC3000 (*avrB*) (Figure 3.5.4a and b). The presence of anthocyanin does not appear to affect the production of H_2O_2 associated with the oxidative burst. It may be possible that the strong and rapid induction of defence responses through *R* genes may over-ride the suppression of basal defences in *ads2*. This hypothesis was investigated by spraying mutant lines with BTH, an SA analogue, a week before challenge with virulent *Pst* DC3000. The induction of SAR by BTH treatment may reverse the suppression of basal defence responses which causes the susceptibility to *Pst* DC3000 displayed by *ads2*. Figure 3.6.1 reveals that BTH spraying could induce a level of resistance in *ads2* to virulent *Pst* DC3000, comparable with untreated Col-0. However, like *ads1*, BTH treatment of *ads2* did not induce a level of resistance as BTH-treated Col-0. This

suggests that the suppression of basal defences in *ads2* can be reversed by *R* gene activation and induction of SAR by BTH treatment.

The susceptibility of *ads2* to virulent *Pst* DC3000 may be due to activity in the ET- or JA-dependent pathways which can be overcome upon triggering of *R* gene mediated resistance. To investigate whether *ads2* possess increased or decreased sensitivity to ET seedlings were grown on ACC plates (exogenous ethylene donor) in a method adapted from Guzman and Ecker (1990). Figure 3.6.2 reveals that *ads2* behaves like wild type *Arabidopsis* in response to ACC. In addition, the effect of Me-JA on the root growth of *ads2* was measured. Figure 3.6.3 reveals that *ads2* roots were inhibited by 80 % by 150 μ M of Me-JA compared to 70 % in *ads1* and Col-0 plants. In consideration of the earlier described phenotypes of shorter roots and anthocyanin production it is possible that measurements of Me-JA or expression analysis of *PDF1.2* may reveal that the *ads2* mutant may have a modestly perturbed JA-dependent pathway.

TAIL-PCR of homozygous *ads2* genomic DNA resulted in the production of a specific band that decreased in size with the use of nested primers specific for 35S enhancers at the right border of pSKI015. Sequencing of two of these PCR products revealed a fragment from the last exon of a rubber elongation factor gene (At3g05500). In the 20 kb region surrounding this gene, there are five other genes annotated in the same orientation and a tRNA in the opposite orientation. To identify an up-regulated gene or genes in *ads2*, RT-PCR analysis of gene expression was carried out in untreated Col-0 and *ads2*. Figure 3.8.4 reveals that two genes are expressed in *ads2* that are not expressed in Col-0. At1g05480 encodes a protein that has a RAD9-like domain and an N-myristoylation transferase domain (Qi *et al.*, 2000; Lee *et al.*, 2003). RAD9 is a cell cycle check point protein that is cleaved by caspase-3 during apoptosis (Lee *et al.*, 2003). The cleavage of RAD9 results in its translocation from the nucleus to the cytosol where it promotes apoptosis by interacting with Bcl-XL (Lee *et al.*, 2003). N-myristoylation transferase is an enzyme responsible for the post-translational modification of the N-terminus of a diverse range of proteins by addition of myristic acid onto glycine residues (Qi *et al.*, 2000). The N-myristoylation of proteins is thought

to promote their affinity with membranes (de Jonge *et al.*, 2000). The post-translational N-myristoylation of caspase substrates have been shown to target cleavage products to mitochondria during apoptosis (Shrivastav *et al.*, 2003). In addition, the pro-apoptotic protein BID is also proteolytically cleaved, post-translationally myristoylated and relocalised to the mitochondrial membrane, releasing cytochrome C and promoting PCD (Zha *et al.*, 2000). In plants, many proteins possess predicted N-myristoylation motifs although few have been confirmed to be myristoylated experimentally. Putative N-myristoylation sites in kinases, Pto and Fen, may be associated with their *R* gene-related function by facilitating their plasma membrane localisation (Thompson and Okuyama, 2000). N-myristoylation has also been shown to be required for the response to salt-stress (Ishitani *et al.*, 2000). At1g05500 encodes a rubber elongation factor (Kim *et al.*, 2003) which is up-regulated in response to wounding in *Ficus carica* and in response to other abiotic stresses and that peroxidase activity has been associated with REF activity (Kim *et al.*, 2003). Both these genes could have a role in disease resistance. In order to confirm which of these genes could be responsible for increased susceptibility to virulent *Pst*, each of these genes could be fused to a 35S CaMV promoter and over-expressed in Col-0 by stable transformation.

Unfortunately, the genes responsible for either of these mutants were not characterised in time for investigation in *N. benthamiana* and *S. tuberosum*. Therefore, a gene previously identified through activation tagging in *Arabidopsis*, *ADR1*, was selected for functional investigation during the other plant-pathogen interactions studied during this project (Grant *et al.*, 2003; Chini *et al.*, 2004). It was of interest to investigate whether *ADR1* had a conserved function in other plant species.

3.10. Conclusions

Two *Arabidopsis* activation tagged (pSKI015) mutants with activated disease susceptibility (*ads*) were identified. Both mutants segregated 3:1 for the presence of the insert and appeared co-dominant for associated phenotypes. The *ads1* mutant was shown to be susceptible to virulent and avirulent *Pst* DC3000, although large amounts of H₂O₂, an HR inducing molecule, constitutively accumulated. The genomic location of the activation tag in this mutant has been located to a genomic region adjacent to the centromere on chromosome III although it remains to be confirmed that all BAC clones contain the same region.

The *ads2* mutant was shown to be susceptible to *Pst* DC3000 but possessed a functional *R* gene-mediated response to *Pst* DC3000 (*avrB*). The homozygous *ads2* also accumulated discernable amounts of the stress-related pigment, anthocyanin. TAIL-PCR identified the location of pSKI015 in *ads2* plants to gene At3g05500. Expression analysis of all six genes in this region revealed that two genes are overexpressed (At3g05480 and At3g05500).

4. Role of Cathepsins in the Hypersensitive Response

The HR is a rapid, inducible form of PCD that shares some conserved hallmarks, mechanisms and enzyme activities with mammalian apoptosis. However, the complex series of molecular interactions that lead to the HR are still poorly understood. The HR appears to be fundamental for resistance in countless plant interactions with biotrophic pathogens and most probably involved in necrotrophic disease development. Therefore there is intense interest in elucidating the major components that induce and regulate the HR.

4.1. *Role of Caspases in Mammalian PCD*

Mammalian apoptosis is associated with the cleavage of a specific subset of cellular polypeptides and degradation of chromosomal DNA (Chang and Yang, 2000). Cysteine-dependent aspartate-directed proteases (caspases) have been shown to be directly or indirectly responsible for many cleavage patterns that are hallmarks of apoptosis (Yuan *et al.*, 1993; Earnshaw *et al.*, 1999). Caspases are translated as zymogens, known as procaspases, and are predominantly processed by other caspases, forming an activation cascade (Earnshaw *et al.*, 1999). Initiator caspases are activated following either the extrinsic activation of cell death receptors, e.g. procaspase-8 and -10, or intrinsically by release of cytochrome C from the mitochondria, e.g. procaspase-9 and -2 (Chang and Yang, 2000). However, recent research demonstrates that cleavage is not required or sufficient for initiator caspase activity and proposes they are active as obligate dimers (Stennicke *et al.*, 1999; Boatright and Salvesen, 2003). Once activated, initiator caspases consequently process common downstream effector caspases that already exist as dimers (e.g. caspase-3, -6 and -7), which leads to PCD.

4.2. *Caspase-Independent PCD*

Early studies in the *ced-3 C. elegans* mutant provided the first indication that PCD could occur without caspases and was revealed to involve the proteases calpains and cathepsins

(Abraham and Shaham, 2004). In mammals also there is evidence supporting caspase-independent PCD, such as during autophagy, tumour necrosis factor (TNF)-mediated liver injury and during development of neurons and chondrocytes (Leist and Jäättelä, 2003). A range of research indicates many non-caspase proteases, such as cathepsins, calpains and serine proteases, play important roles during apoptosis but also trigger PCD in a caspase-independent manner (Abraham and Shaham, 2004). Calpains are calcium-dependent proteases involved in processing various enzymes and cytoskeletal components, thereby linking their activity to a variety of intracellular events (Johnson and Guttmann, 1997). Cathepsins are aspartate and cysteine proteases and thus far, cysteine cathepsins B and L and aspartate cathepsin D have been most clearly associated with PCD from studies in mammalian models. Cathepsin B deficient mice show increased resistance to apoptosis (Mathiasen and Jäättelä, 2002; Foghsgaard *et al.*, 2001) and other experiments have shown that cathepsin B can activate caspases (Kingham *et al.*, 2001; Vancompernelle *et al.*, 1998). The serine protease, granzyme B, has also been demonstrated to be required for rapid caspase-mediated PCD and a slower caspase-independent necrosis-like PCD (Mathiasen and Jäättelä, 2002).

4.3. *Proteolytic Machinery in Plant HR*

Although plant PCD shares many morphological characteristics with caspase-dependent cell death in mammals, no direct caspase homologues have been identified thus far (Chichkova *et al.*, 2004). Interestingly, several examples of caspase-like activities involved during the HR have been identified in oat, tobacco and *Arabidopsis*, however these activities do not correspond with the regulatory initiator caspase activities (del Pozo and Lam, 1998; Coffeen and Wolpert, 2004; Chichkova *et al.*, 2004; Rojo *et al.*, 2004). A caspase-like family of *Arabidopsis* proteases includes nine metacaspases and, although these proteins have significantly similar tertiary structure to caspases, there is no experimental evidence confirming a similar function (Woltering, 2004; Sanmartín *et al.*, 2005). However, the expression of one tomato metacaspase, *LeMCA1*, has been shown to be up-regulated during infection with *B. cinerea* and another metacaspase may

be responsible for the VEIDase activity essential for PCD during plant embryogenesis (Hoeberichts *et al.*, 2003; Bozhkov *et al.*, 2004). In addition, vacuolar processing enzymes (VPEs) belong to the caspase-like protease family and may show similar protein folding (Kinoshita *et al.*, 1999; Hayashi *et al.*, 2001; Rojo *et al.*, 2004).

Nevertheless, PCD regulation and caspase-like activities will most probably be associated with some of the 488 annotated protease genes in the *Arabidopsis* genome (van der Hoorn and Jones, 2004). In addition to the caspase-like proteins, larger protease families are associated with the HR: the papain-like, subtilisin-like and pepsin-like proteases (van der Hoorn and Jones, 2004). One papain-like protease from tomato, RCR3, has been shown to be required for resistance to *C. fulvum*, mediated through Cf-2 (Beers *et al.*, 2000; Krüger *et al.*, 2002; Rooney *et al.*, 2005). In addition, the potato homologues of *CYP1* and cathepsin B are also up-regulated in response to an incompatible isolate of *P. infestans* (Avrova *et al.*, 1999; Avrova *et al.*, 2004). Subtilisin-like proteases are the largest protease family in *Arabidopsis*, with varied roles, including defence responses, e.g. the potential PR protein, P69 (Tornero *et al.*, 1997; Beers *et al.*, 2004). In addition, some subtilisin-like proteases possess caspase-like activities (saspases) involved during victorin-induced PCD (Coffeen and Wolpert, 2004). Fewer aspartic pepsin-like proteases have been characterised in comparison with the other plant protease families mentioned thus far. Nevertheless, the apoplastic aspartic protease, constitutive disease resistance 1 (*CDR1*) was discovered by screening activation-tagged *Arabidopsis* lines for resistance to virulent *P. syringae*, which shares regions of homology with human cathepsin D (Xia *et al.*, 2004). *CDR1/nahG* crosses revealed that CDR1 may function by cleaving endogenous targets in an SA-dependent manner (Xia *et al.*, 2004). The large number of proteases likely to be involved during the HR has obscured the search for elusive major initiator proteases. However, the benefits from understanding the HR in plant-microbe interactions will continue to drive the search.

4.4. *Potato Genes Identified in Resistance to P. infestans*

The HR appears to be essential in potato for *R* gene-mediated and non-race-specific (quantitative or field) resistance against the oomycete, *Phytophthora infestans* (Birch and Whisson, 2001; Kamoun *et al.*, 1999). Elucidating the genes up-regulated in resistant potato cultivars in response to *P. infestans* is a key step towards understanding the subsequent complex signalling events and identification of possible targets for breeding durable disease resistance. Suppression subtractive hybridisation (SSH) is a technique used to enrich for ESTs in tester cDNA material not present in cDNA synthesised from driver RNA (Birch *et al.*, 1999). This technique was used to create a cDNA library enriched for genes from leaves of resistant potato cv Stirling (tester) 24 hpi with an avirulent strain of *P. infestans*, employing cDNA from infected leaves of the susceptible cv. Bintje (driver) to remove common sequences (Birch *et al.*, 1999). Several classes of genes identified encode proteins that are implicated in PCD in other organisms (Birch *et al.*, 1999; Avrova *et al.*, 1999), including a cysteine protease-encoding sequence (Accession number AJ245924) with significant homology to a tomato cysteine protease (*CYP1*) (Accession number AJ003137) at both the DNA and amino acid level (93.7 % and 95.7 %, respectively) (Avrova *et al.*, 1999). The protein sequence reveals two conserved domains; a cysteine protease domain conserved with all plant and cathepsin family of mammalian cysteine proteases. In addition, a C-terminal granulin-like domain is present on the potato cysteine protease which is not conserved with mammalian cathepsins. Interestingly granulins are a separate class of proteins involved in regulating cell growth in animals (Bateman and Bennett, 1998). Other cysteine proteases that contain granulin-like domains have only been identified in plants, such as oryzain α and β in rice (Avrova *et al.*, 1999). In addition, the potato *CYP1* gene is a likely homologue of the *Arabidopsis* gene *RD21* (At1g47128) which was shown to play a role in drought induced senescence (Yamada *et al.*, 2001).

Suppressor of G2 allele of SKP1 (*SGT1*) was a gene included as a control because in barley, *Arabidopsis* and *N. benthamiana* it is essential for *R* gene-mediated resistance

(Azevedo *et al.*, 2002; Tor *et al.*, 2002; Austin *et al.*, 2002). Published work involving the silencing of *SGT1* in *N. benthamiana* (Peart *et al.*, 2002) made it an interesting gene to investigate during the potato-*P. infestans* interaction and to utilise as a positive control of VIGS for later silencing experiments in *N. benthamiana* and potato.

As part of the collaboration between SCRI and Edinburgh University, a novel gene discovered from an activation-tagging screen in *Arabidopsis* was to be included in the functional screens in potato and *N. benthamiana*. Genes responsible for activated mutant phenotype, as described in Chapter 3, had not been identified early enough for inclusion. A gene identified from an earlier activation-tagged mutant, acquired disease resistance 1 (*ADR1*) (At1g33560) has had its role in both biotic and abiotic stress well characterised (Chini *et al.*, 2004; Grant *et al.*, 2003). *ADR1* is a putative CC-NBS-LRR which possesses an additional but partial kinase domain. Constitutively over-expressing *ADR1* in *Arabidopsis* confers resistance to virulent pathogens including the oomycete, *Peronospora parasitica* (Grant *et al.*, 2003). It was therefore of interest to investigate whether a gene identified from the sequenced model plant could be involved in the potato-potato late blight interaction. The potato orthologue of *ADR1* (BG596098) was identified through TBlastX analysis of the TAIR higher plant EST dataset.

4.4.1. Expression of *CYP1*, *ADR1* and *SGT1* in *S. tuberosum* cultivars

Initially, Northern blot analysis of potato plants treated with an incompatible race of *P. infestans* was performed to investigate the expression of genes, *StCYP1*, *StADR1* and *StSGT1*, during potato responses to *P. infestans*. Cv Stirling possesses both race-specific and non-race-specific resistance to *P. infestans* whereas cv Bintje and Maris Piper possess little resistance. Both race-specific and non-race-specific resistance have been shown to involve the HR. Race-specific resistance is usually conveyed by a single *R* gene that rapidly induces HR at the site of attempted infection. However, non-race-specific resistance is hypothesised to be controlled by multiple but weak or partial *R* genes and others that are spread over many QTL which are much slower to trigger PCD resulting in a trailing HR behind the spreading of the pathogen (Avrova *et al.*, 2004). To

genetically separate race-specific and non-race-specific resistances, Stirling was crossed with the susceptible cv Maris Piper and, after extensive screening, a line with only race-specific resistance (# 29) and another with only non-race-specific resistance (# 53) were identified (Avrova *et al.*, 2004). Subsequently, potato cvs Stirling, Bintje and the two Maris Piper/Stirling progeny clones were sprayed with a race of *P. infestans* collected from the field the year before, which was shown to be incompatible on Stirling. Leaves were collected from uninfected plants (0) and plants 12, 24, 48 and 72 hpi. This provides an overview of gene expression throughout the life cycle of *P. infestans*, which is biotrophic until 36 hpi when it switches to a necrotrophic infection strategy. An HR successfully induced during the first 36 hpi prevents potato blight disease spread and therefore genes involved in defence against *P. infestans* should be up-regulated before this time point in a resistant potato plant. Leaf samples had been previously collected by a former PhD student, Nawsheen Taleb. RNA was extracted from these samples and 5 µg per sample was run on a gel for Northern blot analysis (Figure 4.4.1).

StCYP1 gene expression was found to be up-regulated within the first 36 hpi in Stirling and clone # 29 both of which possess an *R* gene which recognises this strain of *P. infestans*. Interestingly, although the HR is involved in non-race-specific resistance, cathepsin-like expression was not detectable in the clone # 53, suggesting that *StCYP1* is not involved in both defence responses. *StCYP1* was also slightly up-regulated by 24 hpi in Bintje which may have been caused by the high titre of the *P. infestans* spore suspension.

The expression of *StSGT1b* (BG599125) was expressed during the first 48 hr of the incompatible interaction in Stirling and in the first 24 hpi in the *R* gene containing # 29. However, in Bintje it also appears to be expressed during the first 48 hpi, although the expression is induced to a lesser extent in comparison with Stirling. This suggests that *StSGT1* expression is not specific to the incompatible interaction.

Gene expression of *StADR1* was present during the first 48 hpi of the incompatible interaction in both Stirling, # 29 and Bintje suggesting that it may not have a role in *R* gene-dependant resistance to *P. infestans* in potato. *StADR1* appeared to be only weakly

up-regulated in the non-race-specific interaction (# 53). Basal expression of *StADR1* is higher in Stirling compared with clone # 29 and # 53 suggesting that unknown components of non-race-specific resistance, inherited by # 53, could be involved in regulating *StADR1* expression.

The northern blot analyses above indicate that *StCYP1* is differentially expressed between an incompatible and an compatible *P.infestans*-potato interaction. These Northern show that the involvement of *StADR1* and *StSGT1* do not appear to be specific to the incompatible interaction. Northern blots would have to be repeated to demonstrate that *StADR1* and *StSGT1* function during a incompatible *P.infestans*-potato interaction.

The expression analyses mentioned above were being performed simultaneously with an additional SSH library performed by other members of the group. The SSH was constructed using tester material from potato cv 1512 c(16), which possesses resistance gene *R2*, collected 15 hpi with *P. infestans* race containing the avirulence gene *Avr2* (Avrova *et al.*, 2004). This resulted in the identification of a further ten genes which maybe involved in the regulation of the HR (Avrova *et al.*, 2004). One of these genes showed high homology at the nucleotide and amino acid level with a Cathepsin B-like protein from *Nicotiana rustica* (Accession no. X81995). Cathepsin B proteins also appear highly conserved in all plant representatives present in databases and therefore potato cathepsin B was named *StCathB* (AY450641) (Avrova *et al.*, 2004). In addition, the full length amino acid sequence of potato *StCathB* displayed 48 % identity with human preprocathepsin B (Appendix I). As discussed in 4.2, cathepsin B has been linked directly with caspases and PCD in mammalian models and was therefore predicted to play a role during the HR (Kingham *et al.*, 2001; Vancompernelle *et al.*, 1998).

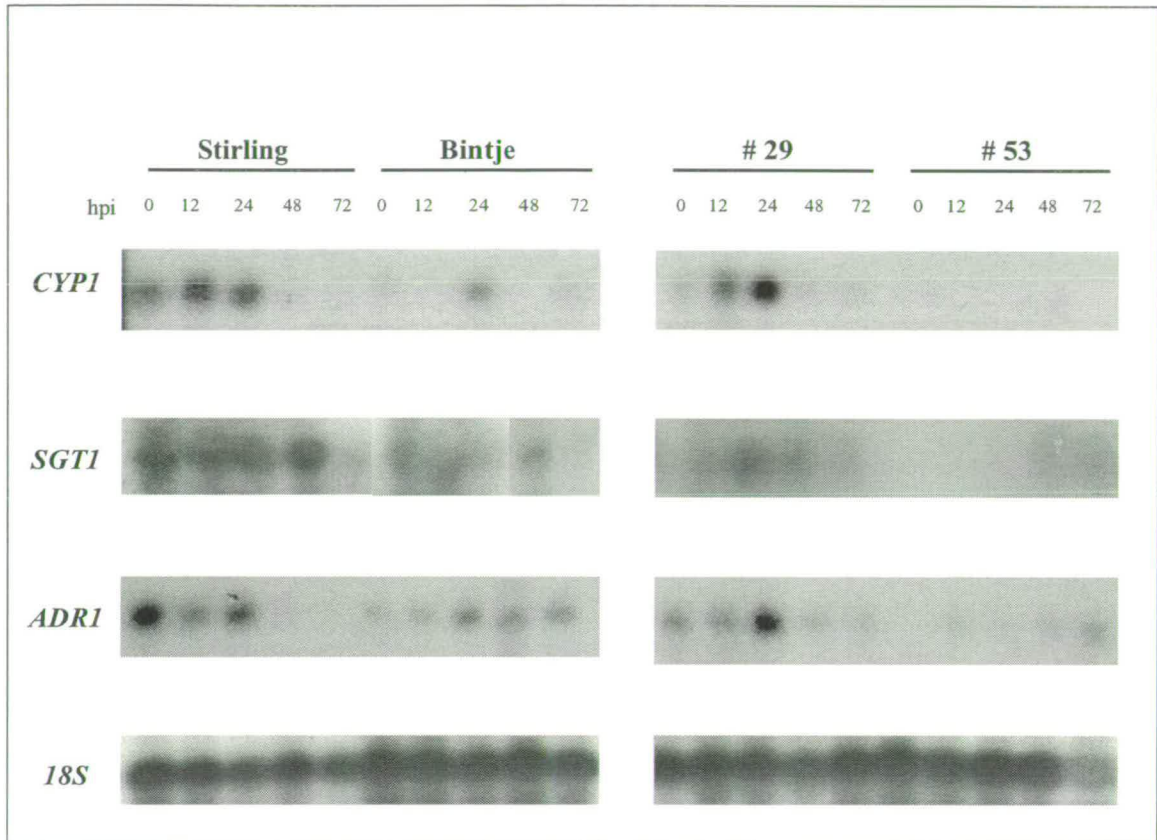


Figure 4.4.1: Expression of candidate genes of interest in *S. tuberosum* cultivars during infection with *P. infestans*. RNA was extracted from leaf samples collected 0, 12, 24, 48 and 72 hours post-inoculation. Stirling possess both race-specific and non-race-specific resistance to *P. infestans* whereas Bintje represents a compatible interaction. Maris Piper/Stirling cross # 29 possess just race-specific resistance and # 53 possesses just non-race-specific resistance. *CYPI*, Cathepsin-like candidate from SSH; *SGT1*, positive example of a gene involved in the HR in other plant species; *ADRI*, gene identified from *Arabidopsis* mutant line through activation tagging and shown to convey resistance to oomycete pathogens when overexpressed. Barley 18S probe was used to reveal RNA loading.

4.4.2. Expression of Cathepsin B in *S. tuberosum* cultivars

Expression analysis of this gene was performed by I. Hein and V. Rokka using filters prepared for Figure 4.4.1. A potato cathepsin B gene (Accession no. BG590588) was found to be induced by 15 hpi during the incompatible interaction and by 48 hpi during the compatible interaction as with *StCYPI* (Figure 4.4.2). The expression pattern of cathepsin B was consistent with all incompatible interactions using potato cvs Stirling, Maris Piper/ Stirling clone # 29 and 1512 c(16). The expression of cathepsin B in compatible interactions was also consistent in potato cvs Maris Piper, Bintje and with the field resistant clone # 53 (Avrova *et al.*, 2004).

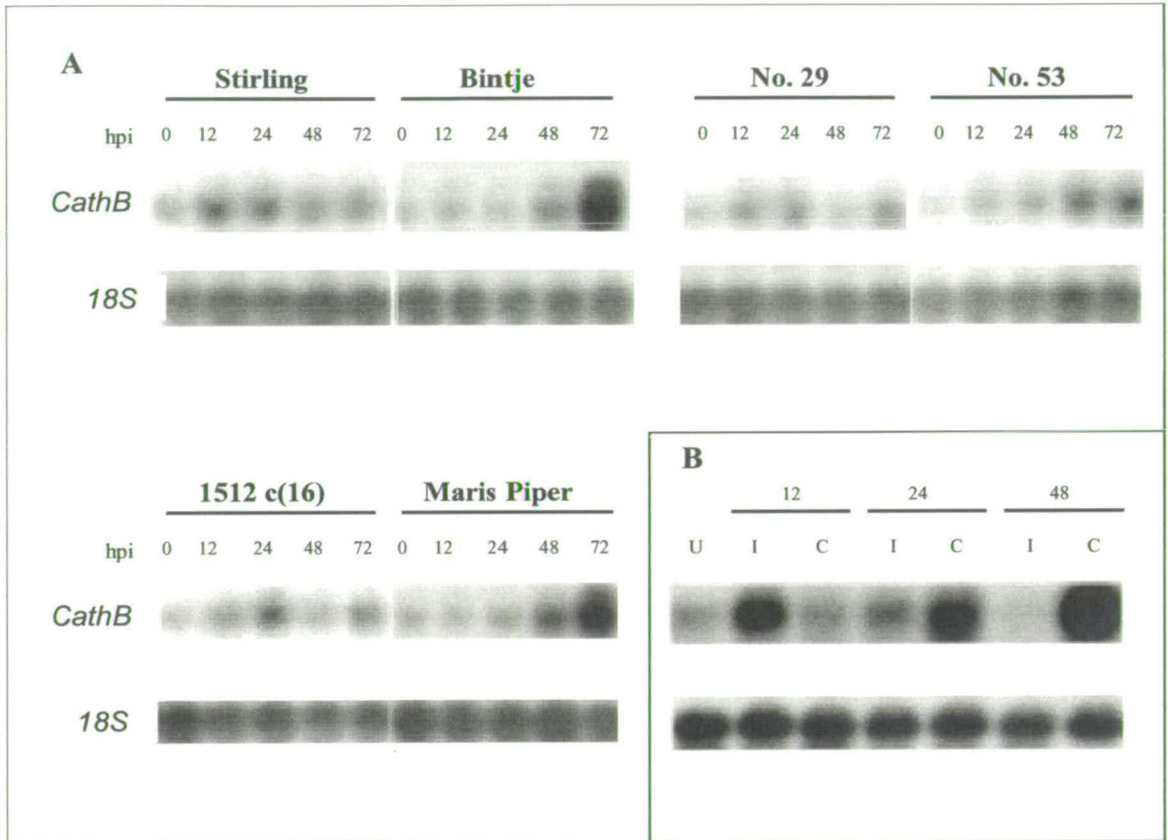


Figure 4.4.2: Expression of cathepsin B in compatible and incompatible potato-*P. infestans* interactions. A, Cathepsin B expression was up-regulated by 12 hpi in a race-specific incompatible interaction with Stirling, no. 29 and 1512 c (16). Maris Piper and Bintje time courses represent cathepsin B expression during a compatible interaction. The field resistant, No. 53 time-course reveals the expression of cathepsin B during non-race specific resistance. B, Shows cathepsin B expression at 12, 24 and 48 hpi. I, 1512 c(16) which carries the *R2* resistance gene and an incompatible race of *P. infestans* carrying *Avr2*; C, Maris Piper with the same race of *P. infestans*; U, represents untreated 1512 c(16) potato plants.

4.5. *CathB* and *SGT1* Gene Silencing in *N. benthamiana*

N. benthamiana is a well established model for studying the HR and virus-induced gene silencing (VIGS). TRV VIGS vectors have been widely used to functionally analyse genes of interest and in this project a TRV vector (MacFarlane and Popovich, 2000) was used to clone genes of interest in anti-sense orientation. *Nicotiana* species are close relatives of potato and therefore homologues of the potato *CYP1*, *CathB*, *ADR1* and *SGT1* genes could be easily sought in order to study the function through gene silencing whilst an additional VIGS system was being developed for potato (Chapter 5). HR in *N. benthamiana* can be triggered using the non-host bacterial pathogens, *Erwinia amylovora* (*Eam*) and *Pst* DC3000 (*avrB*). The effect of silenced gene of interest could then be investigated in terms of HR phenotype, extent resistance is compromised, gene expression and protein activity. The differential expression patterns of *StCYP1*, *StCathB*, *StADR1* and *StSGT1* indicated that each of these genes are involved in *R* gene-mediated resistance against *P. infestans* in the potato cultivar Stirling. However, due to time constraints, *CathB* was prioritised for in depth functional analysis because of its PCD-related role in mammals. *SGT1* was used predominantly as a positive control of a HR-related gene well characterised by VIGS in *N. benthamiana* (Peart *et al.*, 2002).

EST homologues of *StCathB* and *StSGT1* were identified from *N. benthamiana*, *N. tabacum*, *N. rustica*, and tomato (Appendix I: cDNA alignment of Cathepsin B). Blast tools and sequence alignments were used to highlight gene- or gene family-specific regions with relatively high conservation between the species. A 607 bp region of *NbSGT1* (Accession no. AF516181) was cloned into TRV vector in anti-sense using *AvrII* and *KpnI* restriction sites (Table 2.5.1 and Figure 4.5). A 376 bp region of *NbCathB* was amplified using *N. benthamiana* cDNA with primers designed from alignment of *N. tabacum*, *S. tuberosum* and *N. rustica* (AF359422, BG590588 and X81995 respectively). *NbCathB* fragment was then cloned in anti-sense orientation into TRV also using *AvrII* and *KpnI* restriction sites. *N. benthamiana* plants were inoculated at 2 weeks old with sap from a virus-infected *N. benthamiana* plant and left for a further 3-4 weeks for a silencing phenotype to develop. In addition, a previously constructed

TRV::GFP vector was inoculated with every experiment involving TRV::SGT1_{as} and TRV::CathB_{as}, as a control of *N. benthamiana* plants infected with a transgenic TRV vector.

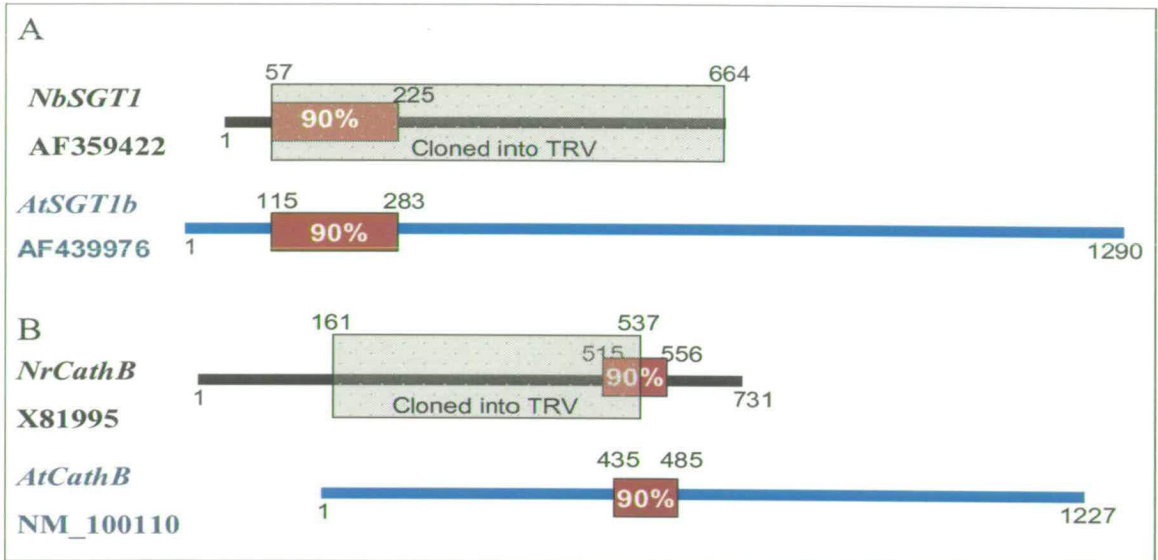


Figure 4.5: Regions of *Sgt1* and *CathB* coding sequences cloned in anti-sense into TRV RNA 2. Dark grey blocks represents region of 90 % nt homology between *Nicotiana* ESTs and *Arabidopsis* coding DNA. Light grey blocks represents the region cloned into TRV. **A**, 609 bp region of *NbSGT1* cloned into TRV in anti-sense including regions encoding the TPR and SGS domains. **B**, 376 bp region of *CathB* cloned into TRV including N-terminal region of papain-like protease domain.

4.5.1. Silencing and HR Phenotype

In order to investigate the HR-related phenotype caused by silencing *NbSGT1* and *NbCathB*, *N. benthamiana* plants were inoculated with TRV::GFP, TRV::SGT1_{as} or TRV::CathB_{as} and left for 4 weeks for silencing to develop. TRV::GFP, TRV::SGT1_{as} or TRV::CathB_{as} *N. benthamiana* plants were then inoculated with two different bacterial pathogens: The avirulent bacterium *Pst* DC3000 (*avrB*), and the apple pathogen *E. amylovora* (*Eam*) which induces a strong non-host HR in *N. benthamiana*. Initially an optimal inoculum concentration had to be found and therefore VIGSed plants were inoculated with bacterial suspensions ranging between 10⁵ and 10⁸ CFU/ml in 10 mM MgCl₂. Suspension of both bacteria at 10⁶ CFU/ml was repeatedly found to be optimal as it produced the expected HR response in TRV::GFP plants and delayed or absent HR symptoms in TRV::SGT1_{as} and TRV::CathB_{as} infected plants (Figure 4.5.1). The

bacterial suspensions of 10^6 CFU/ml are in accordance with those used by Peart *et al.*, 2002 to investigate *SGT1*. Therefore, the suspension of 10^6 CFU/ml was used for all further experiments involving TRV infected *N. benthamiana*.

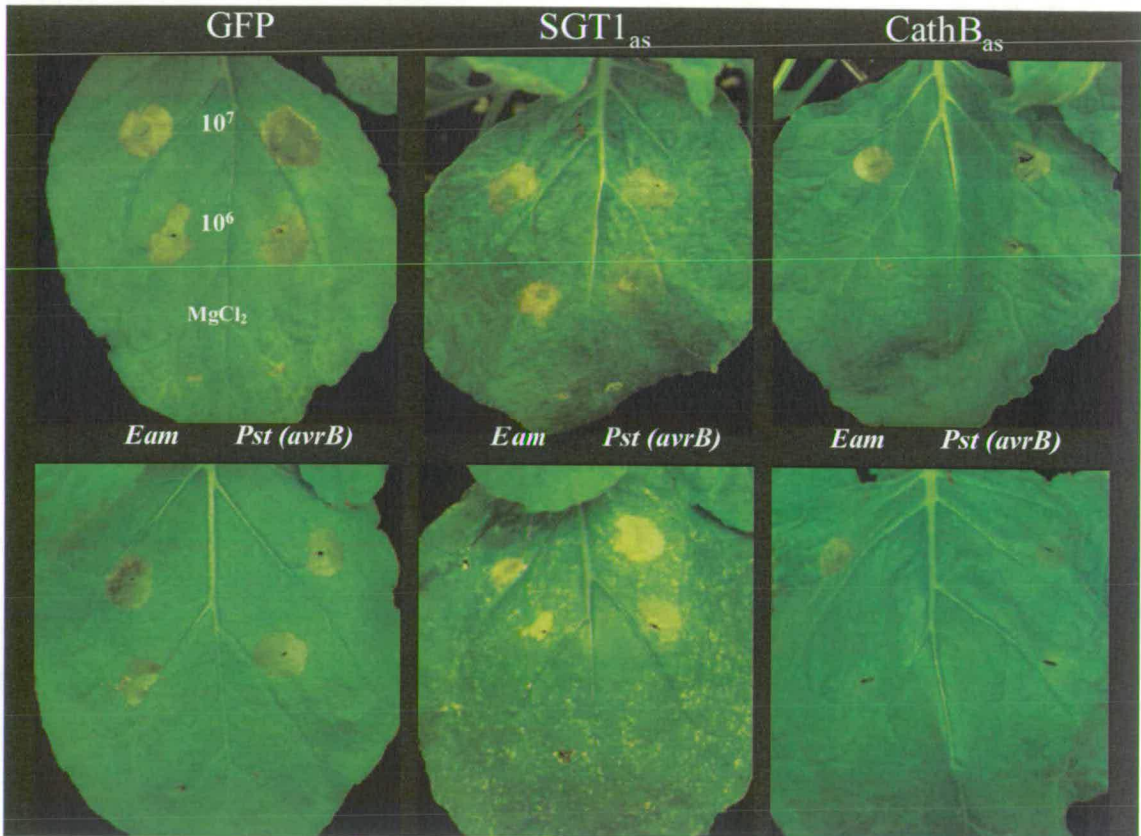


Figure 4.5.1: HR phenotype in TRV::GFP, SGT1_{as} and CathB_{as} *Nicotiana benthamiana*. Plants inoculated with bacterial suspensions 10^6 and 10^7 CFU/ml with *E. amylovora* on left hand side of the leaf and *P. syringae* pv *tomato* DC3000 (*avrB*) on the right. Inoculation buffer (10 mM MgCl₂) was inoculated at the bottom of the leaf on both sides of the vein as a control of any damage caused by the infiltration process. Photographs were taken 48 hpi.

4.5.2. Silencing and Viable Bacterial Numbers

The encouraging discovery that silencing of *NbSGT1* and *NbCathB* could delay and in some cases inhibit an HR induced by avirulent bacterial suspensions of 10^6 CFU/ml led to experiments to analyse the numbers of viable bacteria that could be recovered from the inoculated region. Leaf discs of 1 cm² were cut from leaves 4 dpi with both *Eam* and *Pst* DC3000 (*avrB*) at 10^6 CFU/ml, diluted to 10^{-4} and spread on LB plates. In Figure 4.5.2a, there was more than a log difference in *Pst* growth in SGT1_{as} *N. benthamiana* compared

with control GFP plants. Growth of *Eam* was one log greater in SGT1_{as} plants compared with GFP *N. benthamiana*. Figure 4.5.2b, displays the colony forming units of *Eam* and *Pst* DC3000 (*avrB*) recovered from TRV::GFP and TRV::CathB_{as} *N. benthamiana* leaves. Greater than 1-log more viable *Pst* DC3000 (*avrB*) were recovered from CathB_{as} plants than GFP plants and approximately 1-log greater *Eam* was recovery from CathB_{as} plants than GFP plants. Therefore, silencing of *CathB* allows greater avirulent and non-host pathogen growth than GFP and is comparable with *SGT1* silenced *N. benthamiana*.

Recovery of bacteria from inoculated leaves was repeated at least three times with *Eam* with similar results and the mean values are shown in Figure 4.5.2b. In TRV::GFP plants, 5×10^5 CFU/ml *Eam* were recovered compared with $25\text{-}30 \times 10^5$ CFU/ml from TRV::SGT1_{as} and TRV::CathB_{as} *N. benthamiana*.

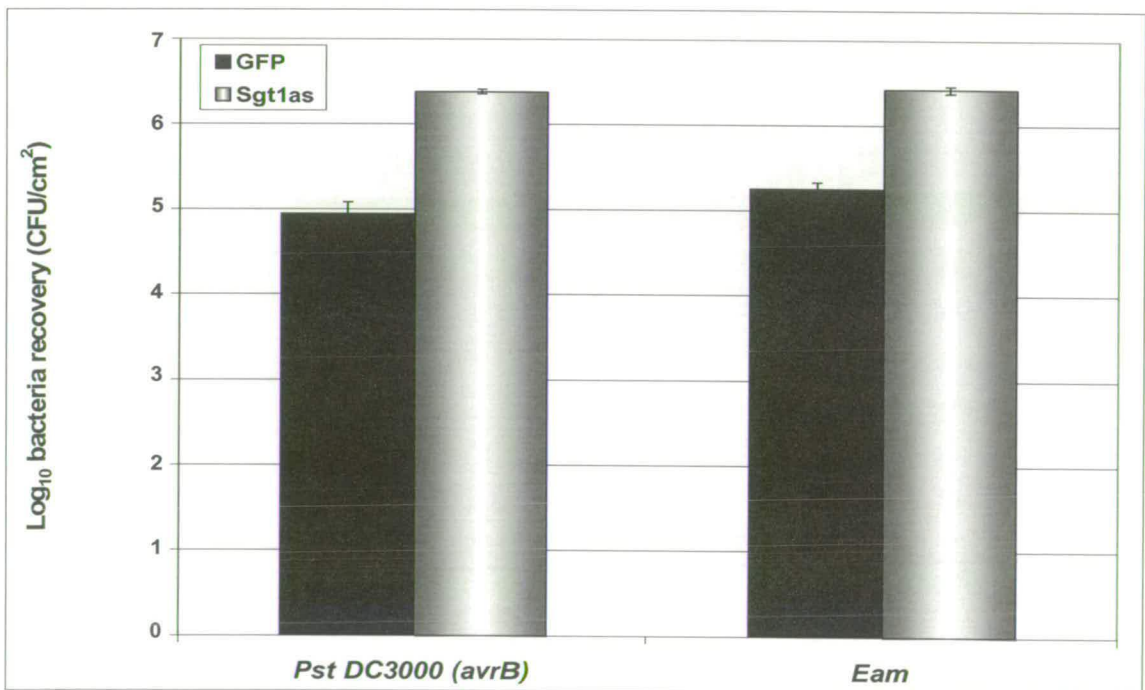


Figure 4.5.2a: *Pst* DC3000 (*avrB*) and *Eam* colony counts in TRV::GFP and TRV::SGT1_{as} plants. Comparison of colony forming units (CFU) recovered from TRV::GFP and TRV::SGT1_{as} *N. benthamiana*. Error bars represent standard error (SE) from experiment involving eight leaf discs from six different plants, each disc plated twice.

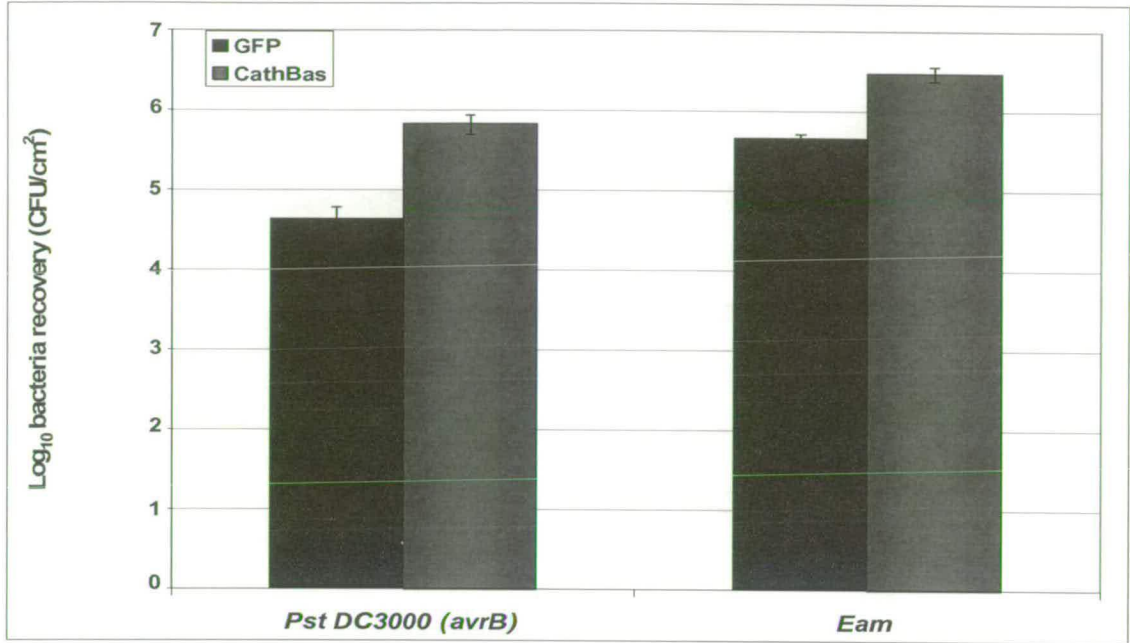


Figure 4.5.2b: *Pst* DC3000 (*avrB*) and *Eam* colony counts in TRV::GFP and TRV::CathBas plants. Comparison of colony forming units (CFU) recovered from TRV::GFP and TRV::SGT1_{as} *N. benthamiana*. Error bars represent standard error (SE) from experiment involving eight leaf discs from six different plants, each disc plated twice.

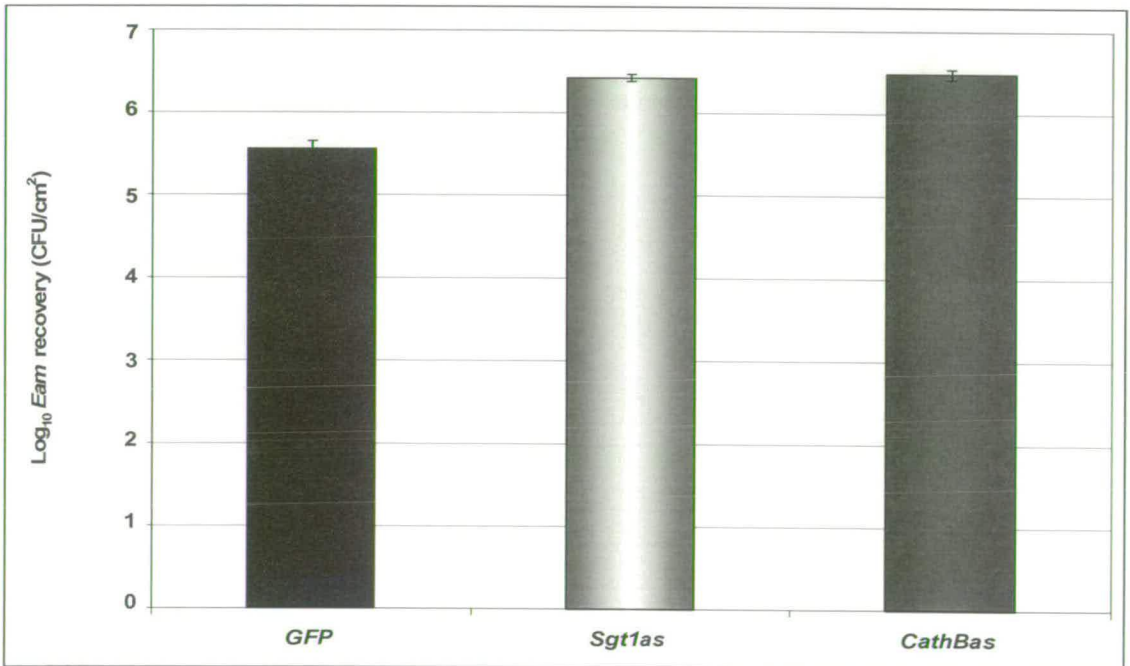


Figure 4.5.2c: Bacterial colony counts in TRV silenced plants 4 dpi. Mean *Eam* recovery CFU/cm² of three biological replicates comparing CFU recovered from TRV::GFP, TRV::CathBas and TRV::SGT1_{as} *N. benthamiana*. Error bars represent combined SE from three biological replicates.

4.5.3. Silencing of *SGT1* in *N. benthamiana*

TRV::SGT1_{as} plants displayed both a morphological and a delayed HR phenotype when compared to TRV::GFP (Figure 4.5.1) indicating that silencing of the *SGT1* transcript was occurring. However, to confirm that silencing was occurring in TRV::SGT1_{as} *N. benthamiana* western blot analysis was performed on these plants at four weeks post-inoculation with viral constructs. Proteins were extracted from three leaf layers (top, middle and lower) from three individual plants for both GFP and SGT1_{as} and one non-inoculated control plant. Rat polyclonal antibody, raised to the SGT1-specific (SGS) domain of *Arabidopsis* SGT1, was kindly provided by Ken Shirasu and used to probe *N. benthamiana* protein samples (Figure 4.5.3). The 48 kDa SGT1 protein was detectable in the non-inoculated and in the TRV::GFP plants but not in TRV::SGT1_{as} infected plants. This provides evidence of successful inhibition of SGT1 protein synthesis in the TRV::SGT1_{as} *N. benthamiana*.

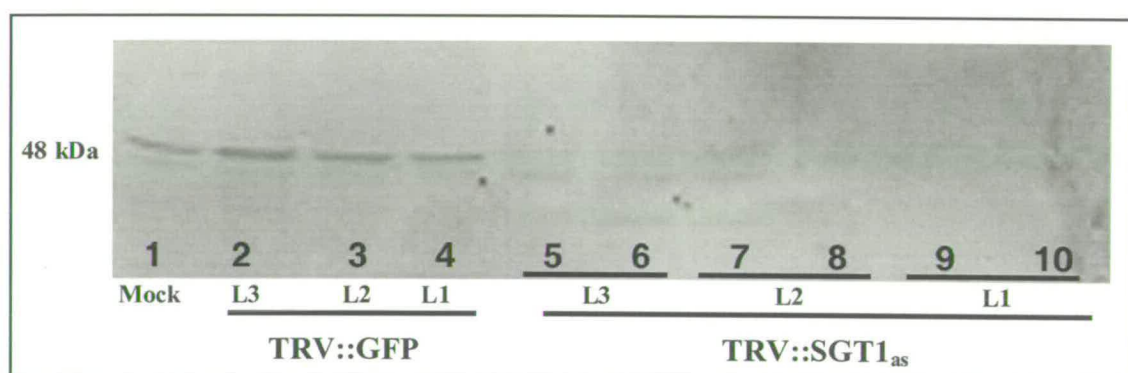


Figure 4.5.3: SGT1 protein in TRV::GFP and TRV::SGT1_{as} *N. benthamiana*. Lane 1 contains uninoculated *N. benthamiana*; 2-4 samples from 3 leaf layers of TRV::GFP; 5-10, 3 leaf layer samples extracted from TRV::SGT1_{as} plant in two separate experiments. 5, 7, 9 leaf layers from experiment 1 and 6,8,10 are leaf layers from experiment 2. L3, top; L2, middle and L1, oldest leaves 4 weeks post-inoculation with TRV (Western blot analysis performed with Christophe Lacomme).

4.5.4. Quantification of Cathepsin B Silencing

To confirm and quantify the silencing of *NbCathB* mRNA in TRV::CathK_{as} *N. benthamiana* plants, quantitative real-time RT-PCR was used as the most sensitive method of RNA detection. The levels of *NbCathB* mRNA were assessed relative to the levels of 25S rRNA present in each sample taken at 0, 6, 12 and 18 hpi with 10⁶ CFU/ml of *Eam*. RNA was extracted from leaf samples taken from at least one leaf disc from six individual plants. 25S was used as an endogenous control for constitutive gene

expression. 25S RNA does not possess a poly-A tail and therefore cDNA was synthesised using random hexamers using leaf samples taken from two previously performed biological replicates. Taqman primers were designed to a region of the *NbCathB* downstream of the sequence cloned into TRV. The levels of *NbCathB* RNA were quantified and for each time point, samples were normalised to the *NbCathB* mRNA levels in the GFP 0 hpi time point (Figure 4.5.4). A 1.8-fold up-regulation of *NbCathB* was observed as a peak at 6 hpi with TRV::GFP plants inoculated with *Eam*. In TRV::CathB_{as} *N. benthamiana*, *NbCathB* expression did increase slightly at 6 hpi with *Eam* but 7-fold lower than that observed in TRV::GFP plants at 6 hpi. The silencing of *NbCathB* was maintained through 6 and 12 hpi in comparison with *NbCathB* expression in TRV::GFP plants. It can therefore be concluded that *NbCathB* expression is being knocked-down to around 20 % of the equivalent GFP plants during the most critical early time-points following *Eam* inoculation in TRV::CathB_{as} *N. benthamiana*.

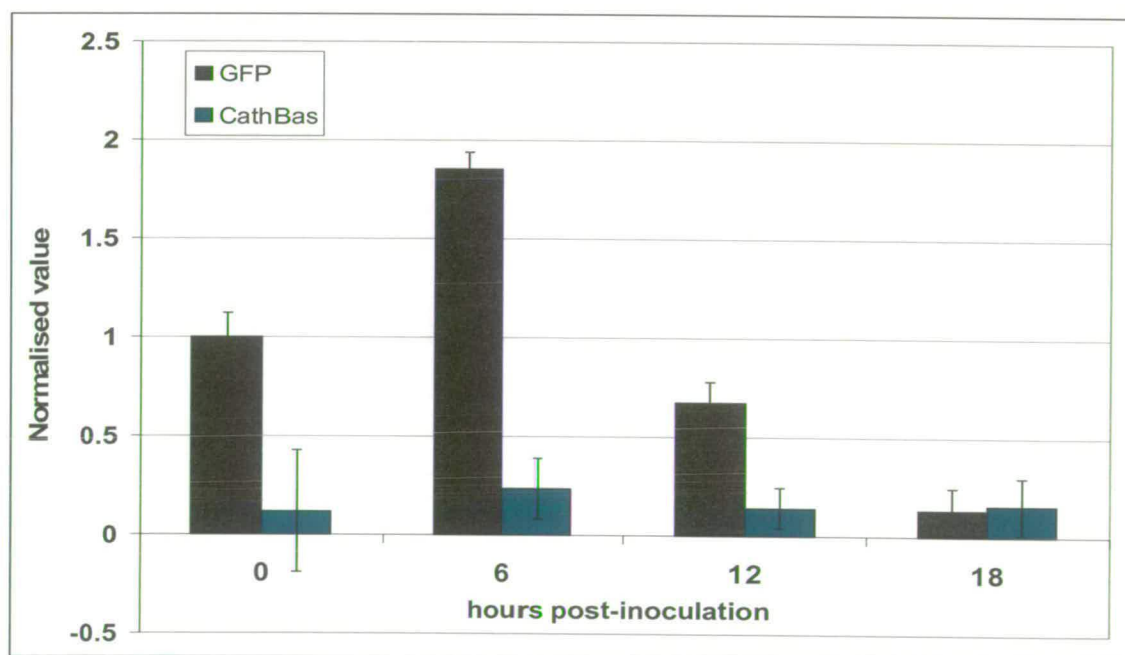


Figure 4.5.4: Quantification of *NbCathB* transcript levels in TRV::GFP and TRV::CathB_{as} plants. Graph displays levels of *NbCathB* transcript in TRV::GFP and TRV::CathB_{as} plants between 0-18 hpi with 10^6 CFU/ml suspension of *Eam*. Amount of cDNA in each sample was normalised using level of 25S transcript. Values represent mean of two biological replicates, each sample being performed in triplicate both times and shown relative to the GFP 0 hpi time-point, plus and minus SE. *NbCathB* taqman primers were designed under the guidance of Ingo Hein. Random hexamer cDNA synthesis was performed by Eduard Venter and the RT-PCR by Ingo Hein.

4.5.5. Protein Activity in Silenced Plants

To further investigate the affect of silencing *NbCathB*, a cathepsin B colorimetric substrate (Z-Arg-Arg-pNA) was used to assay cathepsin B activity between 0 and 24 hpi with *Eam* bacterial suspension of 10^6 CFU/ml. Optimal procedure was based on additional information requested from Calbiochem (Merck Biosciences, UK) At least three leaves from three individual plants were collected for each time point. The concentration of total protein in each sample was measured using a standard Bradford assay and results obtained using the cathepsin B substrate were adjusted to mg/ml of total protein (Figure 4.5.5). In plants inoculated with TRV::GFP, a sharp increase in cathepsin B activity was observed at 6 hpi, mirroring the peak of expression measured in Figure 4.5.4. In contrast, the TRV::CathBas inoculated *N. benthamiana* showed no increase in cathepsin B activity at 6 hpi. The close association between cathepsin B activity and expression in TRV::GFP and TRV::CathBas *N. benthamiana* indicates that protein turnover of cathepsin B is most likely rapid. This experiment was repeated twice further and gave similar results.

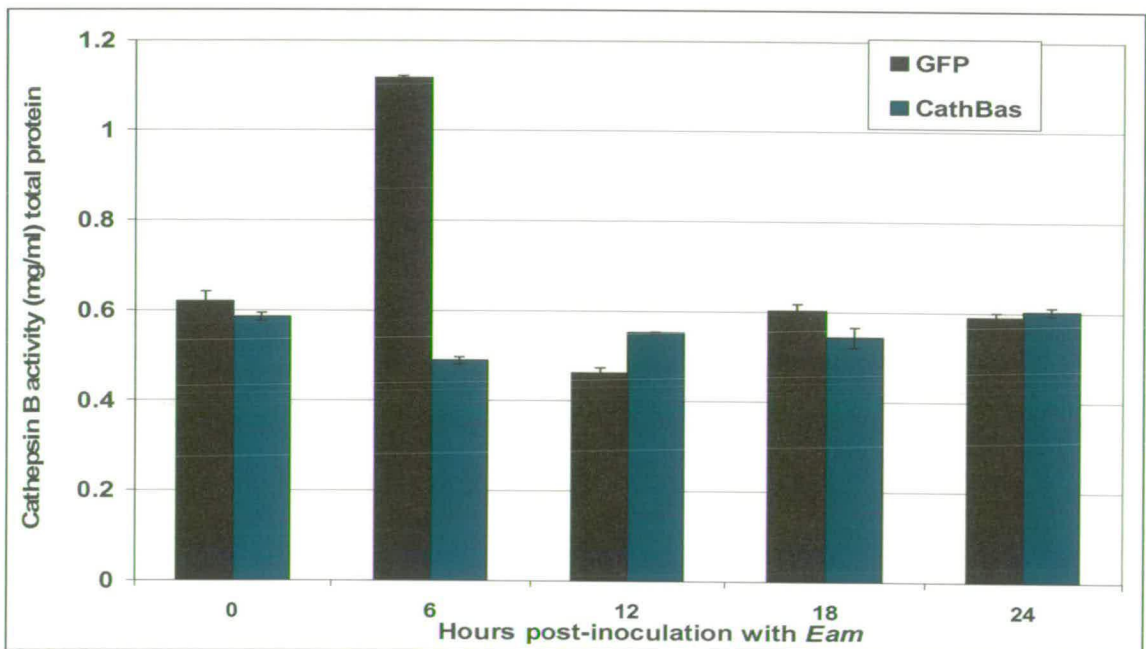


Figure 4.5.5: Cathepsin B activity in TRV::GFP and TRV::CathBas *N. benthamiana*. The graph displays mean values of pooled protein samples analysed in triplicate for both the Bradford assay and the Cathepsin B activity assay. Leaf samples were collected at 6 hr intervals post-inoculation with *Eam*. Values shown represent the mean and the error bars indicate the SE of triplicate readings for each sample.

4.6. *Cathepsin B inhibitors in N. benthamiana*

4.6.1. Inhibitors and HR phenotype

To further investigate the involvement of cathepsin B during *Eam*-induced HR, a range of mammalian cathepsin B protein inhibitors were ordered from Calbiochem (Merck Biosciences, UK) (Table 4.6). As human cathepsin B and plant cathepsin B share 47 % homology at the amino acid level, it was possible that these inhibitors would inhibit plant cathepsin B activity (Appendix I: Protein Alignment). Early experiments use Z-FGNHO-Bz and Ac-LVK-CHO inhibitors diluted to 1-150 μM with *Eam* bacteria suspensions of between 10^5 - 10^8 CFU/ml in MES or MgCl_2 buffer.

Table 4.6: Cathepsin B Inhibitors

Inhibitor	Full Name	Reference
Cathepsin B, S, L and Papain: Z-FGNHO-Bz	benzyloxycarbonyl-Phe-Gly-hydroxylamine-benzoyl	Demuth et al., 1996
Cathepsin B I: z-FA.fmk	benzyloxycarbonyl-Phe-Ala-fluoromethylketone	Schotte et al., 2001
Cathepsin B II: Ac-LVK-CHO	Acetyl-Leu-Val-lysinal	McConnell et al., 1993
Cathepsin B IV: CA-074 Me	[L-3-trans-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline Methyl Ester	Foghsgaard et al., 2004

In accordance with the optimum bacterial suspension concentration found for VIGSed plants (4.5.1), 10^6 CFU/ml was found to consistently induce the HR (Figure 4.6.1a). The observed HR induction could be inhibited repeatedly with 150 μM of either cathepsin inhibitor in 5 mM MES and therefore 5 mM MES buffer was used for subsequent inoculations. Figure 4.6.1a shows the leaves inoculated with 10^6 CFU/ml of *Pst* DC3000 (*avrB*) with 150 μM inhibitor resulted in a patch of chlorosis, characteristic of disease symptoms caused by virulent *Pst*. In addition, HR symptoms in some leaves inoculated with 10^6 CFU/ml of *Eam* appeared reduced compared to control inoculation sites suggesting the inhibitors were delaying the HR. Henceforth, the concentration of inhibitors was increased to an optimal concentration of 1 mM. Inhibitor concentration of 1 mM reproducibly repressed the HR symptoms using all four inhibitors (Figure 4.6.1b). In this experiment, the volume inoculated was carefully inoculated so that the 10 μl of each suspension was pressure infiltrated as equally as possible.

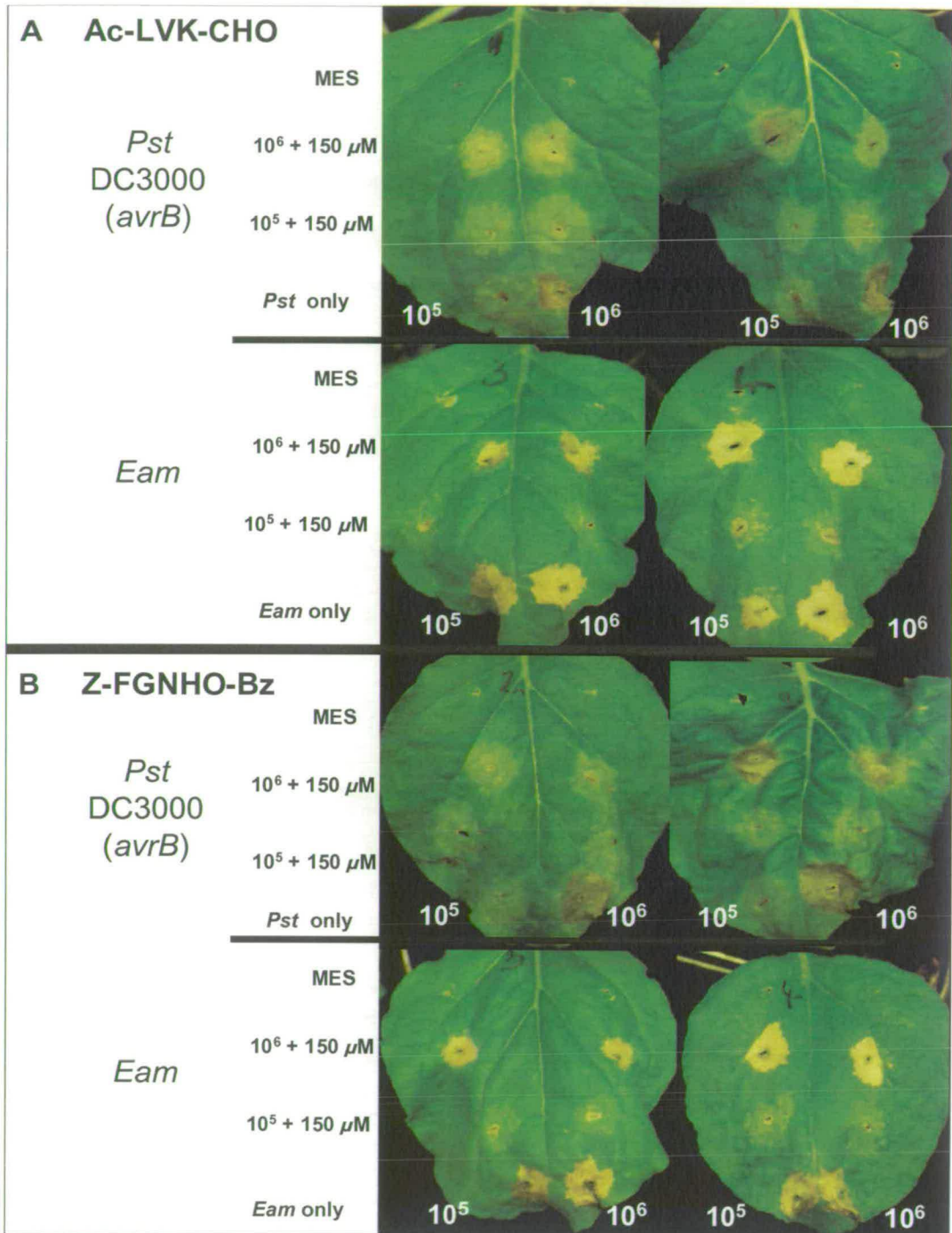


Figure 4.6.1a: Optimizing CFU/ml bacterial suspensions with 150 μM of cathepsin inhibitors. A, Cathepsin B-specific Inhibitor II (Ac-LVK-CHO) and B, general cathepsin inhibitor (Z-FGNHO-Bz). *Eam* and *Pst* (*avrB*) bacterial suspensions at 10^5 and 10^6 CFU/ml inoculated with and without cathepsin B inhibitors.

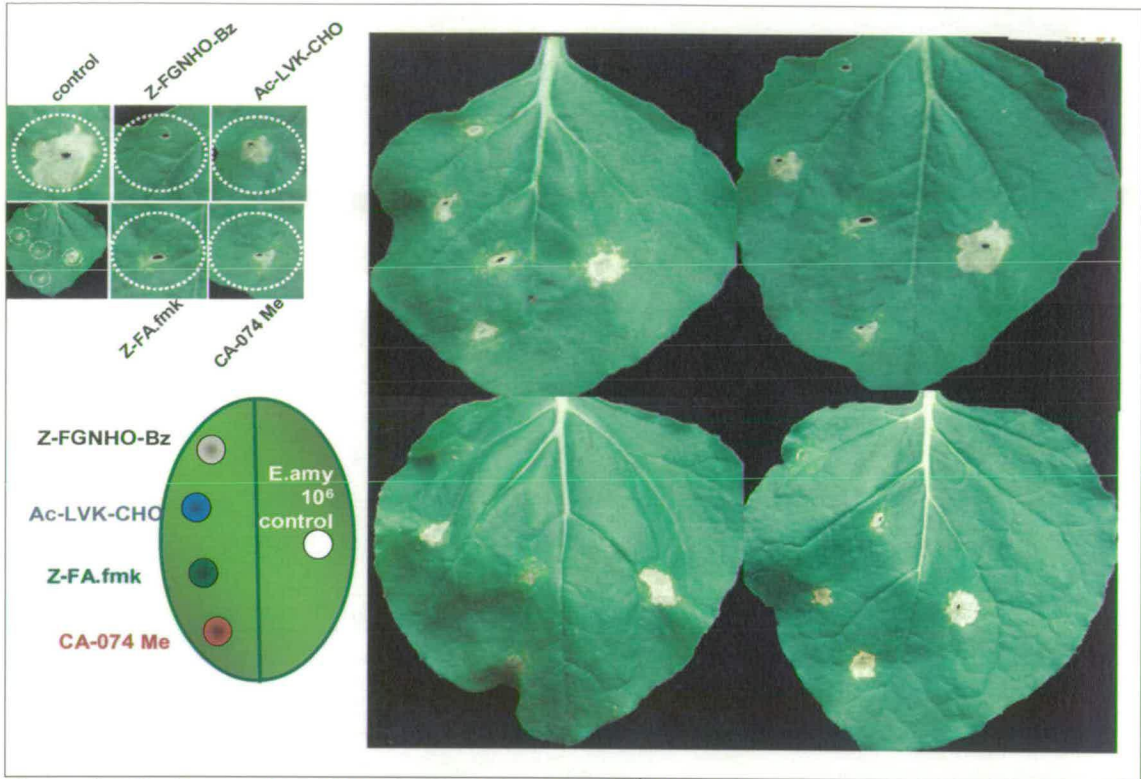


Figure 4.6.1b: Repression of HR induced by *Eam* (10^6 CFU/ml) with 1 mM Cathepsin inhibitors. Photographs of four leaves inoculated with $10 \mu\text{l}$ of *Eam* suspension (10^6 CFU/ml) on the right side of the leaf and with 1 mM of each of the inhibitors on the left.

4.6.2. Inhibitors and Viable Bacteria Counts

Following visual observations that the cathepsin B inhibitors could repress the HR phenotypes, it was necessary to measure any difference in bacterial growth. Leaf discs were cut from the *Eam* (10^6 CFU/ml) inoculated plants with and without inhibitor. Recovery of viable *Eam* inoculated with each cathepsin B inhibitor was performed and revealed a 10-fold increase in bacteria numbers with Ac-LVK-CHO and CA-074 Me cathepsin inhibitors and 5-fold increase with z-FA.fmk (Figure 4.6.2, A). This implies a link between suppression of the HR phenotype and the number of HR-inducing bacteria that could be recovered from inoculated leaf discs. To investigate whether the inhibitors affected *Eam* growth, LB liquid cultures supplemented with 1 mM inhibitor were inoculated with *Eam* and grown overnight. The resulting optical densities of these overnight cultures are shown in Figure 4.6.2B which illustrates that these inhibitors are not toxic to the bacteria although does not reveal differences in growth rate over time. It

is therefore most likely that the suppression of cathepsin B activity was responsible for the repression of HR phenotypes in Figure 4.6.1B and not reduced bacterial viability.

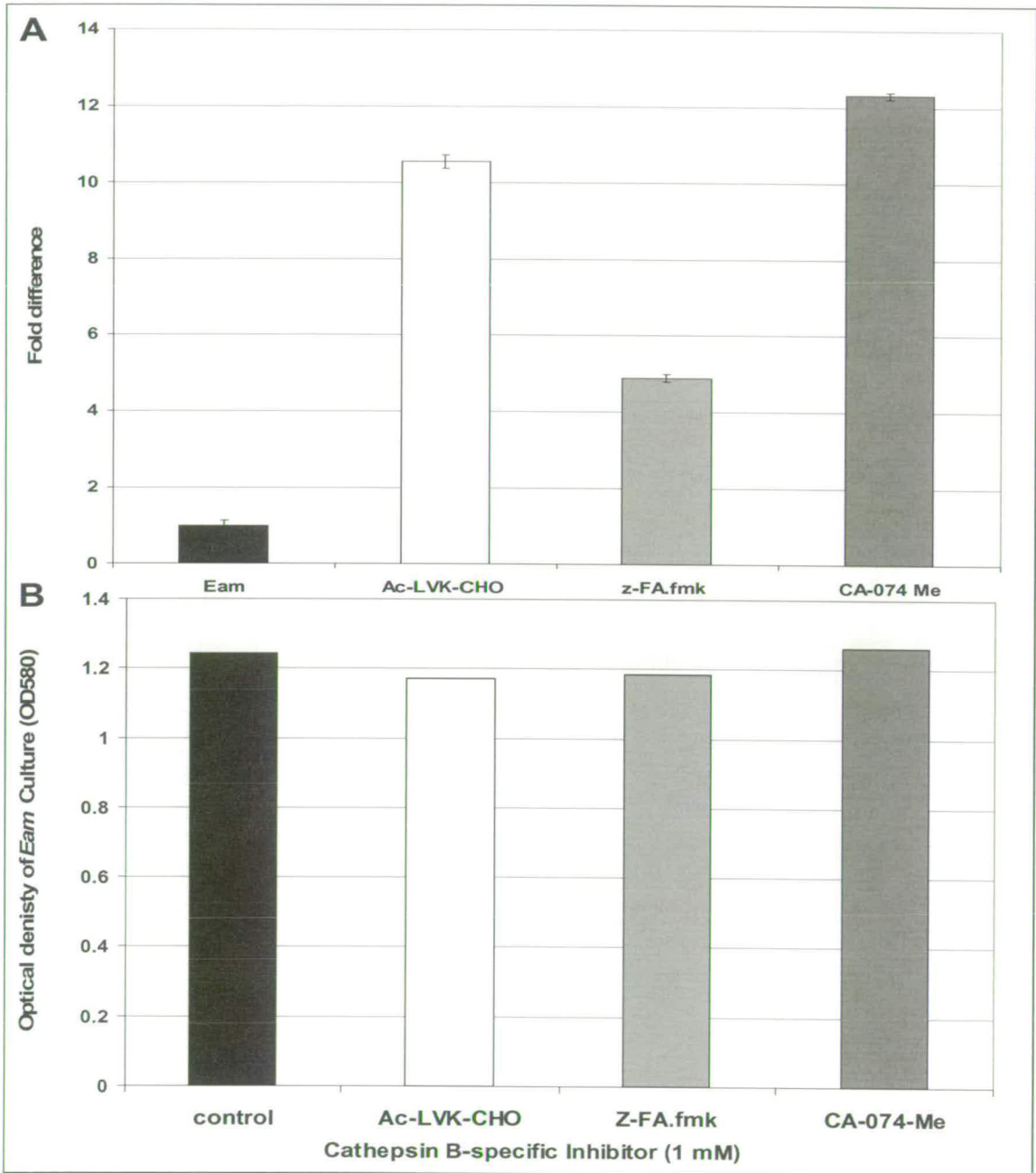


Figure 4.6.2: Effect of cathepsin B-specific inhibitors on *in vivo* and *in vitro* pathogen growth. A, Fold increase in *Eam* CFU/cm² recovered from leaves co-inoculated with 1 mM cathepsin B-specific inhibitors compared with control inoculations with no inhibitor. Results are the mean of two biological replicates and error bars represent the combined SE. **B,** optical densities (OD₅₈₀) of 1 ml of overnight liquid cultures of *Eam* in LB broth at 30 °C in presence of 1 mM cathepsin B-specific inhibitors.

4.6.3. Inhibitors and Cathepsin B Activity

The next experiment was designed to investigate the effect the inhibitors had on endogenous cathepsin B activity. Therefore the activity of cathepsin was measured prior to infiltration and 6 hpi with 5 mM MES buffer, *Eam* suspension (10^6 CFU/ml) and same bacterial suspension with 1 mM of Ac-LVK-CHO or z-FA.fmk cathepsin B-specific inhibitors Figure 4.6.3. In agreement with the results presented in Figure 4.5.5, an increase in cathepsin B activity was observed at 6 hpi with *Eam* but not induced in the MES inoculated control. This cathepsin B activity at 6 hpi was substantially blocked by both cathepsin B-specific inhibitors. This supports the hypothesis that cathepsin B protein activity at 6 hpi with the non-host pathogen *Eam* is necessary for successful HR development and that co-infiltrated inhibitors block the cathepsin B activity at 6 hpi.

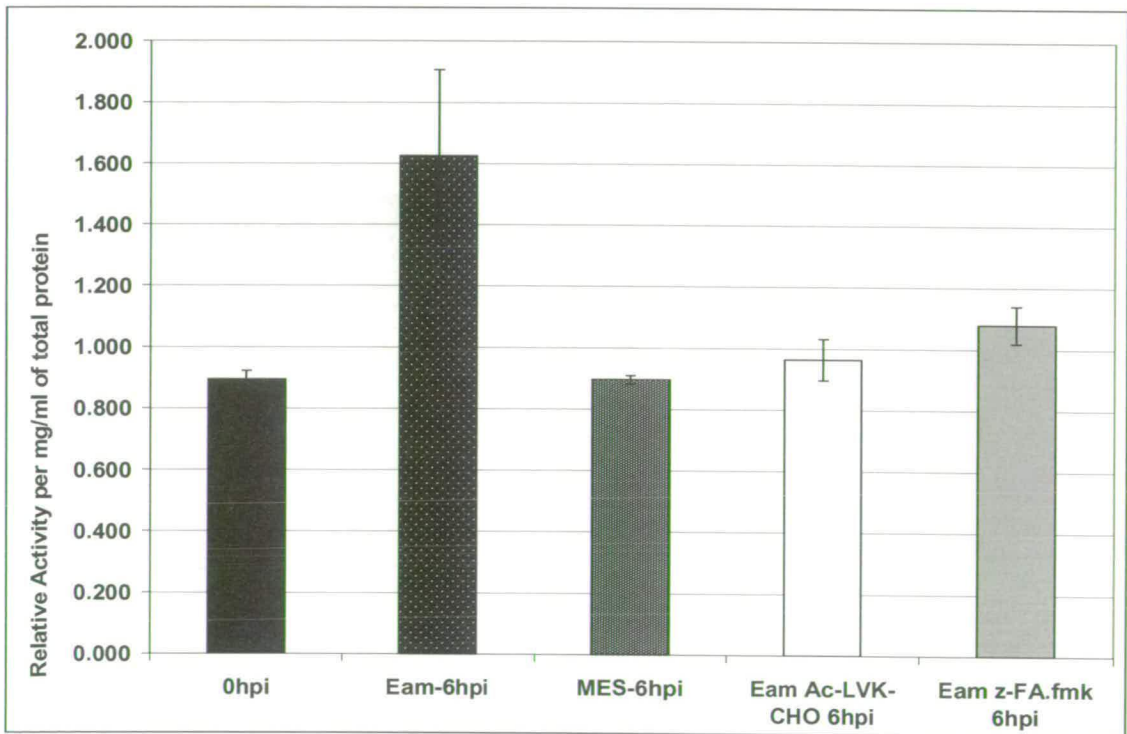


Figure 4.6.3: Cathepsin B protein activity in inhibitor treated *N. benthamiana* 6 hpi with *Eam*. Cathepsin B activity was measured using a colorimetric assay and made relative to mg/ml of total protein using Bradford Protein assay. Values represent the mean of two biological replicates, with samples of each treatment taken from 6 replicate plants. Error bars represent the combined SE from both experiments. Experiment was repeated many times although the data presented here were measured by Eduard Venter.

4.7. *Cathepsin B and PARP Cleavage*

During mammalian apoptosis, a hallmark of PCD is the cleavage of poly (ADP-ribose) polymerase (PARP) (Boucher *et al.*, 2001). PARP is a monomeric, 116 kDa protein with unusual zinc-fingers that are thought to recognise and be activated by DNA breaks. It modifies protein targets (e.g. histones and topoisomerase) (D'Amours *et al.*, 1999). PARP possess 62 Asp residues, some of which have been shown to be cleavage sites for caspases (DXXD) (Duriez and Shah, 1997). Caspase-3 cleavage of PARP typically produces a 25 kDa N-terminal fragment and an 85 kDa fragment (Kaufmann *et al.*, 1993). In addition, active lysosomal cathepsin B has also been shown to cleave PARP in the presence of broad spectrum caspase inhibitor, zVAD-fmk (Gobeil *et al.*, 2001). Caspase-1 activity has been identified during the HR although it is not known to cleave PARP and the mammalian orthologue is involved in cytokine activation and is not an initiator or effector of apoptosis (Hatsugai *et al.*, 2004; Chang and Yang, 2000). It is possible that plant cathepsin B may be responsible for the cleavage of PARP during the HR. D'Silva *et al.*, (1998) used bovine PARP-1 to identify cysteine protease activity and describe the production of four cleavage products during the cowpea HR, with molecular masses of 77, 52, 47 and 45 kDa (Figure 4.7A). Furthermore, their research demonstrated that only a papain, cathepsin and calpain inhibitor E-64, could block formation of the 45 kDa, N-terminal PARP fragment.

Western blot analysis was performed using bovine cathepsin B, bovine PARP-1 and protein extracts based on methodology described in D'Silva *et al.*, (1998). Firstly, the PARP-1 cleavage pattern produced by bovine cathepsin B was investigated with and without 1 mM Ac-LVK-CHO or z-FA.fmk. Following a 30 min incubation of bovine cathepsin B and bovine PARP-1, a band of 45 kDa was identified which must be the N-terminal DNA-binding domain recognised by the antibody. Cathepsin B inhibitor, Ac-LVK-CHO appeared to block the formation of this band and produced a pattern similar to undigested PARP-1. The inhibitor, z-FA.fmk, on the other hand, did not block the formation of the 45 kDa fragment. This suggests that the reaction rate of PARP-1 cleavage by bovine cathepsin B can be partially inhibited by z-FA.fmk and almost

completely inhibited by Ac-LVK CHO. Figure 4.7C, lane 2 was included as a negative control but reveals that endogenous plant PARP cross reacts with the bovine PARP antibody. However, formation of a 45 kDa PARP fragment in the total protein extracted from TRV::GFP at 6 hpi with *Eam* supports the presence of cathepsin B activity. Lane 3 reveals the same 45 kDa band produced by the bovine cathepsin B and bovine PARP. Lane 4, shows that 1 mM Ac-LVK-CHO can prevent formation of the 45 kDa band in a bovine cathepsin B and bovine PARP incubation. Lane 5 was designed to identify cathepsin B activity in the crude protein extract from TRV::GFP plants at 6 hpi. However as lane 2 has already revealed cross reaction between endogenous PARP and the antibody the activity of *N. benthamiana* cathepsin B on bovine PARP cannot be determined. The effect of Ac-LVK-CHO on cathepsin B activity in total protein extracts from TRV::GFP plants was investigated (lane 6). The inhibitor, Ac-LVK-CHO appears to have not prevented the formation of the 45 kDa fragment in this experiment. Interestingly, the incubation of TRV::CathB_{as} 6 hpi total protein extract with bovine PARP reveals a massive degradation of the PARP and accumulation of 6 and 14.5 kDa fragments. Interestingly, the same fragments can be identified in lane 4, in which bovine cathepsin B and bovine PARP are incubated with 1 mM Ac-LVK-CHO, suggesting a similar loss of cathepsin B activity but perhaps an increase in activity of other proteases. However, the absence of 116 kDa fragment in lane 7 suggests that there is less protein loaded and that the PARP may be degraded in this sample. Since the bovine PARP-1 antibody cross reacts with endogenous plant PARP the activity of endogenous *N. benthamiana* cathepsin B cannot be properly investigated until it has been tagged, purified and investigated *in vitro*.

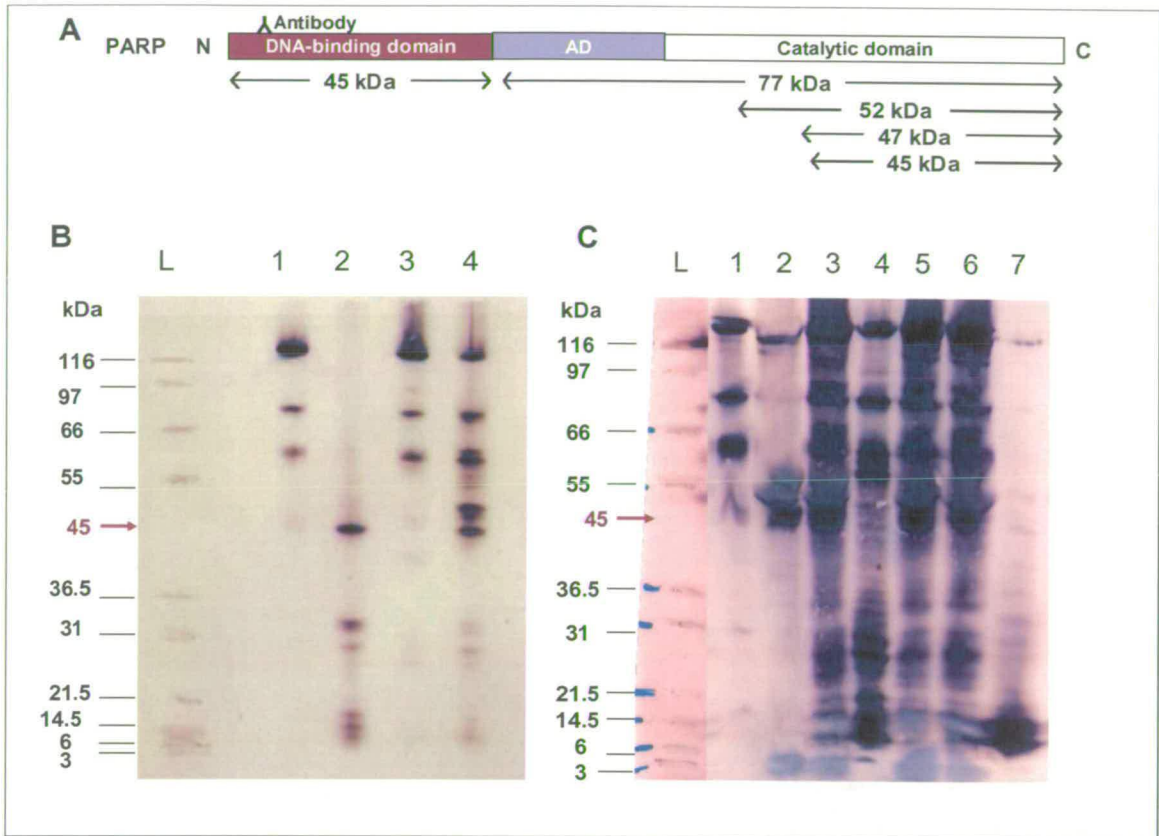


Figure 4.7: Cleavage of PARP with bovine cathepsin B, inhibitors and protein extracts. Samples run on 4-8 % SDS-PAGE gel. Western blots probed with polyclonal antibody raised against 2-20 aa of bovine PARP and visualised using secondary antibody conjugated with alkaline phosphatase. **A**, domains of bovine PARP, antibody binding and previously described cleavage products (D'Silva *et al.*, 1998); AD, automodification domain. **B**, L represents protein standard ladder. 80 ng of Bovine PARP used when mentioned. incubation time of 30 min. Lane 1, bovine PARP; Lane 2, bovine PARP and bovine cathepsin B; Lane 3, bovine PARP, bovine cathepsin B and 1 mM Ac-LVK-CHO; Lane 4, bovine PARP, bovine cathepsin B and 1 mM Z-FA.fmk. **C**, Lane 1, bovine PARP; Lane 2, protein extract from TRV::GFP 6 hpi with *Eam*; Lane 3, bovine PARP and bovine cathepsin B; Lane 4, bovine PARP, bovine cathepsin B and 1 mM Ac-LVK-CHO; Lane 5, bovine PARP and protein extract from TRV::GFP 6 hpi with *Eam*; Lane 6, bovine PARP and extract from TRV::GFP 6 hpi and 1 mM Ac-LVK-CHO; Lane 7, bovine PARP and protein extract from TRV::CathBas 6 hpi with *Eam*.

4.8. Effect of Inhibitors on Caspase-like Activity

As mentioned previously, several examples of caspase-like activity have been measured during the HR (del Pozo and Lam, 1998; Coffeen and Wolpert, 2004; Chichkova *et al.*, 2004; Rojo *et al.*, 2004) and during developmental PCD (Belenghi *et al.*, 2003). In addition, studies of caspase-independent cell death in the mammalian system have

implicated some cysteine proteases as an alternative route to PCD (Abraham and Shaham, 2004). It was therefore interesting to examine whether cathepsin B functioned upstream or independently of the caspase-like activity measured by Chichkova *et al.*, (2004) during HR induced by *N*-TMV interaction. Chichkova *et al.*, 2004, utilised two caspase-3 cleavage sites present in the VirD2 protein (TATD and GEQD) to construct a fusion protein that localises to the nucleus until caspase-like activity cleaves the nuclear localisation signal (NLS) from VirD2 releasing GFP into the cytoplasm (Figure 4.8A). Linearised TMV GFP::VirD2 vector was transcribed and added to a TMV strain U1 coat protein for reassembly. TMV GFP::VirD2 was then inoculated into *N. tabacum* CB9 which contains a viral movement protein which helps the spread of the virus. Plants were incubated at 24 °C for 48 hr to allow viral lesions to develop. Lesions were examined under the scanning laser confocal microscope, location marked and then photographs taken before inoculation of *Eam* (10^6 CFU/ml) with and without 1 mM Ac-LVK-CHO inhibitor and TATD inhibitor. The same lesions were inspected every hour until cytoplasmic GFP was identified in the *Eam* inoculated lesions, followed by the *Eam* and inhibitor treated lesions (usually around 6 hpi) (Figure 4.8B). At 6 hpi with *Eam* suspension resulted in cleaved GFP::VirD2 re-localization to the cytoplasm of epidermal cells suggesting caspase activity was induced. Co-inoculation of *Eam* with the caspase-3 inhibitor, TATD, inhibited the cleavage and re-localisation of the GFP::VirD2 protein fusion. Unexpectedly, this result was also observed using the cathepsin B-specific inhibitor, Ac-LVK-CHO. Therefore, inhibition of cathepsin B activity at 6 hpi with *Eam* also causes inhibition or delay of caspase-3 activity during the plant HR. However, this is preliminary data and the experiment needs to be repeated to draw definite conclusions. In addition, the GFP::VirD2 construct could be used for Western blot analysis using purified cathepsin B, bovine cathepsin B and total protein extracts with an antibody raised to GFP to detect a size change caused by VirD2 cleavage.

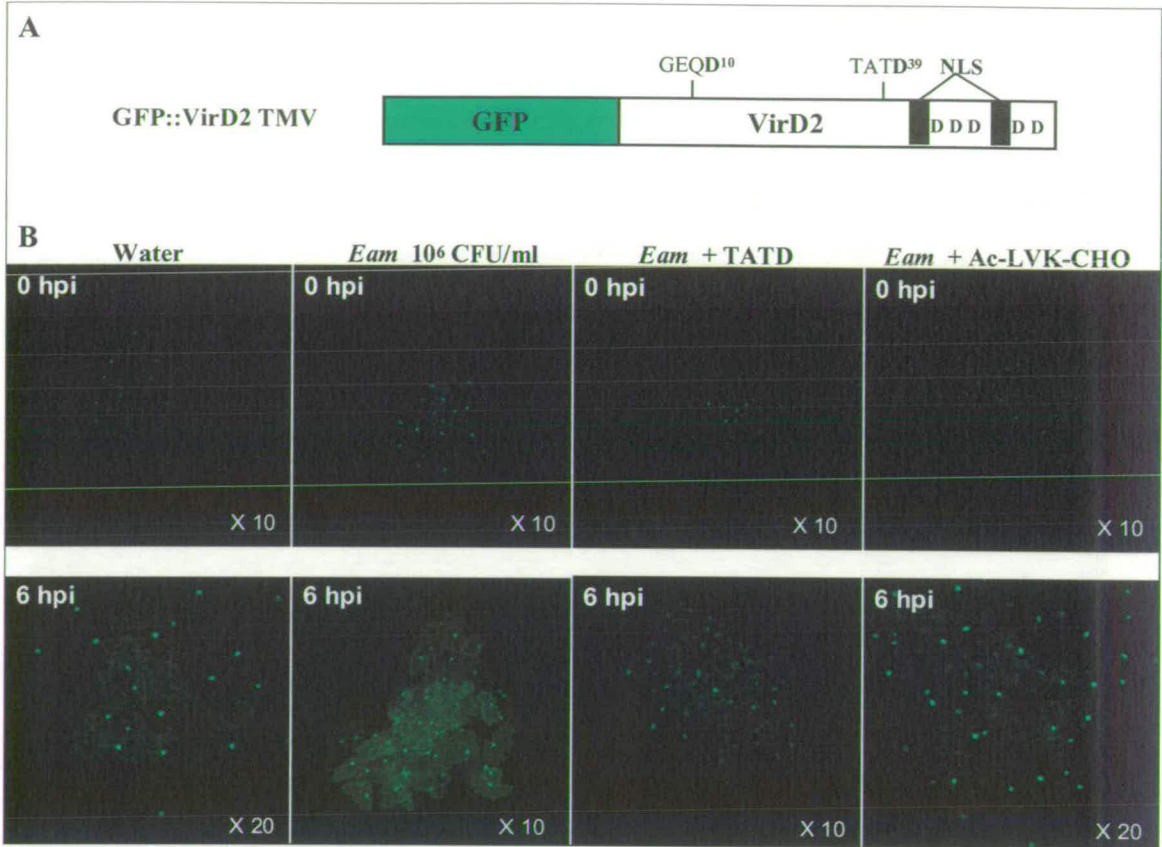


Figure 4.8: GFP::VirD2 localisation in *Eam*-induced HR in tobacco. **A**, Map of TMV GFP::VirD2 construct and position of caspase cleavage sites. **B**, Each TMV GFP::VirD2 lesion was imaged before and 6 hpi after treatment. Water treatment, 0 hpi and 6 hpi; *Eam* 10⁶ CFU/ml 0 hpi and 6 hpi; *Eam* (10⁶ CFU/ml) co-inoculated with 1 mM caspase inhibitor, TATD 0 hpi and 6 hpi; *Eam* (10⁶ CFU/ml) co-inoculated with 1 mM cathepsin B, Ac-LVK-CHO. Trudi Gillespie provided instruction on the scanning laser confocal microscope.

4.9. *Arabidopsis* Cathepsin T-DNA Insert Lines

To take advantage of the sequenced model *Arabidopsis* and the wealth of resources available to the research community, all three *Arabidopsis* cathepsin B gene homologues (At1g02300, At1g02305 and At4g01610) and one *CYP1* homologue, known as *RD21*, (At1g47128) were identified. Insertion lines in these genes (as annotated at time of ordering) were obtained from Nottingham Arabidopsis Stock Centre (NASC) and Arabidopsis Biological Resource Centre (ABRC) through the SIGnAL website (Alonso *et al.*, 2003) (See Table 4.9 and 2.15).

Table 4.9: Insertion loci, SALK seed line number and accession number of flanking sequence

Locus	SALK Insertion No.	Left Border Flanking Seq. Accession
At1g02300	SALK_049118	BH755420
At1g02300/ At1g02305	SALK_138261	BZ767041
At4g01610	SALK_019639	BH752998
At1g47128	SALK_090550	BZ762147

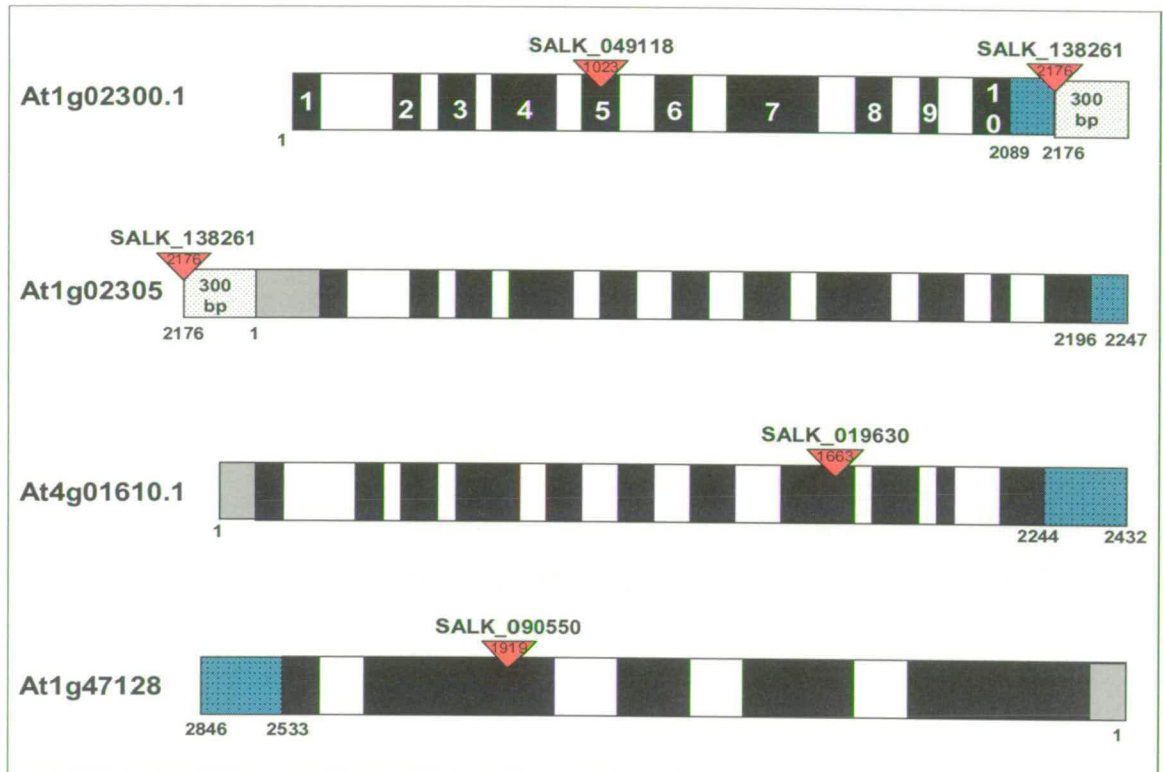


Figure 4.9: Cathepsin B insertion lines from ABRC. Grey areas represent 5' and 3' UTRs, black regions are exons and white regions are introns. The red triangle represents the pROK2 T-DNA vector and the location of its insertion within the gene found using left border flanking sequences.

4.9.1. Genotyping and expression analysis of mutant lines

The T-DNA insertion lines were sent as the progeny of a heterozygous insertion Col-0 *Arabidopsis* parent and therefore required genotyping to identify homozygous offspring. Homozygous plants were identified by a PCR reaction with three primers, two gene-specific primers that produce a PCR product of 800 bp and one primer (LBb1) that anneals to the pROK2 T-DNA insert (2.8.3). Gene-specific primers were designed through the SIGnAL website appropriate for each insertion site (See 2.8.3). When the insert was present, a PCR product of 400 bp was produced. By this means, insertion

lines were genotyped until a generation of completely homozygous progeny was identified. In order to confirm the knock out of gene expression in these mutants, primers were designed to the 3'UTR of the three cathepsin B homologues and could be used to distinguish their expression in the insertion lines.

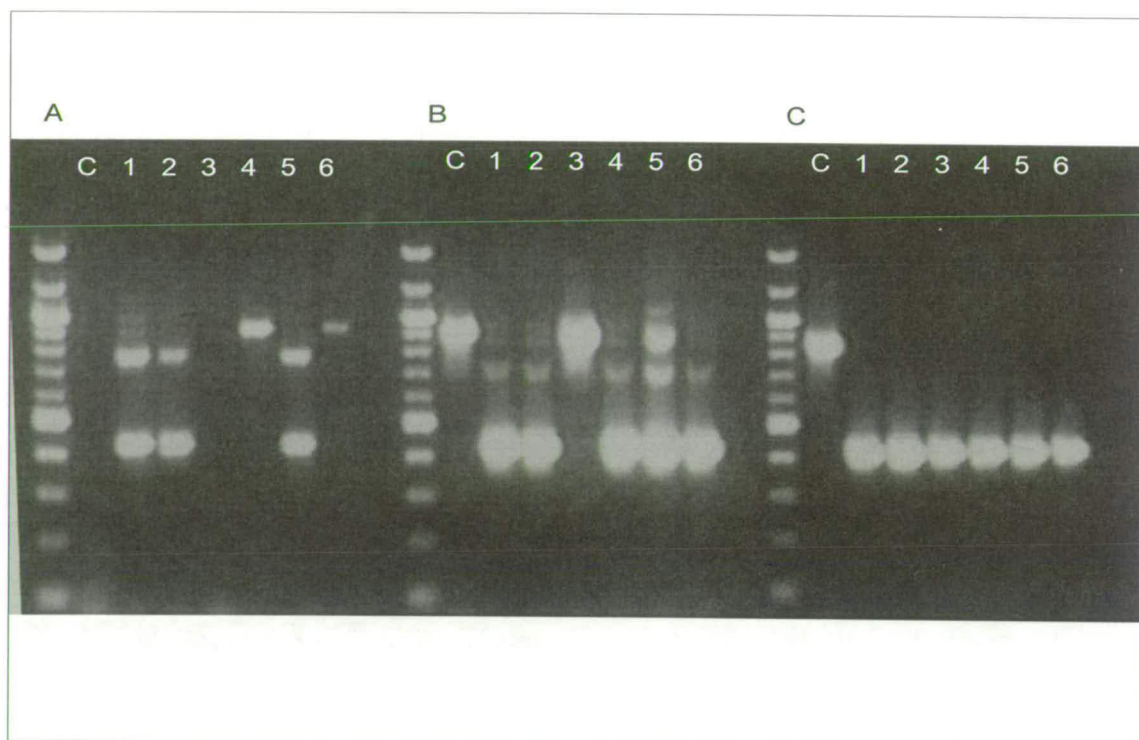


Figure 4.9.1a: Genotyping products of PCR of SALK T-DNA insertion lines. Combination of a gene-specific primer and a primer specific for the left border of the pROK2 produces a band of 400 bp. Two gene-specific primers are designed to produce a band of 800 bp. A, T₁ generation of SALK_049118, C represents Col-0, 1-6, numbered T₁ progeny of a homozygous mutant identified in the same manner. 1, 2 and 5 are homozygous for the T-DNA insert, 4 and 6 being homozygous wild-type. B, T₁ generation of SALK_138261. 1, 2, 4 and 6 are homozygous for the pROK2 insert. 3 is homozygous wild type and 5 is heterozygous for the insert. C, T₁ progeny of SALK_090550 all revealed to be homozygous for the insert.

Cathepsin B insertion lines SALK_049118 and SALK_138261 and the *CYPI* (*RD21*) insertion line SALK_090550 did not display any morphological phenotypes. However, insertion line SALK_019630, which corresponds to the third cathepsin B locus, At4g01610 did display a growth phenotype (Figure 4.9.1b). Under long day conditions, used to grow all *Arabidopsis* plants, SALK_019630 mutants appeared to have the growth characteristics of plants responding to low red to far-red ratios typically experienced by plants situated in the shade (Fankhauser and Casal, 2004). The leaves were smaller and a

paler shade of green with longer petioles. Wild type *Arabidopsis* leaves generally lie parallel with the soil whereas the leaves of SALK_019630 pointed upwards, erect at almost 45 ° angles with the soil. This phenotype has been previously described in phytochrome B (*phyB*) mutants and referred to as a constitutive shade-avoidance phenotype (Reed *et al.*, 1993; Whitelam and Devlin, 1997).



Figure 4.9.1b: Morphology of cathepsin insertion lines. SALK insertion lines displayed no obvious morphological phenotypes when compared to Col-0, except SALK_019630. This line displayed longer petioles, smaller, paler leaves which pointed upwards towards the light.

4.9.2. Cathepsin mutants response to virulent *Pst* DC3000

Wild type, *nahG* transgenic (with perturbed SA-dependent signalling pathway) and the cathepsin insertion line *Arabidopsis* plants were inoculated with 10^6 CFU/ml of *Pst* DC3000 to investigate whether any mutants were perturbed in basal defence responses. Histochemical analysis experiments were performed with at least 6 leaves from individual plants, representative of each plant genotype and from two biological replicates. Staining of hydrogen peroxide in virulent *Pst* DC3000 challenged plants revealed that SALK_019630 (light-sensing phenotype) accumulated the same minimal amount of H_2O_2 as Col-0 by 4 hpi (Figure 4.9.2a). However, staining leaves from the other cathepsin B insertion lines and the *CYP1* insertion line showed that these mutants did not accumulate the small amount of H_2O_2 as detected in Col-0 (Figure 4.9.2a). Dead cell staining at 12 hpi revealed that *Pst* DC3000 did not induce cell death in any of the *Arabidopsis* plants inoculated.

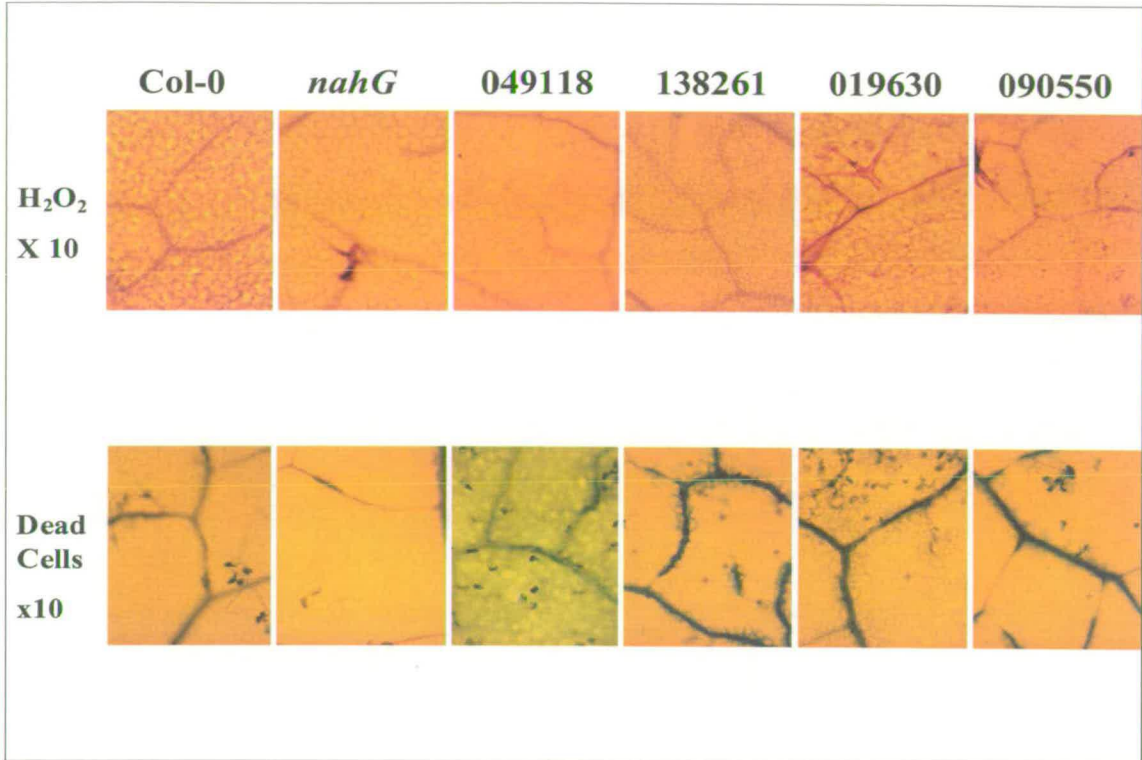


Figure 4.9.2a: Hydrogen peroxide and dead cell staining post-inoculation with *Pst* DC3000. DAB staining for hydrogen peroxide was performed 4 hpi with 10^6 CFU/ml virulent *Pst*. Trypan blue staining for dead cells was performed at 12 hpi with *Pst* DC3000.

To quantify the numbers of virulent bacteria that could grow within these mutant lines, leaves were ground in buffer, diluted and spread on KB plates supplemented with rifampicin. Due to time constraints experiment could only be repeated twice with similar results. Preliminary colony counts revealed that cathepsin B insertion lines SALK_138261, SALK_019630 and SALK_49118 allowed more than 10-fold greater increase in virulent *Pst* DC3000 growth, comparable with *nahG* transgenic plant. *CYP1* knockout line 090550 on the other hand displayed a similar level of growth as Col-0.

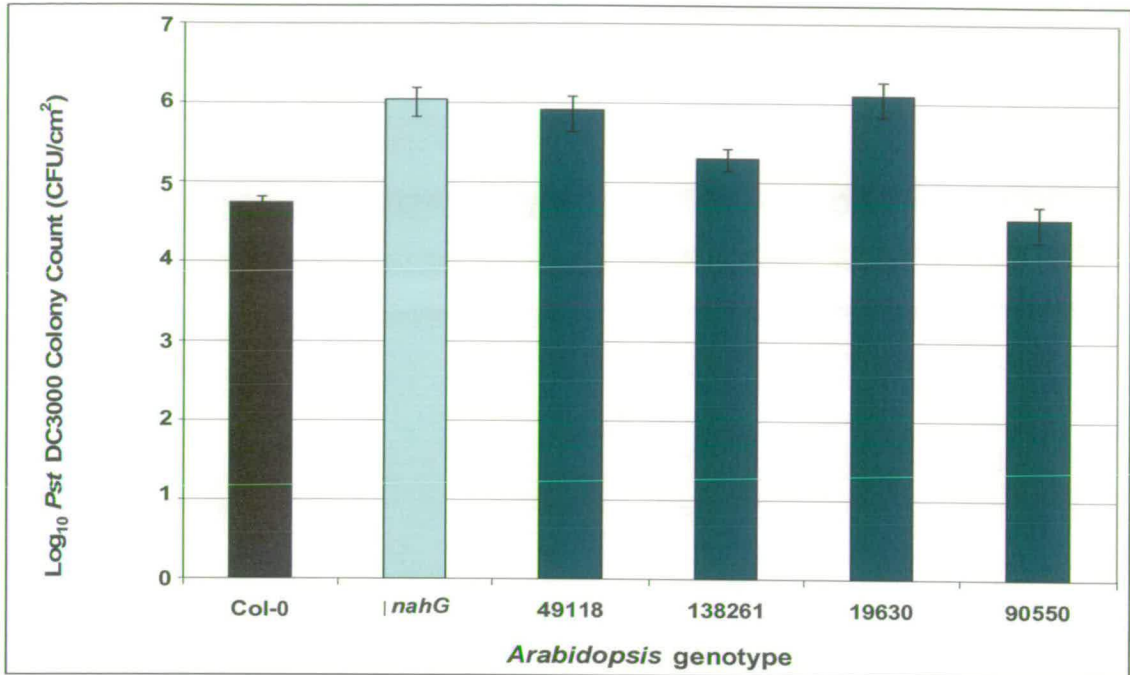


Figure 4.9.2b: *Pst* DC3000 colony counts in SALK T-DNA insertion lines 4 dpi. Graph shows mean values of colony forming units recovered from leaves expressed as a percentage of Col-0. Error bars represent SE of 4 individual plants from one biological replicate.

4.9.3. Cathepsin mutants response to *Pst* DC3000 (*avrB*)

Pst DC3000 (*avrB*) should be recognised by RPM1 triggering an HR in *Arabidopsis*. Wild type, the *nahG* transgenic and cathepsin insertion line *Arabidopsis* plants were inoculated with 10^6 CFU/ml of *Pst* DC3000 (*avrB*) to investigate whether any mutants were perturbed in RPM1-triggered defence responses. Histochemical analysis experiments were performed with at least 6 leaves from individual plants, representative of each plant genotype and from two biological replicates. Staining of hydrogen peroxide in *Pst* (*avrB*) challenged plants revealed that SALK_019630 (light-sensing phenotype) accumulated more H₂O₂ than Col-0 by 4 hpi (Figure 4.9.3a). Staining leaves from the other cathepsin B insertion lines and the *CYP1* insertion line showed that these mutants accumulate less H₂O₂ than Col-0 and similar amounts compared with *nahG* transgenic (Figure 4.9.2a). Dead cell staining at 12 hpi revealed that *Pst* DC3000 (*avrB*) did not induce extensive cell death in cathepsin B insertion mutants SALK_049118 and SALK_138261. In addition, the *CYP1* insertion line and the third cathepsin B homologue (SALK_019630) showed moderate amounts of cell death. The H₂O₂ leaf

staining revealed that cathepsin B SALK_049118 and SALK_138261 lines and *CYPI* SALK_090550 line show a phenotype similar to *nahG* suggesting that they are perturbed in CC-NBS-LRR triggered oxidative burst. The dead cell stain revealed that all mutants had fewer dead cells than Col-0 at 12 hpi. Leaves were also stained with trypan blue at 24 hpi with similar results (data not shown).

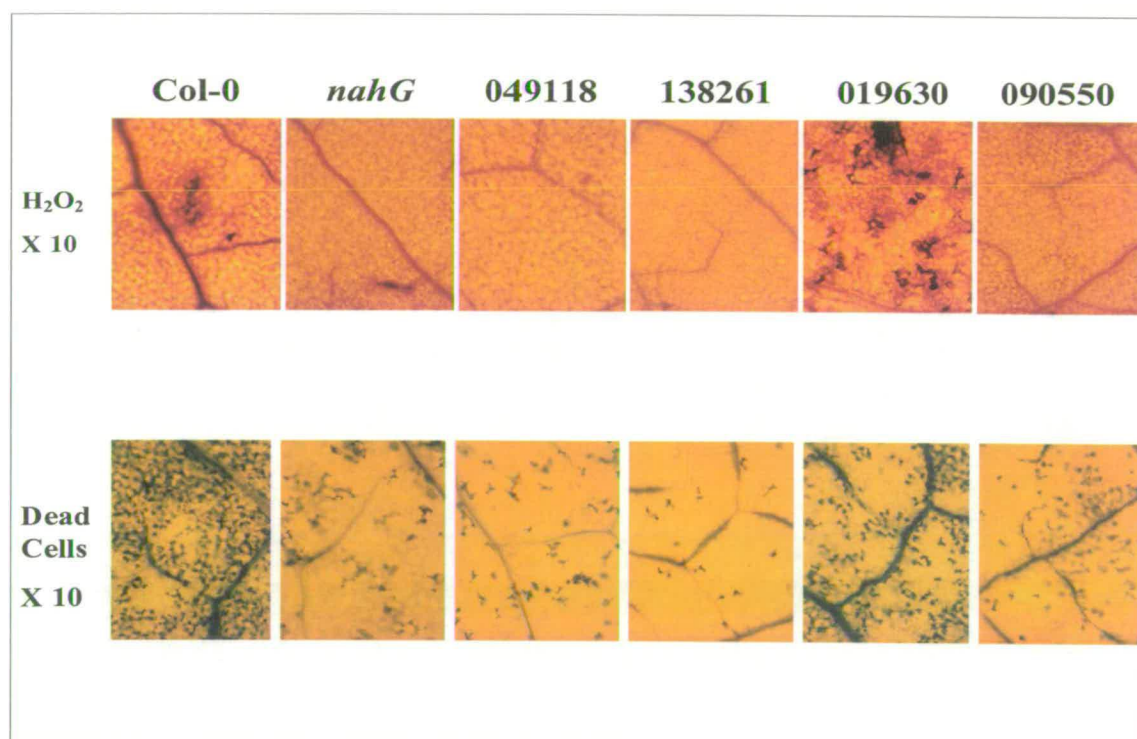


Figure 4.9.3a: Hydrogen peroxide and dead cell staining post-inoculation with *Pst* DC3000 (*avrB*). DAB staining for hydrogen peroxide was performed 4 hpi with 10^6 CFU/ml suspension. Trypan blue staining for dead cells was performed at 12 hpi with *Pst* DC3000 (*avrB*).

To quantify the numbers of avirulent bacteria that could grow after CC-NBS-LRR triggered HR in these mutant lines, leaves were ground in buffer, diluted and spread on KB plates supplemented with rifampicin and kanamycin (Figure 4.9.3b). Due to time constraints, colony counts were performed twice but with similar results. Preliminary colony count data revealed a trend that cathepsin B insertion lines, SALK_138261 and SALK_019630, and *CYPI* insertion line, SALK_090550 may have allowed 5-fold more *Pst* to proliferate by 9 dpi. However, taking into consideration the standard error, the difference between these insertion lines and Col-0 is not statistically robust. The *nahG* plants, that cannot accumulate SA-signalling and therefore cannot induce an effective

HR, showed 15-fold more avirulent *Pst* growth compared with Col-0. Interestingly, cathepsin B insertion line 049118 was found to allow more than 20-fold greater bacterial growth in comparison with Col-0. This suggests that cathepsin B homologue, At1g02300, but not necessarily the other cathepsin B homologues are required for induction of the HR through RPM1.

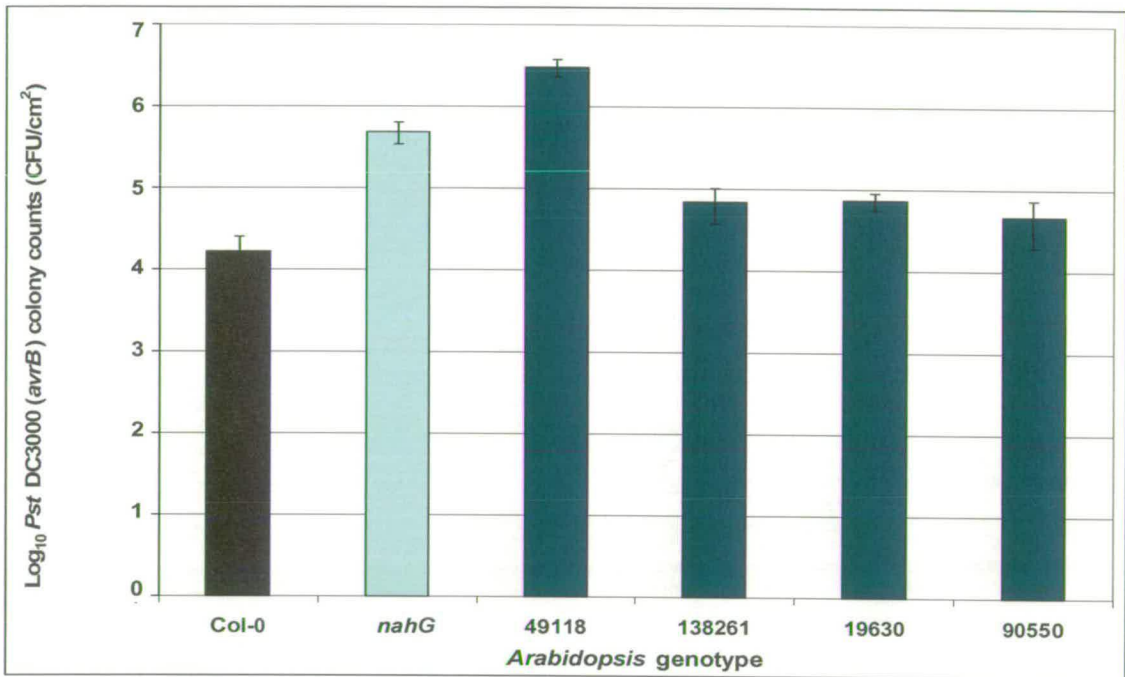


Figure 4.9.3b: *Pst* DC3000 (*avrB*) colony counts in SALK T-DNA insertion lines 9 dpi. Graph shows mean values of colony forming units recovered from leaves from 4 individual plants. Error bars represent SE of one biological replicate.

4.10. Discussion

Some cathepsins and other proteases have been shown to play a role in both caspase-dependent and caspase-independent PCD in mammalian systems (Abraham and Shaham, 2004). Although caspase-like activities have been identified in plants, initiator caspase genes or proteins responsible for those activities have not been identified thus far (Chichkova *et al.*, 2004; Belenghi *et al.*, 2004; Hatsugai *et al.*, 2004; Sanmartín *et al.*, 2005). The *Arabidopsis* genome encodes 488 annotated protease genes, many of which will most probably be associated with PCD and defence (van der Hooft and Jones, 2004). The discovery of two cysteine proteases up-regulated during the potato-*P.*

infestans interaction gave an indication that these genes may be involved in regulating the HR. A major aim of this project was therefore to elucidate whether either of these cathepsin-like genes could be a key regulator of the HR.

Northern blot analysis was used to provide an indication of the expression pattern of candidate genes in potato and also in *N. benthamiana* (data not shown). Two cathepsin genes of interest were up-regulated during the early stages of an incompatible potato-*P. infestans* interaction. The *CYPI* gene was also up-regulated rapidly following an INF1 elicitor treatment and *E. amylovora* infection in *N. benthamiana* which also made it a positive candidate for future gene silencing experiments (data not shown). However, the decision not to use this gene for imminent gene silencing studies during this project was based on evidence for its probable mode of activation. Previous research reveals that the *Arabidopsis* homolog, RD21 accumulates as an intermediate form (iRD21), that lacks the N-terminal propeptide (NTPP) form but still possesses the C-terminal granulin domain, in the endoplasmic reticulum (ER) bodies and vacuoles (Hayashi *et al.*, 2001; Yamada *et al.*, 2001). The mature and active RD21 protein (mRD21) was shown to be activated during senescence and lacked the C-terminal granulin domain. Further experiments by Yamada *et al.*, (2001) also showed that RD21 was not self-catalytically converted from proRD21 to iRD21 or from iRD21 to mRD21 and that α VPE and γ VPE, responsible for maturing various vacuolar proteins within vacuoles (Yamada *et al.*, 1999), were not sufficient for its processing. This data suggests that *StCYPI* is most likely to be an effector protease that is matured as a down stream target of other regulatory proteases. On the other hand, cathepsin B is one of the best studied mammalian cysteine proteases (Muntener *et al.*, 2004). Cathepsin B knockout mice have been shown to be resistant to apoptosis (Guicciardi *et al.*, 2001; Zeiss, 2003). Cathepsin B has also been demonstrated to act both independently or directly upstream of caspases (Vancompernelle *et al.*, 1998; Foghsaard *et al.*, 2001; Kingham *et al.*, 2001). This data supports a possible role for cathepsin B as a key regulator protease in the plant HR and therefore cathepsin B was selected for further in depth functional analysis over *CYPI* due to insufficient time to study both genes in such detail.

N. benthamiana is a close relative of potato and a commonly utilised model system for studying the HR in which silencing of disease resistance genes has been well documented (Peart *et al.*, 2002). TRV silencing vectors with *SGT1* and *CathB* gene fragments cloned in anti-sense were designed, constructed and inoculated as RNA transcripts into *N. benthamiana* plants. Four weeks after viral infection, silenced plants inoculated with 10^6 CFU/ml suspensions of the non-host apple bacterium *E. amylovora* (*Eam*) and *P. syringae* (*Pst*) DC3000 displayed a delay or even complete suppression of the HR symptoms compared with TRV::GFP plants. TRV::SGT1_{as} silenced plants have previously been reported to have a delayed HR triggered by NBS-LRR *R* genes (Peart *et al.*, 2002). It was therefore encouraging to observe a comparable or even more effective inhibition of the HR in TRV::CathB_{as} plants (Figure 4.5.1). In order to quantify the effect the delayed HR had on growth of non-host bacteria, leaf discs were cut from inoculated regions of TRV infected *N. benthamiana*, ground in buffer, diluted and spread on plates. Viable *Pst* recovered from both TRV::SGT1_{as} and TRV::CathB_{as} plants was at least ten-fold greater than TRV::GFP control. Viable *Eam* recovered from the silenced plants was around ten-fold or slightly less than GFP controls. Therefore, silencing of *CathB* suppresses the HR allowing ordinarily incompatible bacterium to proliferate more successfully (Figure 4.5.2a, b and c).

To confirm the extent of silencing, quantitative real-time RT-PCR was attempted through a time course. The difficulty in performing quantification in such a sensitive machine is that induction of normally utilised endogenous standards such as GAPDH and ubiquitin genes is detected. The normalised value therefore appears less or more than expected as calculations assume that the standard value is indicative of the total RNA in samples. Quantification of *CathB* transcript levels in Figure 4.5.3 revealed that expression in TRV::CathB_{as} was knocked down to 20 % of TRV::GFP in equivalent time points up to 12 hpi with *Eam*. TRV::SGT1_{as} *N. benthamiana* plants in these experiments displayed the same shorter, more branched structure in addition to the delayed HR phenotype as previously described in Peart *et al.* (2002). Therefore, silencing of *SGT1* in these plants was believed with some confidence to be occurring. Nevertheless, silencing of *SGT1*

was confirmed at the protein level by western blot analysis of TRV::GFP and TRV::SGT1_{as} leaf samples (Figure 4.5.4).

To investigate whether silencing affected cathepsin B protein activity following non-host pathogen inoculation, cathepsin B activity was examined in TRV::GFP and TRV::CathB_{as} plants. Figure 4.5.5 revealed that silencing of *CathB* transcripts prevented the peak of cathepsin B activity observed at 6 hpi in TRV::GFP plants. Therefore induction of both *CathB* transcript levels and cathepsin B activity were successfully repressed in TRV::CathB_{as} plants which subsequently delayed the HR allowing significantly increased non-host bacterial growth.

To further investigate cathepsin B activity and to support the silencing data, commercially available mammalian cathepsin B inhibitors were ordered (Table 4.6). As human cathepsin B and plant cathepsin B share 47 % homology at the amino acid level, it was possible that these inhibitors may not inhibit plant cathepsin B activity. However, at 150 μ M, cathepsin B inhibitors were shown to suppress the HR induced by bacterial suspensions of 10^5 CFU/ml and Ac-LVK-CHO was also shown to suppress the HR induced by 10^6 CFU/ml (Figure 4.6.1a). Once the optimal inhibitor concentration of 1 mM was utilised with 10^6 CFU/ml a reproducible suppression of the HR was observed with all inhibitors tested (Figure 4.6.1b). As with the *CathB* silenced plants, colony counts were used to quantify the delayed HR phenotype. Figure 4.6.2A revealed that 1 mM of cathepsin B specific inhibitors Ac-LVK-CHO and Ca-074 Me allowed 10-fold and z-FA.fmk allowed 5-fold more non-host bacterial growth. To evaluate whether the inhibitors may be toxic to the bacteria, overnight cultures were grown in LB with 1 mM inhibitor which did appear to have an effect on the growth of *Eam* although rate of growth was not examined (Figure 4.6.2B). Since more than one mammalian cathepsin B inhibitor appears to suppress cathepsin B activity in *N. benthamiana* it can be suggested that plant cathepsin B possesses similar active sites and therefore activity as mammalian cathepsin B.

Cathepsin B inhibitors were co-infiltrated into the extracellular spaces of the *N. benthamiana* leaves and whether or not they can diffuse into the cell has not been tested.

However, cathepsin B activity of protein extracts in leaf discs cut from the inoculated regions was measured to estimate their inhibitory effect *in vivo*. Figure 4.6.3 reveals that the peak in activity observed at 6 hpi in *Eam* (10^6 CFU/ml) treated leaves was suppressed to the level of uninoculated and buffer inoculated samples by cathepsin B specific inhibitors Ac-LVK-CHO and z-FA.fmk. The suppression of cathepsin B activity agrees with the data presenting the observed delayed HR phenotype and increase in pathogen growth. In addition, cathepsin B inhibitors have suppressed the HR and cathepsin B activity to an almost identical extent as *CathB* silencing, supporting the role of cathepsin B as a regulator of the HR.

The cleavage of PARP is a commonly used marker of apoptotic cell death in mammalian apoptosis and interestingly lysosomal cathepsin B has been shown to cleave PARP, to produce a major fragment of 50 kDa (Boucher *et al.*, 2001; Gobeil *et al.*, 2001). Cleavage of the PARP DNA-binding domain to produce a C-terminal fragment of 45 kDa has been previously described during the HR (Figure 4.7A) (D'Silva *et al.*, 1998). Although the PARP cleaving activity of endogenous plant cathepsin B cannot be thoroughly tested without purified protein, the effect of cathepsin B-specific inhibitors on bovine cathepsin B activity and in the crude protein extract from leaf samples from TRV::GFP 6 hpi with *Eam* was investigated. Interestingly, the major cleavage product of PARP with bovine cathepsin B was a C-terminal fragment of 45 kDa in size, Figure 4.7B, revealed that that Ac-LVK-CHO could almost completely inhibit the cleavage of PARP after 30 min incubation with bovine cathepsin B. In addition, 1 mM z-FA.fmk could prevent the complete cleavage of PARP by bovine cathepsin B. It is therefore possible that plant cathepsin B cleaves PARP to produce this major cleavage product of 45 kDa *in vitro*. However, Figure 4.7C shows that an increase of incubation time (1 hr) of bovine cathepsin B, 1 mM cathepsin B-specific inhibitors and PARP decreases the observed extent of PARP cleavage inhibition by the inhibitors. In addition, crude protein extract from TRV::GFP 6 hpi was shown to cross react with the bovine PARP antibody and although a 45 kDa band was present, the major cleavage product in this sample was closer to 50 kDa in size. This larger band was also identified in the lane which included both PARP and TRV::GFP 6 hpi protein extract. This 50 kDa product corresponds with

the *in vivo* and *in vitro* experiments performed using lysosomal extracts and purified lysosomal cathepsin B (Gobeil *et al.*, 2001). Therefore, it remains unclear whether plant cathepsin B could be responsible for cleaving PARP to produce both a 45 kDa found by D'Silva *et al.* 2001 and the 50 kDa fragment produced by lysosomal cathepsin B (Gobeil *et al.*, 2001). Then again, from evidence described in current literature, the role of cathepsin B should be more akin to initiator caspases that activate downstream proteases rather than effector caspases which proteolytically dismantle the cell (Kingham *et al.*, 2001; Vancompernelle *et al.*, 1998). Therefore, the appearance of a larger PARP cleavage product in TRV::GFP 6 hpi protein samples maybe indicative of other protease activity present at this time point. Repetition of this experiment, including incubation of PARP with purified plant cathepsin B would help answer this question.

Caspase-3-like activity has been previously measured by Michael Taliansky who works at SCRI. He kindly provided his TMV vector containing GFP fused to VirD2 that has two caspase-3 cleavage sites (GEQD and TATD) (Chickova *et al.*, 2004). This allowed preliminary examination of whether cathepsin B inhibition could suppress the activation of the caspase-3 activity linked to the cleavage of the VirD2 nuclear localisation sequence. Therefore, TMV GFP::VirD2 lesions were inoculated with *Eam* (10^6 CFU/ml), *Eam* with 1 mM TATD inhibitor (caspase-3 inhibitor) and *Eam* with 1 mM Ac-LVK-CHO (cathepsin B inhibitor). Figure 4.8 shows preliminary data which suggests that plant cathepsin B activity maybe necessary for activating the previously described caspase-3-like activity identified by Chickova *et al.* (2004). However, it cannot be completely excluded that Ac-LVK-CHO may inhibit the activity of the caspase-3-like protein. The effect of cathepsin B-specific inhibitor Ac-LVK-CHO on the measured caspase-3-like activity could be performed once both proteins have been purified. The protein responsible for caspase-3 activity is presently being investigated by collaborators at Moscow State University.

Arabidopsis gene homologues of *CYP1* and cathepsin B were identified and availability of T-DNA insertion mutants in these genes at the SIGnAL website were checked. Fortunately, at the time of ordering there were T-DNA knockouts available in an exon or

UTR of all three cathepsin B genes. However, the annotation of At1g02300 and At1g02305 was revised a year later and consequently SALK_138261 insertion appears to lie at the end of At1g02300's ORF. This suggests that this insertion line may not have affected the At1g02305 gene. Subsequent to this discovery, other T-DNA insertions had become available and ordered although the genotyping of these seeds could not be performed within the time frame of this project. Expression of all three cathepsin B genes in all three mutants would require careful primer design as all three genes are 80-90 % identical at the nucleotide level.

Inoculation of the *Arabidopsis* insertion lines with virulent *Pst* DC3000 was performed to investigate whether the T-DNA inserts in these genes had perturbed basal defence responses. The transgenic *nahG* was used as a control, as the inhibition of SA accumulation in this plant has been well documented to affect basal defence responses, HR and SAR (Lawton *et al.*, 1995). Mutants with T-DNA inserts displayed no unusual H₂O₂ accumulation and production of dead cell in response to *Pst* DC3000 (Figure 4.9.2a). Recovery of *Pst* DC3000 4 dpi revealed that mutants with inserts in gene loci At1g02300 and At4g01610 allowed 10-fold greater growth than Col-0 and to a comparable level as *nahG* (Figure 4.9.2b). T-DNA insertions in these cathepsin B genes appear to have disrupted basal resistance implying that cathepsin B is involved in basal resistance. The data presented in Figure 4.9.2b suggests that the cathepsin B gene present at the At1g02305 locus does not play a role in basal resistance. Since re-annotation of this region has positioned the T-DNA insert out with the ORF and UTRs of At1g02305 it is possible that this line possesses a functional cathepsin B gene at the At1g02305 locus. The same experiment will have to be repeated with another *Arabidopsis* SALK line that possesses a T-DNA insert in the ORF of this locus to conclude whether or not At1g02305 has a role in basal resistance.

Cathepsin insertion lines were inoculated with avirulent *Pst* DC3000 (*avrB*) to investigate whether these lines could induce the HR characterised in Col-0 by a biphasic H₂O₂ burst between 1-6 hpi followed by PCD by 12 hpi (Shirasu and Schulze-Lefert, 2000; Grant and Loake, 2000). The transgenic *nahG*, which cannot accumulate SA,

cannot induce an HR to avirulent pathogens (Lawton *et al.*, 1995). Staining Col-0, *nahG* and cathepsin insert lines revealed that all genes except At4g01610 may be required for the early H₂O₂ burst (Figure 4.9.3a). Trypan blue staining revealed that those mutants which could not accumulate H₂O₂ could subsequently not induce PCD by 12 hpi (Figure 4.9.3a) or by 24 hpi (data not shown). Recovery of *Pst* DC3000 (*avrB*) from the same plants at 9 dpi revealed that the *Arabidopsis* line with an insert in the ORF of At1g02300 allowed 20-fold greater *Pst* growth than Col-0. This suggests that one of three cathepsin B homologues in *Arabidopsis* is required for RPM1 triggered resistance to *Pst* DC3000 (*avrB*). Examination of the function of the cathepsin B at At1g02300 during resistance responses triggered by different *R* genes to other types of pathogens would reveal whether it is essential for HR or only for HR induced by *avrB*.

Evidence that the three cathepsin B genes have varying roles in *Arabidopsis* is also supported by the phenotype clearly linked with At4g01610 insertion line (SALK_019630). This cathepsin B knockout line appeared to have the growth characteristics of plants responding to low red to far-red ratios typically experienced in the shade (Frankhauser and Casal, 2004). This phenotype has been previously described in phytochrome B (*phyB*) mutants and referred to as a constitutive shade-avoidance phenotype (Reed *et al.*, 1993; Devlin *et al.*, 1997). Since active *phyB* (Pfr form) is required to limit growth of stems and petioles, in the absence of *phyB*, this growth is not suppressed by light (Frankhauser and Casal, 2004). It is therefore possible that the cathepsin B protein encoded by At4g01610 may be involved in proteolytic cleavage of phytochromes during light perception and signalling.

The role of the cathepsin B gene at locus At1g02305 cannot be determined until this knockout line has been shown to have no At1g02305 expression or experiments are repeated with an alternative homozygous T-DNA insertion line in the ORF. Nevertheless, the presence of T-DNA within At1g02300 affects the ability of this *Arabidopsis* plant to induce the HR which mirrors the silencing and inhibitor experiments performed in *N. benthamiana*. It will be interesting to observe how many cathepsin B genes will be revealed by the tomato sequencing project, which should

provide an indication of the number of cathepsin B genes present in *N. benthamiana* and *S. tuberosum* genomes.

4.11. Conclusions

The potato homologues of *CathB*, *CYP1*, *SGT1* and *ADR1* genes were shown to be up-regulated early in the *P. infestans* incompatible interaction. *CathB* was selected for further investigation in the model plant, *N. benthamiana*, because there have been many reports of cathepsin B playing a role in mammalian PCD (Vancompernelle *et al.*, 1998; Mathiasen and Jäätelä, 2002; Foghsgaard *et al.*, 2001; Kingham and Pocock, 2001). *NbCathB* expression was shown to be approximately 80 % reduced in TRV::CathB_{as} plants, preventing a peak of protein activity at 6 hpi in comparison with TRV::GFP plants. *NbCathB* silencing appears to have delayed the HR, consequently allowing 10-fold greater bacterial recovery of *Eam* and *Pst* DC3000 (*avrB*).

One general cathepsin inhibitor and three cathepsin B-specific inhibitors were used to confirm the gene silencing experiments. Two cathepsin B protein inhibitors prevented the peak of cathepsin B activity at 6 hpi, also suppressing the HR and allowing 10-fold greater bacteria to be recovered from inoculated area. This suggests that *N. benthamiana* cathepsin B has similar active sites as mammalian cathepsin B and that blocking this activity delays the HR. Cathepsin B may also play a role in PCD by cleaving PARP which is a commonly used marker of apoptosis. Preliminary data investigating cathepsin inhibitors on the cleavage of VirD2 during PCD suggests that cathepsin B may be upstream of or required for caspase-3-like activity.

The *Arabidopsis* genome contains three homologues of Cathepsin B. T-DNA insertion mutants were ordered from the NASC and genotyped until all progeny were homozygous for the insert. Early results indicate that only one of the three cathepsin B genes (At1g02300) is required for both basal and *Pst* DC3000 (*avrB*) induced HR.

5. Development of a Virus-Induced Gene Silencing (VIGS) System for *Solanum tuberosum*

5.1. Introduction

Many genes involved in crop disease resistance are identified through research into diseases caused by economically significant pathogens. Therefore it is important that effective reverse genetic techniques are developed for crop species (Osterlund and Paterson, 2002). Candidate HR-related genes were previously identified from SSH cDNA libraries in *S. tuberosum* cultivars resistant to *P. infestans*. It would therefore be most appropriate to study the function of these genes by VIGS in potato. However, no VIGS system had been developed for *Solanum* species and therefore part of this research was to develop a VIGS system for *S. tuberosum* cultivars resistant and susceptible to potato blight.

5.1.1. Discovery of Gene-Silencing Technology

Gene silencing was initially discovered serendipitously by three separate groups using transgenic over-expression constructs containing either chalcone synthase (*Chs*) or polygalacturonase (*Pg*) in plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; Smith *et al.*, 1990). This phenomenon is also known as co-suppression or RNA interference (RNAi) (Lawrence and Pikaard, 2003). In *Arabidopsis*, stable transformation has become especially valuable for expressing RNA forms that trigger post-transcription gene-silencing (PTGS) for functional analysis of genes of interest (Cogoni and Macino, 2000). In addition, single-stranded anti-sense RNA and hairpin RNA forms of a candidate RNA transcript have also been shown to be sufficient for knocking down the expression of a gene of interest through PTGS (Lacomme *et al.*, 2003). Gene silencing was also found to be triggered by positive stranded RNA viruses. The high copy numbers of the positive strand form of the genome and/or by the dsRNA intermediates that putatively form while replicating their genome could possibly be the triggers of a PTGS-like defence mechanism that protects the plants against viruses and virus-like retrotransposons

(Lindbo *et al.*, 1993). By inserting an anti-sense copy of an endogenous plant gene into the genome of a positive strand RNA virus vector e.g. Tobacco rattle virus (TRV), the antiviral defence mechanism can be established against the endogenous copy of the gene. VIGS for phenotypic analysis was first used to manipulate the carotenoid biosynthetic pathway in *N. benthamiana* (Kumagai *et al.*, 1995). The hybrid tobacco mosaic virus (TMV) vector contained elements from tomato mosaic virus (ToMV) and the plant phytoene desaturase (*PDS*) cDNA. Increased levels of the enzyme substrate, phytoene and systemic susceptibility to leaf photobleaching resulted as a consequence of the gene silencing. It was later shown using Tobacco Etch Virus (TEV) that the viral RNA, not the viral proteins, trigger gene silencing in host plants (Lindbo *et al.*, 1993). Subsequently, VIGS triggered by cytoplasmic, autonomously replicating viruses have been successfully utilised to modify a range of metabolic pathways (Kumagai *et al.*, 1995), including defence responses (Romeis *et al.*, 2001; Ekengren *et al.*, 2003; Yoshioka *et al.*, 2003).

5.1.2. Mechanism of Gene Silencing

At present, the molecules responsible for sensing aberrant levels of positive, anti-sense or dsRNA transcripts leading to sequence-specific degradation of homologous transcripts in plants remain enigmatic, although may involve some widely conserved components (Voinnet, 2001). In *Drosophila*, aberrant RNA transcripts are cleaved by RNase III-like enzyme named Dicer into 21 bp RNA duplexes known as “short interfering” RNAs (siRNA). The siRNA duplexes are then incorporated into a multi-subunit endonuclease, the RNA-induced silencing complex (RISC), that cleaves all RNA sharing identity with the dsRNA (Voinnet, 2003). A plant homolog of the *Drosophila* Dicer, carpel factory dicer-like 1 (*AtCAF1*), has recently been demonstrated to bind dsRNA and generate siRNAs (Kurihara and Watanabe, 2004). The mammalian RNA-induced silencing complex (RISC) is composed of siRNAs and the Argonaute family of proteins. Argonaute proteins are thought to unwind the siRNA to provide a template for interactions with mRNA which leads to degradation of homologous transcripts (Susi *et al.*, 2004). An *Arabidopsis* Argonaute 1 (AGO1) has been identified and may act within

the plant RNA-induced silencing complex (RISC) (Vaucheret *et al.*, 2004). Gene silencing-associated low molecular weight RNAs were also first detected in plants by Smith *et al.* (1990).

5.1.3. Properties of VIGS Vectors

In higher plants, over 90 % of plant viruses have ssRNA genomes that are replicated with a RNA-dependent RNA polymerase (RdRP) (Voinnet, 2001). The most efficient positive stranded viral vectors for VIGS are those that can accumulate to appropriately high levels within the plant. However, a replication threshold exists, above which silencing defences are induced to such an extent that recombinant viral infection is almost completely extinguished, allowing newly emerging tissue to become virus-free (Ratcliff *et al.*, 1997). In addition, some viral genomes encode silencing suppressors, for example the HC-Pro proteins of TEV and potato virus Y (PVY) have been shown to prevent initiation and even revert transgene silencing of β -D-glucuronidase (GUS) and GFP (Marathe *et al.*, 2000; Voinnet *et al.*, 1999; Beclin *et al.*, 1998). Moreover, the symptoms of some viral infections such as chlorosis, leaf distortion and necrosis may complicate the interpretation of silencing phenotypes (Ratcliff *et al.*, 2001). One of the few disadvantages of VIGS is that virus infection and spread of the systemic silencing signal is unlikely to affect every cell's expression, leading to patchy silencing phenotypes (Ratcliff *et al.*, 2001). Nonetheless, VIGS has become progressively more popular for loss-of-function studies because it is more rapid than traditional stable transformation techniques for the same plant species (Baulcome, 1999b,c).

5.2. *Tobacco Rattle Virus (TRV) Vector*

5.2.1. TRV vector

VIGS has been successfully employed using a small selection of viruses, e.g. TRV, in a restricted number of hosts, primarily *Nicotiana* species (Ratcliff *et al.*, 2001). However, recent advances on other plant species such as tomato have indicated that TRV may have potential for developing gene silencing systems in other dicots (Dinesh-Kumar *et al.*, 2003; Liu *et al.*, 2002). TRV possesses a bipartite, positive sense single-stranded RNA genome

and can infect a wide number of hosts including potato where it is most commonly transmitted by nematodes (Visser *et al.*, 1999). The major advantages of TRV are that the virus can infect active meristems and large patches of adjacent cells (Ratcliff *et al.*, 2001). The TRV RNA 1 is known to encode genes essential for the infection and proliferation of the virus within a plant and can cause infection in the absence of RNA 2 (MacFarlane and Popovich, 2000). RNA 2 encodes the viral coat protein (CP) and *2b* which is required for nematode transmission but may also be required for increased virulence (Valentine *et al.*, 2004). RNA 2 has been successfully modified and utilized as a transgenic vector that can induce silencing in tobacco and *Arabidopsis* (MacFarlane and Popovich, 2000). RNA 2 vectors commonly have all non-essential genes removed to reduce the genetic load on recombinant viruses and to block possible nematode transmission of the virus (MacFarlane, 1996).

It was hypothesized here that the presence of TRV RNA 2-encoded *2b* gene, known to be essential for nematode transmission, may aid the induction of gene silencing in potato where other commonly used TRV vectors have failed thus far (Valentine *et al.*, 2004). The RNA 2 genome (AJ2504880) was engineered to contain just the non-essential viral gene *2b*, TRV CP, the tomato phytoene desaturase (*PDS*) gene (X59948) or green fluorescent protein (*GFP*) (MacFarlane and Popovich, 2000; Baulcombe *et al.*, 1995). TRV::GFP was included as a positive indicator of TRV genome replication and systemic spread. RNA 1 and the modified RNA 2 (Figure 5.2.1) of TRV were transcribed and inoculated into *N. benthamiana* to amplify the construct as described in MacFarlane (1996). Leaf sap from TRV infected plants was rubbed on to aluoxite dusted leaves of 2 week old *N. benthamiana*, *N. tabacum* cv Samsun, *N. tabacum* cv Xanthi and *Solanum tuberosum* L. cv Spey, Stirling and Wilja (See 2.5.1). Spey and Stirling are cultivars bred as SCRI with resistance to *E. carotovora* and *P. infestans*, respectively. Both would therefore be useful cultivars in which to utilise gene silencing for investigating plant disease resistance. Wilja is a cultivar which is noted for its ease of *Agrobacterium*-based transformation (Hugh Barker, personal communication) and may also be more amenable to virus infections and therefore potentially VIGS.

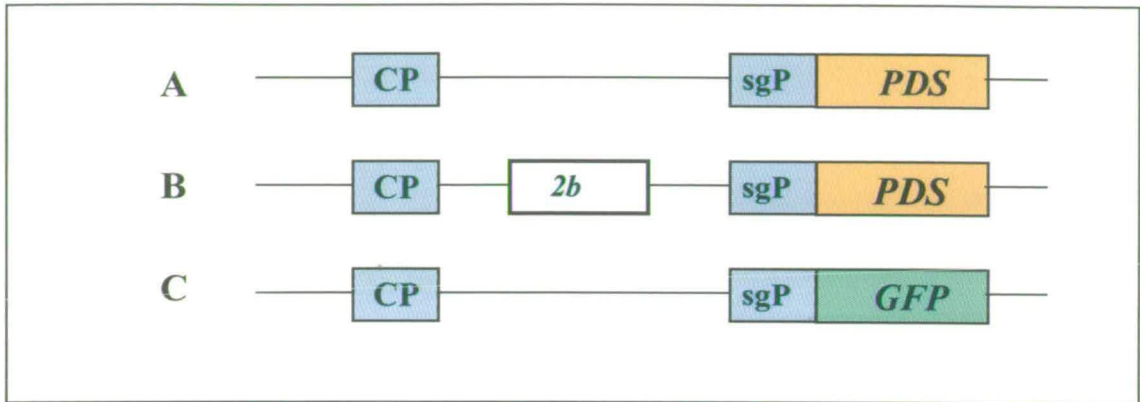


Figure 5.2.1: TRV RNA 2 Constructs. A, RNA 2 vector containing *PDS* cDNA, commonly utilized for gene silencing in *N. benthamiana*. B, RNA 2 vector with (phytoene desaturase) *PDS* cDNA with an additional TRV gene which is non-essential for VIGS, *2b*. C, A control construct containing *GFP* (green fluorescent protein) visible 10-21 dpi in *N. benthamiana*. *Cp*, coat protein; *2b*, non-essential RNA 2 encoded gene; *sgP*, sub-genomic coat protein promoter and *PDS*, phytoene desaturase.

5.2.2. TRV Silencing Symptoms

Plants were examined 5-10 dpi for the easily identifiable photobleaching phenotype or GFP fluorescence. All of the *Nicotiana* plants infected with RNA 2 constructs A and B displayed the photobleaching phenotype associated with *PDS* silencing by 10 dpi in systemic leaves (Photographs in Figure 5.2.2). Also, *Nicotiana* plants infected with the GFP encoding RNA 2 vector fluoresced in green patches on systemic leaves under UV light, revealing the infection pattern of the virus (Figure 5.2.2). Unfortunately, even after 21 dpi, no photobleaching or GFP fluorescence developed in potato plants (photographs not shown). VIGS in *S. tuberosum* may be challenging as a consequence of a tolerance to higher transcript levels due to the number of copies of each gene present in these tetraploid plants or possibly they possess a strong defence mechanism against TRV infection that rapidly inhibits viral proliferation and spread before silencing can occur.



Figure 5.2.2: *Nicotiana* plant symptoms 10 dpi with TRV RNA 2 constructs. Plants infected with three TRV constructs, RNA2 vector with phytoene desaturase (*PDS*), RNA2 vector that contains *PDS* and the *2b* gene and a control construct containing *GFP*. Photographs **A, B, C** show *N. benthamiana* infected with TRV vectors containing *PDS* without *2b*, *PDS* with *2b* and *GFP* respectively. **D, E** and **F** show *N. tabaccum* cv Xanthi infected with TRV vectors -*2b*, with *2b* and *GFP*. The third row (**G, H, I**) shows *N. tabaccum* cv Samsun infected with the same three vectors. *GFP* control on the left in **A, D, E, G** and **H**.

5.2.3. RT-PCR of TRV RNA 2 Coat Protein

To test if viral proliferation and spread was inhibited in potato, the presence of the recombinant RNA 2 in the potato plants was tested using RT-PCR with primers designed to the coat protein (2.8.5). Four young systemic leaves were taken from a variety of potato and *Nicotiana* plants, RNA extracted and cDNA was synthesised. The cDNA was used in a PCR designed to amplify the 1.2 kb RNA 2 CP transcript (Figure 5.2.3). Infected *Nicotiana* plants, as expected, contained TRV RNA 2 in their systemic leaves, confirming the presence of the virus which was not amplified in uninfected *Nicotiana* plants. None of the potato plants revealed a 1.2 kb amplification product suggesting that RNA 2 did not spread effectively to systemic tissue or that it had been rapidly detected and destroyed by a virus-induced defence response. This could explain why TRV did not induce VIGS of *PDS* in *S. tuberosum*.

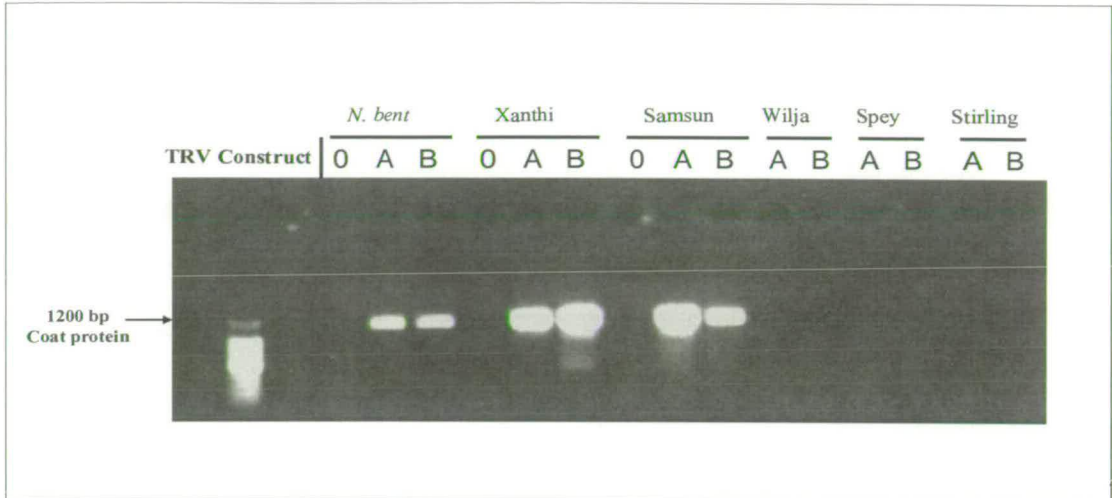


Figure 5.2.3: Reverse transcriptase PCR of TRV RNA2 Coat Protein. *N. benthamiana* negative control, TRV RNA2 vector (A) TRV RNA2 containing *2b* (B). TRV RNA2 constructs A and B also in *N. tabacum* cv Xanthi and *N. tabacum* cv Samsun. The RNA2 CP RNA from constructs A and B did not accumulate in the *S. tuberosum* varieties Wilja, Spey and Stirling.

5.3. *Potato Virus X (PVX) Vector*

5.3.1. PVX Constructs

Since TRV RNA 2 could not replicate or accumulate *in planta* efficiently enough to induce VIGS in potato, it was considered that the next virus tested should accumulate well in Solanaceous species. Potato Virus X (PVX) is a member of the potexvirus group and possesses a monopartite, single-stranded positive sense RNA genome (Chapman *et al.*, 1992). A PVX-based binary expression vector carrying large inserted sequences has been shown to accumulate effectively enough to over-express genes of interest in potato and *Nicotiana* species (Jones *et al.*, 1999; Chapman *et al.*, 1992). It was hypothesised that if PVX could accumulate effectively for efficient gene expression it may function as a silencing vector if potato sequences were cloned in anti-sense or as inverted repeats (Wang and Waterhouse, 2002; Lacomme *et al.*, 2003). In addition, PVX RNA has been shown to induce gene silencing in *Nicotiana* species (Angell and Baulcombe, 1997). Fortunately, a PVX vector (pGR106) had been previously supplied from D. Baulcombe for over-expressing genes from *P. infestans* in a variety of potato cultivars. PVX was

therefore deemed the most suitable virus to test the ability to induce VIGS in *S. tuberosum*. As described previously with TRV, a *GFP* gene was cloned into pGR106 to test the infection symptoms of a transgenic PVX vector in potato and to investigate its infectivity (Figure 5.3.1A). As VIGS depends on high homology between a transgene in the viral vector and the endogenous transcript, a potato *PDS* cDNA (AY484445) fragment was cloned into PVX in anti-sense orientation (section 2.5.2). The cDNA fragment shares 91 % sequence identity with *N. benthamiana* *PDS* cDNA (including stretches of 26, 33 and 47 nt of 100 % identity) (Figure 5.3.1B). This should allow testing of PVX::*PDS*_{as} as a silencing vector in *Solanum* species and *N. benthamiana* in parallel.

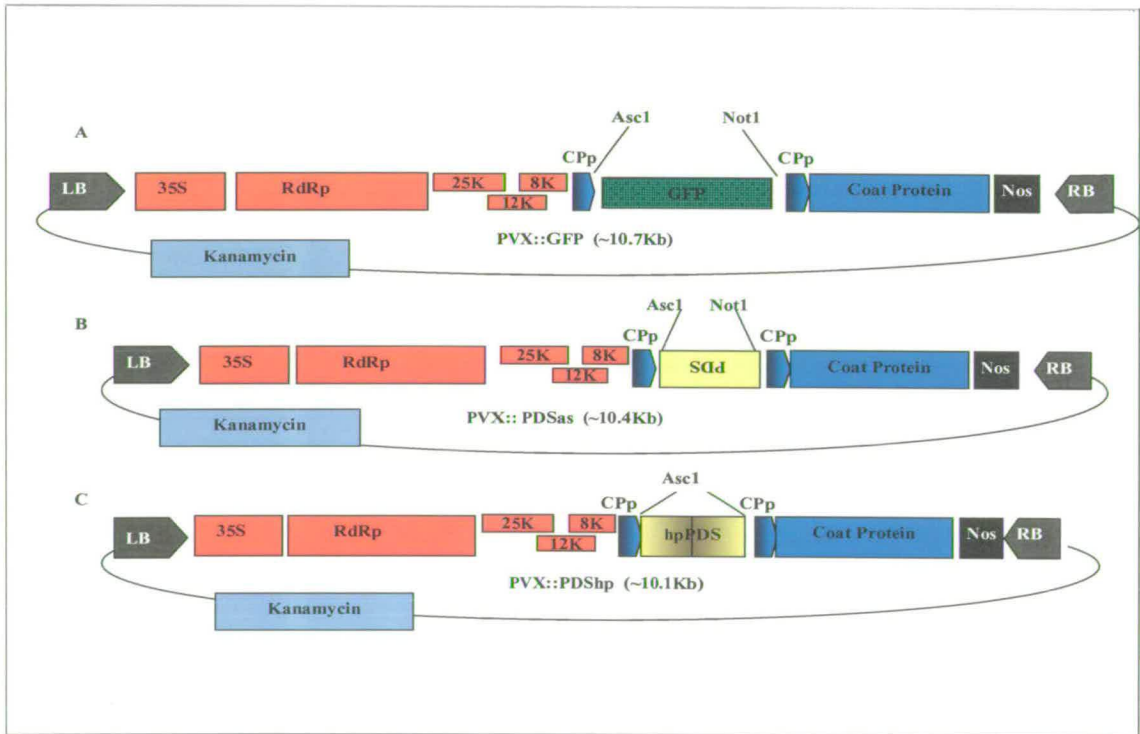


Figure 5.3.1a: PVX Vector Constructs. Construct maps of three pGR106 vector, **A**, with GFP; **B**, with *StPDS*_{as}; **C**, with hairpin *StNiPDS*. See Fairvre-Rampant *et al.*, (2004) for *PDS* region used for hp and as fragments. (For exact details of fragment location, see Appendix II).



Figure 5.3.1b: *PDS* cDNA alignments and regions of high homology. *PDS* alignment of *StPDS* (potato); *LePDS* (tomato); *NtPDS*, *N. tabacum*; *NbPDS*, *N. benthamiana*. Regions shaded in black are 100 % conserved, regions in grey are conserved in 3 of 4 *PDS* cDNAs. *PDS_{as}* fragment (1113-1529 bp on *PDS* AY48445 seq) *PDS_{hp}* fragment (1334-1380 bp on *PDS* AY48445).

5.3.2. PVX Constructs in *N. benthamiana*

Initial tests involved both stab inoculation and agroinfiltration of *N. benthamiana* to determine whether the PVX constructs could replicate efficiently and silence *PDS* successfully in this more receptive model (Takken *et al.*, 2000). Agroinfiltration of PVX appeared to be marginally more effective and therefore this method of PVX delivery was subsequently utilised. In addition, comparisons were drawn between *PDS* silencing induced by construct B (PVX::*PDS_{as}*) and construct C (PVX::*PDS_{hp}*) to evaluate whether construct C exhibited significantly stronger silencing, justifying the more complex and time-consuming cloning steps necessary to construct hairpin vectors. PVX::*PDS_{hp}* did not induce silencing to the extent of PVX::*PDS_{as}* after a series of inoculations (Figure 5.3.2). However, the hairpin was generated from the largest region of 100 % homology shared between *S. tuberosum* and *N. benthamiana PDS* (47 bp) and it is possible that a hairpin of 60 bp may have induced a stronger silencing phenotype as in Lacomme *et al.*

(2003). Larger hairpins (e.g. 200 bp) are thought to have such strong secondary structures that interfere with virus genome replication and commonly recombinant viruses do not replicate well or recombine out the insert containing the hairpin (Lacomme *et al.*, 2003).

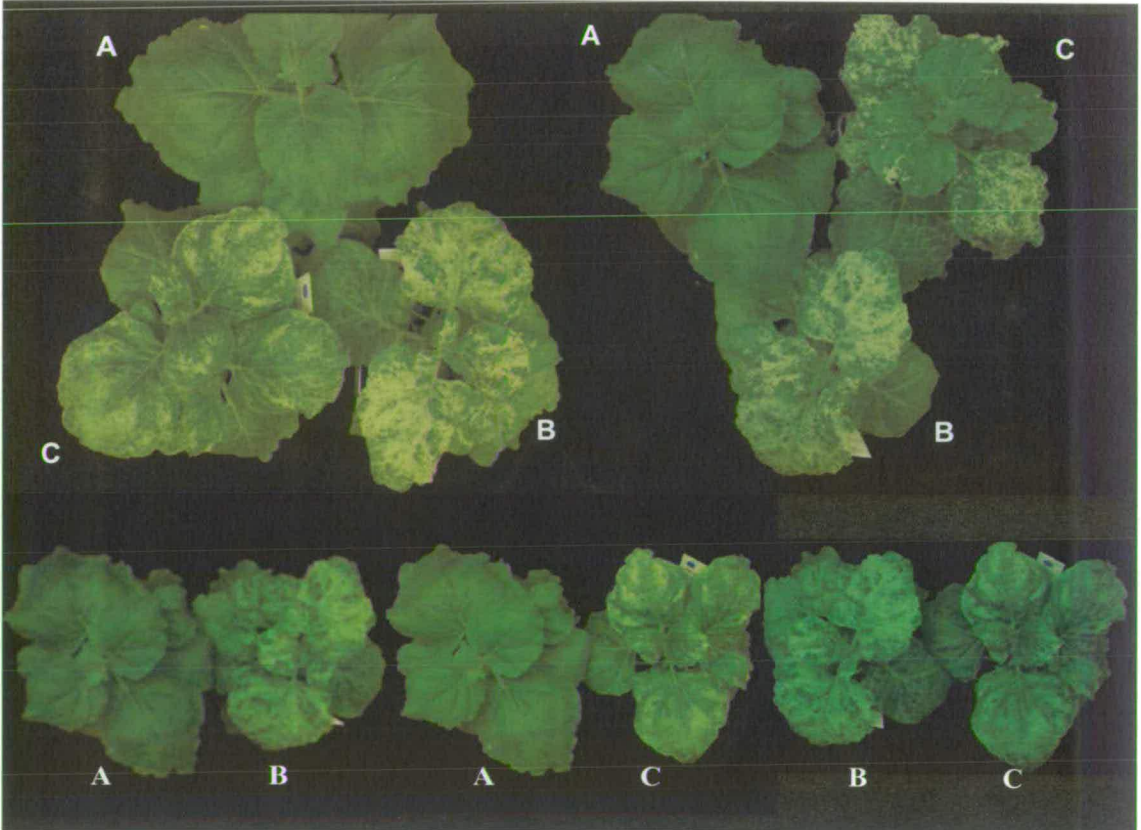


Figure 5.3.2: Comparison between PVX::PDS_{as} and PVX.PDS_{hp} in *N. benthamiana*. A, PVX::GFP, used as control of viral infection symptoms; B, PVX::PDS_{as}; C, PVX::PDS_{hp}. Photographs taken 10 dpi. *N. benthamiana* infected with construct B display larger areas of photobleaching than those infected with construct C.

5.3.3. PVX Constructs in *S. tuberosum*

Following confirmation of the integrity of PVX::PDS constructs B and C in *N. benthamiana*, PDS silencing was subsequently investigated in *S. tuberosum* L. cv Stirling and Bintje. Both cultivars are of interest for investigating resistance to *P. infestans* and lack the *Rx* gene that promotes extreme resistance to PVX (Bendahmane *et al.*, 1995). PVX constructs, B and C, were both stab inoculated and agroinfiltrated to determine

whether either method or construct could induce *PDS* silencing in potato. At 11 dpi, symptoms of *PDS* silencing appeared on the systemic leaves of PVX::*PDS*_{as} agroinfiltrated Stirling and Bintje (Figure 5.3.3A). No corresponding symptoms appeared on PVX::*GFP* or PVX::*PDS*_{hp} indicating that the spots are not symptoms of PVX infection. PVX::*PDS*_{hp} was apparently ineffective at triggering silencing in potato or potato cvs used in this experiment are less permissive for PVX::*PDS*_{hp}. Once again, this could be due to the small sequence length of the hairpin and could be tested by generating a longer inverted repeat (~100 bp) or by utilising a more permissive host (Lacomme *et al.*, 2003). By 18 dpi, systemic *PDS* silencing had developed although significantly more patchy than silencing found in *N. benthamiana* (Figure 5.3.3).

Since PVX::*PDS*_{as} induced *PDS* silencing in Stirling and Bintje, *S. tuberosum* L. cv Desiree and the diploid potato *S. bulbocastanum* were included in subsequent silencing experiments. Desiree was selected because of the relative ease of stable transformation; it is the most common cv used to develop transgenic potatoes and therefore, gene silencing in Desiree was of independent scientific interest. *S. bulbocastanum* is a diploid wild *Solanum* species and was chosen to investigate the effect of ploidy on PVX-induced gene silencing. Fifteen days post-agroinfiltration of all previously mentioned hosts with PVX::*GFP*, infiltrated and systemic leaves were tested for viral accumulation by immunodetection of viral PVX CP using western blot analysis (Figure 5.3.3B). PVX CP accumulation was shown to be more pronounced in both the local and systemic leaves of *N. benthamiana* and the diploid potato compared to the three tetraploid potato cultivars. However, the overall result reveals that all plants tested can tolerate considerable PVX accumulation. It appears, in terms of CP accumulation, that potato is less permissive to PVX accumulation than *N. benthamiana*.

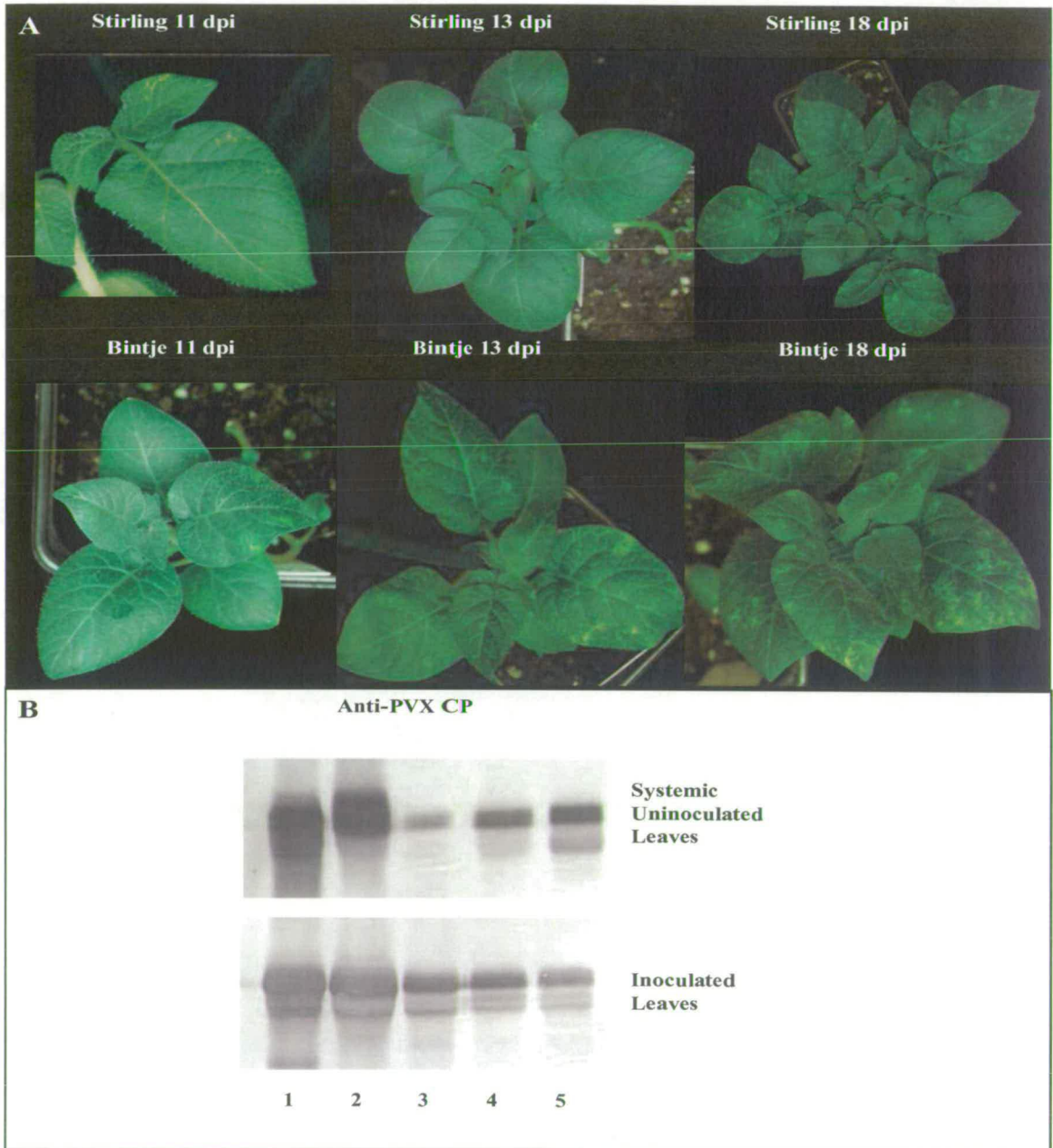


Figure 5.3.3: Progression of *PDS* silencing and PVX CP accumulation in *Solanum* hosts. **A**, Initial discovery and monitoring of *PDS* silencing in tetraploid potato cultivars Stirling and Bintje. **B**, Western blot analysis of PVX CP accumulation in the inoculated and systemic leaves at 15 dpi: 1, *N. benthamiana*; 2, *S. bulbocastanum*; 3, Stirling; 4, Desiree; 5, Bintje. (Western blot displayed was performed by Christophe Lacomme and Katerina Hrubikova).

5.3.4. Quantification of *PDS* Transcripts

Initial *PDS* silencing experiments involving Stirling and Bintje highlighted the potential of PVX as a silencing vector. Even up to 3 months post-agroinfiltration the *pds* silencing

was observed in these tetraploid potato cultivars (photographs not shown). Quantification of the extent of *PDS* silencing was therefore not only of interest in plants 21 dpi but also in the newly emerging leaves of the potatoes at 70 dpi. Therefore, quantitative real-time reverse transcriptase PCR (Taqman, ABI PRISM 7700 Sequence detection system) was performed on 3 leaf layers to determine whether the potato was in the process of PVX “recovery” (Ratcliff *et al.*, 1997). Newly emerging leaves (average of 5 leaflets per leaf) from independent PVX::*PDS*_{as} and PVX::*GFP* Stirling and Bintje plants were collected 70 dpi and named Layer 1 (L1). Leaves collected from the middle of plants infected with PVX::*PDS*_{as} and PVX::*GFP* 70 dpi were named Layer 2 (L2) and leaves collected from middle of PVX::*PDS*_{as} and PVX::*GFP* plants 21 dpi were named Layer 3 (L3). RNA was then extracted from PVX::*GFP* and PVX::*PDS*_{as} leaf layers, RNA quantified and equal μg used for cDNA synthesis as described in 2.8.4. Taqman primers were designed to the region of *S. tuberosum PDS* lying outwith the region cloned into PVX, therefore not confusing the source of *PDS* RNA transcripts.

Quantification of *PDS* transcript was normalised using endogenous control ubiquitin transcript. The level of *PDS* transcript was then made relative to that present in the appropriate *GFP* control plants. Quantification of the transcripts, in two biological experiments (Figure 5.3.4a, A and B), revealed that *PDS* expression was reduced to around 20 % in PVX::*PDS*_{as} plants compared with PVX::*GFP* for both cultivars which is comparable to other VIGS systems (Lacomme *et al.*, 2003). The layer of leaves from which the RNA was extracted did not appear to affect the extent of silencing with one exception in Figure 5.3.4aB, Bintje L1, which did appear to have around 60 % expression, revealing that in some cases the silencing may weaken by 70 dpi. However, it is important that silencing is induced effectively at 21 dpi rather than 70 dpi as potato plants are beginning to flower and would not be useful for further experimentation. A semi-quantitative RT PCR was also performed on the same cDNA samples and the results for Bintje PVX::*PDS*_{as} and PVX::*GFP* at 30 cycles are shown in Figure 5.3.4aC. This RT-PCR highlights the lower amounts of *PDS* PCR product in PVX::*PDS*_{as} Bintje when compared to PVX::*GFP* and the equal amplification of ubiquitin from both samples.

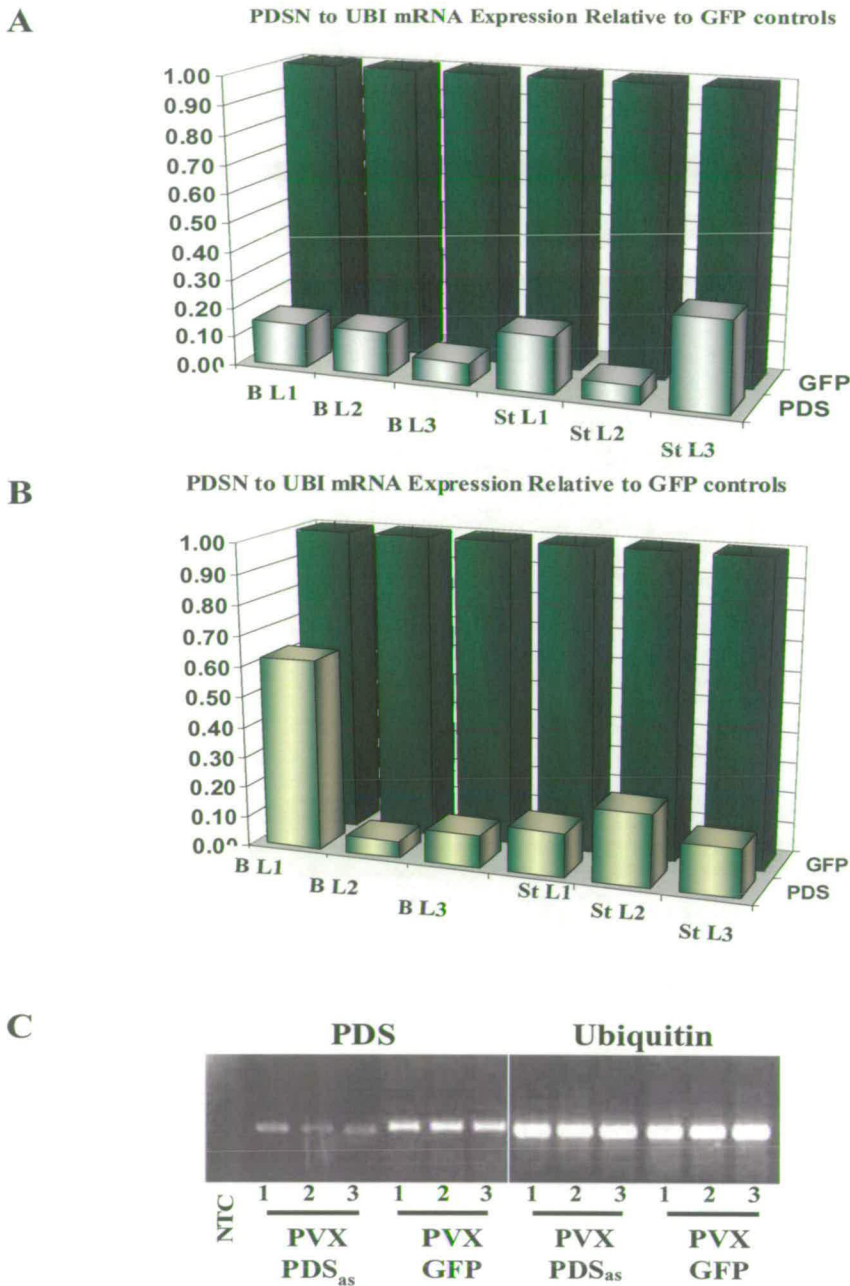


Figure 5.3.4a: Quantification of *PDS* expression in Stirling and Bintje. **A**, Real-time RT-PCR determination of normalised relative amounts of *PDS* mRNA in PVX::PDS_{as} and PVX::GFP challenged plants. B L1, Bintje layer1, L2, layer 2 and L3, layer 3; St, Stirling. **B**, Biological replicate of A. **C**, RT-PCR of *S. tuberosum* Bintje *PDS*-silenced and control plants. 1,2 and 3 corresponding again to the layer number. Each sample used for real-time RT-PCR was carried out in triplicate.

PVX::PDS_{as} silencing in *N. benthamiana* (Figure 5.3.2) appeared to generate a more extensive photobleaching phenotype than in *S. tuberosum* (Figure 5.3.3). It was therefore

of interest to quantify *PDS* silencing induced by PVX::*PDS*_{as} in all host plants to investigate whether the extent of photobleaching correlated with *PDS* transcript levels. Taqman primers for *N. benthamiana* *PDS* were designed, again in a region outwith PVX::*PDS*_{as} (2.8.4). Photographs were taken of PVX::*PDS*_{as} induced silencing of all host plants at 21 dpi (Figure 5.3.4b). Quantitative real-time RT-PCR was performed for *N. benthamiana*, *S. bulbocastanum* and *S. tuberosum* cv Desiree so that the extent of silencing could be compared to that previously measured in Bintje and Stirling (Figure 5.3.4c). In all hosts inoculated with PVX::*PDS*_{as} *PDS* expression was reduced to 20 % of the PVX::*GFP* control plants. This demonstrates that the observed extent of photobleaching does not always correlate with *PDS* mRNA levels, suggesting that although potato is a less permissive host for PVX, the silencing response induced against *PDS* was comparable with *N. benthamiana* (Ratcliff *et al.*, 2001; Lacomme *et al.*, 2003).

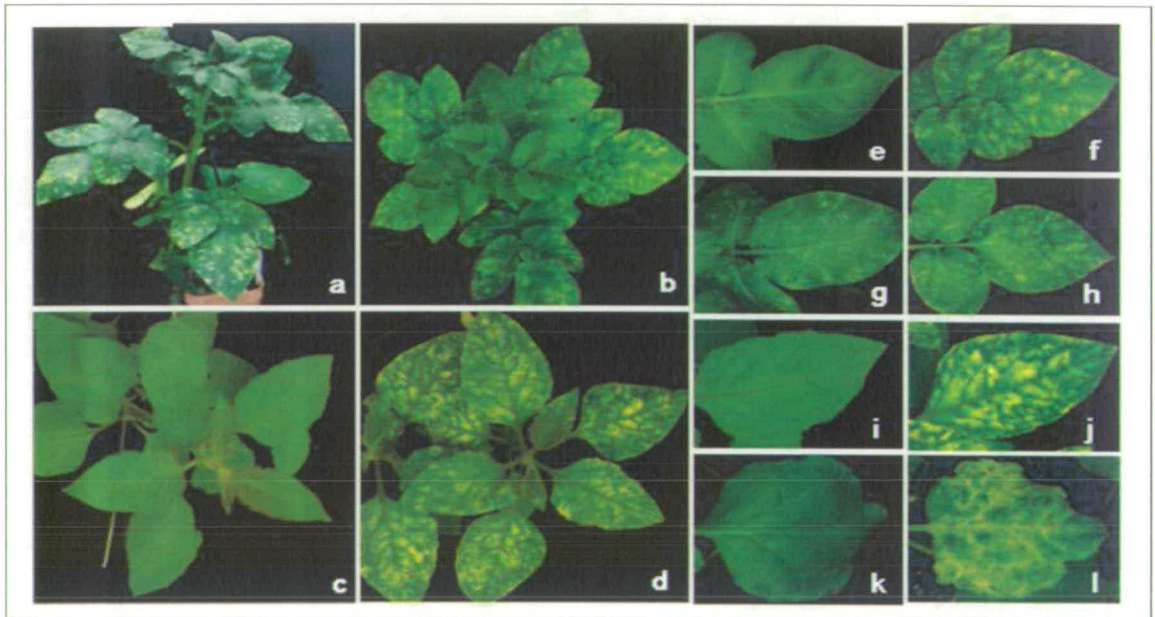


Figure 5.3.4b: Photobleaching phenotype of *Pds* silencing in *Solanum* species. Photobleaching phenotypes observed by 21 dpi. **a** and **b**, tetraploid *S. tuberosum* cv Bintje; **c**, PVX::*GFP* control **d**, diploid *S. bulbocastanum*. Close-up on photobleached leaves: **f**, *S. tuberosum* cvs Bintje; **g**, Stirling; **h**, Desiree; **j**, *S. bulbocastanum*; **l**, *N. benthamiana*. Symptomless PVX::*GFP* infected leaf: **e**, cv Bintje; **f**, *S. bulbocastanum*; **i**, and uninfected *N. benthamiana* leaf (**k**) (Faivre-Rampant *et al.*, 2004).

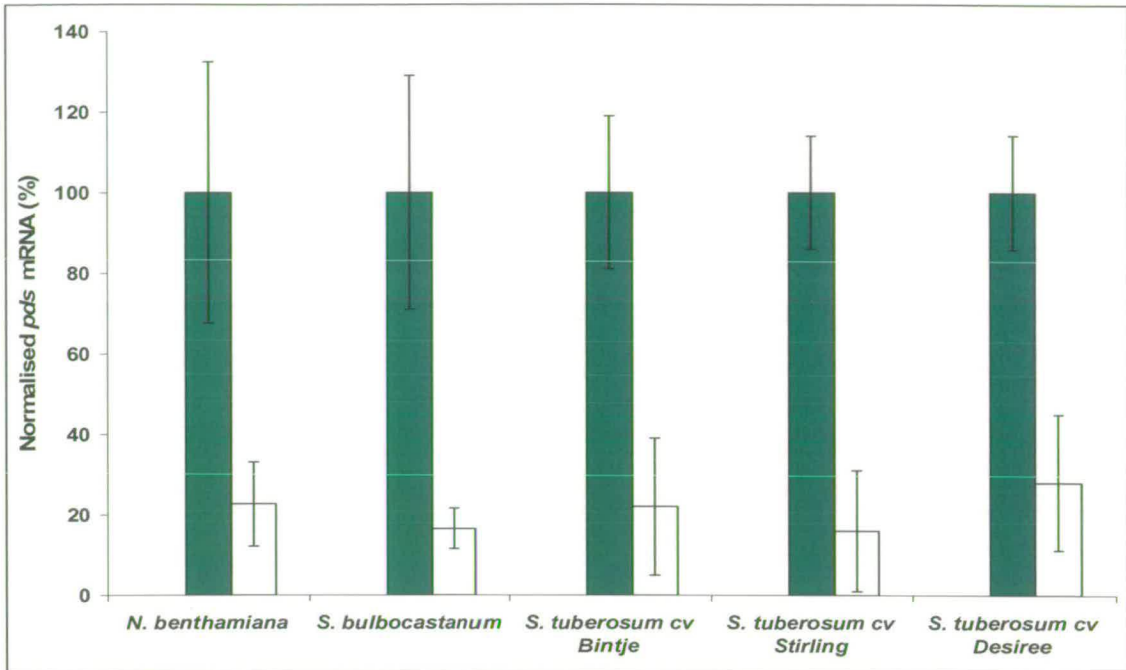


Figure 5.3.4c: Quantification of PVX::PDS_{as} triggered silencing in all hosts. Quantitative real-time RT-PCR of normalised relative amounts of *PDS* mRNA levels in PVX::PDS_{as} and PVX::GFP plants at 21 dpi. For each sample, values represent the means of at least three leaves from different plants per construct. Each sample used for real-time RT-PCR was carried out in triplicate. Quantitative real time RT-PCR of *N. benthamiana*, *S. bulbocastanum* and *S. tuberosum* cv Desiree were performed by K. Hrubikova. Error bars represent standard error of two replicates for two individual biological experiments (Faivre-Rampant *et al.*, 2004).

5.4. Silencing of HR-Related Genes in Potato

PVX was shown to efficiently silence the reporter gene *PDS* in diploid and tetraploid potato. Genes involved in disease resistance were the next targets of PVX-induced gene silencing in *S. tuberosum* cvs Bintje and Stirling. Stirling possesses *R* gene and quantitative resistance to *P. infestans* which both rely on an effectively induced HR. However the genes responsible for these types of resistance are poorly understood. Bintje is a commercially grown variety which is highly susceptible to *P. infestans* and is used as a control of compatible potato-blight interactions.

5.4.1. PVX Vectors Containing HR-Related genes

Fragments of candidate genes from potato *SSH*, *CathB* and *Cyp1* (Avrova *et al.*, 2003; Avrova *et al.*, 2001) were both cloned in anti-sense into PVX (see 2.5.2). In addition,

potato homologues of *Arabidopsis* SAR-inducing *R* gene-like *ADRI* (Grant *et al.*, 2003) and *SGTI*, a gene documented to be required for *R* gene-triggered resistance in other plant-pathogen interactions (See 1.4.1.2), were identified and also cloned in anti-sense into PVX. Stirling and Bintje were agroinfiltrated with PVX defence gene constructs as well as PVX::GFP as control and left for 4 weeks for silencing to develop.

Silencing of two of the four defence genes resulted in morphological phenotypes by 21 dpi with the PVX constructs (Figure 5.4.1). PVX::SGT1_{as} plants displayed a stunted and more branched growth phenotype in comparison with PVX::GFP which is previously described in *SGTI* silenced *N. benthamiana* (Peart *et al.*, 2002). Interestingly, the leaflets also developed a lobed morphology with yellowing around the edges suggesting that silencing of *SGTI* affects aspects of leaf development. PVX::CathB_{as} developed a more elongated and sparse stature with chlorotic foliage compared with PVX::GFP. Interestingly, this phenotype was also displayed in the *CathB* (At4g01610) *Arabidopsis* knock out mutant which is currently under investigation (See Chapter 4). Silencing of *ADRI* and *CYPI* in potato did not result in any obvious morphological phenotypes.

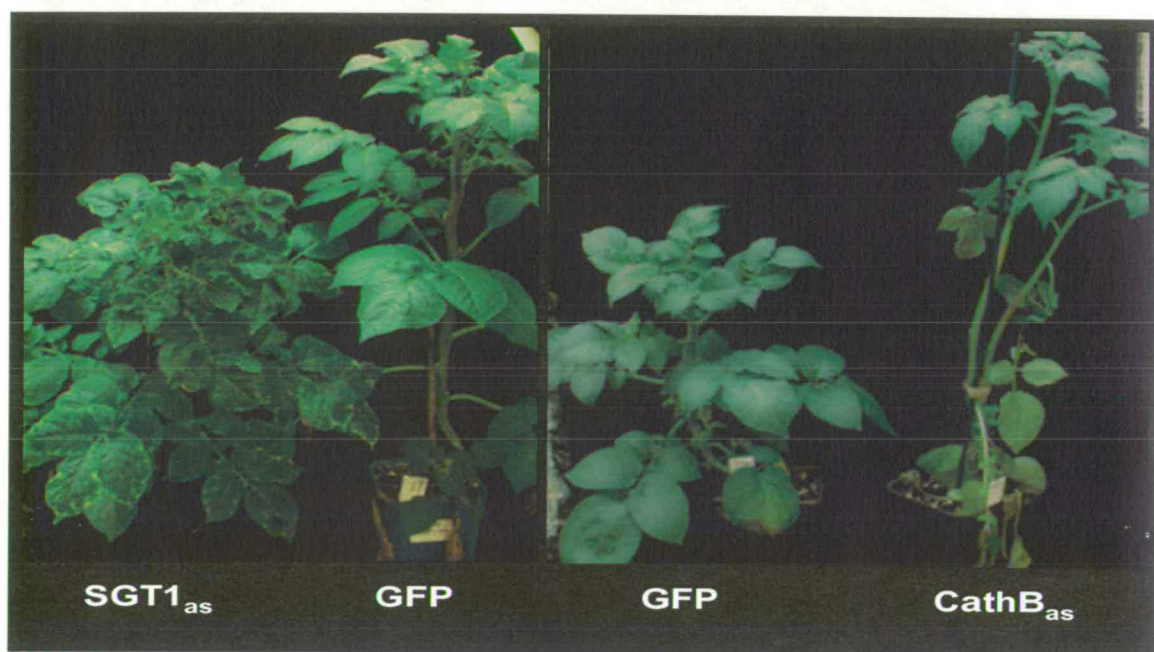


Figure 5.4.1: Phenotype of PVX::SGT1_{as} and PVX::CathB_{as} inoculated Stirling. Stirling inoculated with PVX::SGT1_{as} displayed a stunted and more branched phenotype also documented in *SGTI* silenced *N. benthamiana* (Peart *et al.*, 2002) however leaflets also developed a lobed shape with yellow edges. PVX::CathB_{as} Stirling developed an elongated shape in combination with paler foliage.

5.4.2. Resistance to *P. infestans*

Spore suspensions of a simple (15.5.1) and a complex race (99/23) of *P. infestans* were evenly sprayed over potatoes and kept in almost 100 % humidity overnight. The simple race (1,4) of *P. infestans* is known to overcome *R1*- and *R4*-gene-mediated resistance in potato and is recognised by an unknown *R* gene in Stirling potato plants. The complex race (1,2,3,4, 6 and 7) of *P. infestans* overcomes resistance conveyed by *R1*, *R2*, *R3*, *R4*, *R6* and *R7* and its AVR proteins are not recognised by the *R* protein in Stirling plants. The development of disease symptoms was monitored and recorded between 5-7dpi (Figure 5.4.2). PVX::CYP1_{as} Stirling plants did show increased susceptibility to the complex race of *P. infestans* (99/23). Thus the screen revealed that CathK may be important for resistance in Stirling against complex race 99/23. However, there was no apparent effect of PVX-induced silencing in Stirling challenged with the simple race. Silencing of HR-related genes in Bintje produced a more varied range of results which were not statistically significant. However, PVX.CYP1_{as} Bintje plants appeared slightly more susceptible to the simple race of *P. infestans*. This suggests that spraying *P. infestans* on probably patchy patterns of silencing in these potatoes was not a suitable screen for investigating these genes in potatoes.

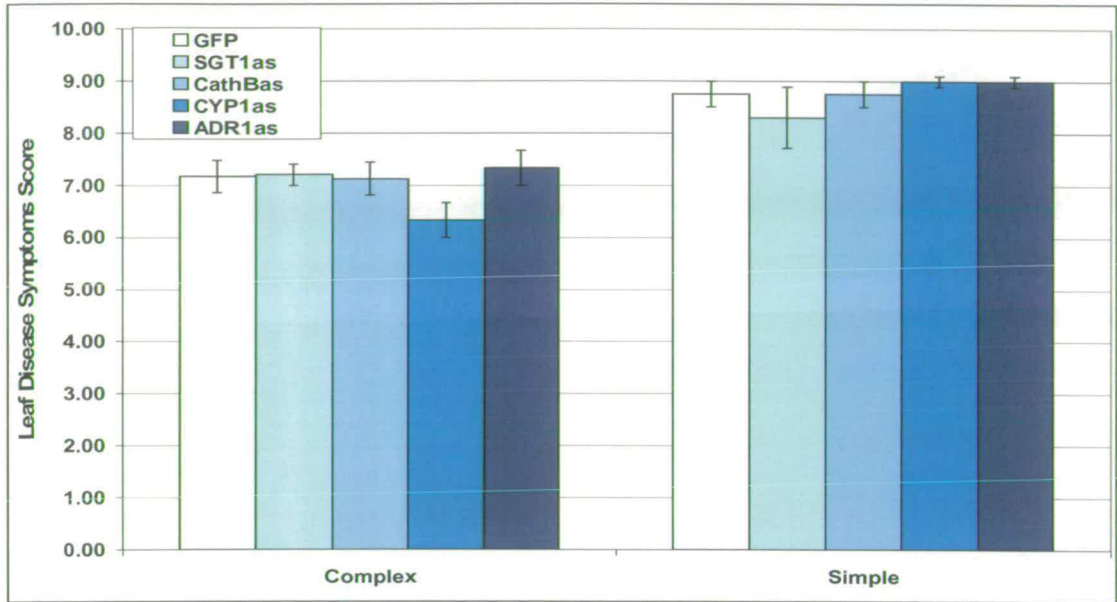


Figure 5.4.2a: Infection of silenced *S. tuberosum* cv Stirling with *P. infestans*. Resistant cultivar Stirling infected with control construct PVX::GFP and 4 HR-related PVX::SGT1_{as}, PVX.CathB_{as}, PVX.CYP1_{as}, PVX::ADR1_{as} were sprayed with the simple race (15.5.1) and the complex race (99/23) of *P. infestans*. Briefly, scoring was as follows: 1= >90 % necrotic tissue, 2= 81–90 % necrotic, 3= 71–80 % necrotic, 4= 61–70 % necrotic, 5= 41–60 % necrotic, 6= 26–40 % necrotic, 7= 11–25 % necrotic and 8= <10% necrotic. Bars represent standard error of one experiment using five plants per silencing construct.

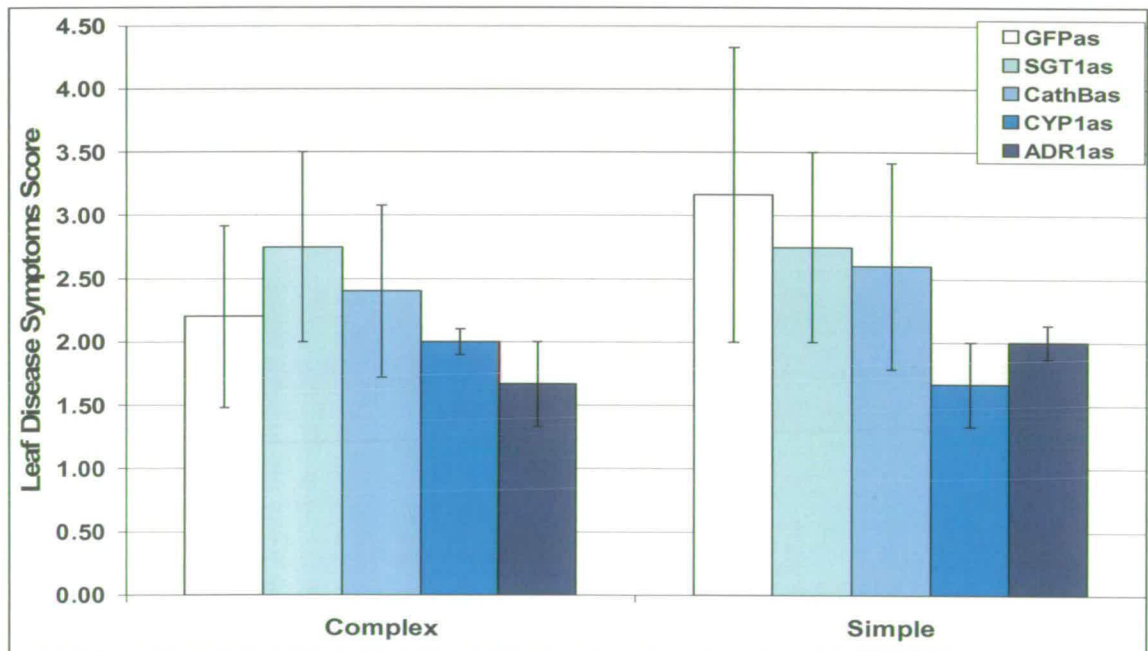


Figure 5.4.2b: Infection of silenced *S. tuberosum* cv Bintje with *P. infestans*. Bintje infected with the same PVX constructs as Stirling and infected with both simple and complex races of *P. infestans*. Briefly, scoring was as follows: 1= >90 % necrotic tissue, 2= 81–90 % necrotic, 3= 71–80 % necrotic, 4= 61–70 % necrotic, 5= 41–60 % necrotic, 6= 26–40 % necrotic, 7= 11–25 % necrotic and 8= <10% necrotic. Bars represent standard error of one experiment using five plants per silencing construct.

5.5. Discussion

HR-related genes had been identified from a SSH performed in tetraploid potato cultivars and the candidate gene, *CathB* has been investigated by TRV-Induced gene silencing in the model system, *N. benthamiana* (See Chapter 4). However, in order to study defence responses in potato to *P. infestans* (and other economically important pathogens of potato) the development of a gene-silencing system was necessary. Gene-silencing in the cultivars from which they were identified would be very useful for studying the functions of these genes. TRV, one of the most commonly utilised silencing vectors, was tested initially for its silencing capabilities in three tetraploid potato cultivars, Wilja, Spey and Stirling. Although TRV vectors efficiently silenced *PDS* in *Nicotiana* species, no accumulation of the transgenic RNA 2 could be detected in the potato cultivars. Since RNA 2 is not essential for TRV virus replication and systemic spread within the plant, it is most likely that there is no selection pressure that necessitates TRV to replicate its RNA 2, especially if it carries a large insert that hinders its replication. It is commonly observed that recombination can occur at subgenomic promoters in viral vectors, especially if present at either side of a transgene (Chapman *et al.*, 1992). However, if the TRV RNA 2 in potato was losing its *PDS* transgene through recombination then the coat protein of the wild-type RNA 2 would still have been detected in the RT-PCR (5.2.3). It is also possible that a sequence of higher homology rather than the tomato *PDS* would have induced silencing (Ryu *et al.*, 2004). Interestingly, a couple of months after the publication of PVX work in this chapter, Brigneti *et al.*, (2004) published the use of bipartite TRV RNA 1 and RNA 2 vectors (Liu *et al.*, 2002) for gene silencing in potato diploids *S. bulbocastanum*, *S. okadae* and tetraploid *S. tuberosum* cvs Cara and Pentland Ivory. Brigneti *et al.* (2004) report the balance between RNA 1 and RNA 2 in potato plants was critically important for inducing sufficient gene-silencing, that TRV-induced gene silencing failed in vegetatively propagated potatoes and success was only achieved by using potato cvs grown from seed. Micropropagation is the most efficient method of multiplying potatoes for research purposes and was used to generate potatoes for all work

mentioned in this chapter and most probably the reason for results presented in Figure 5.2.3.

The PVX-based expression vector has one major advantage over TRV when utilising it as an expression or silencing vector in potato. The coat protein, corresponding subgenomic promoters and transgene cloning site are adjacent to genes essential for replication on a single plasmid. This means that the transgene will automatically be replicated as recombinant PVX replicates its positive strand RNA genome. It is still possible that the transgene could be lost through recombination at the subgenomic promoters. Therefore, gene fragments cloned into PVX in this study were kept below 700 bp, limiting the genetic load of the PVX genome to reduce the risk of PVX losing an insert through recombination while replicating *in planta* (Thomas *et al.*, 2001).

It was apparent that the hairpin construct used in this study did not improve the silencing of *PDS* in *Nicotiana* species and did not silence in *Solanum tuberosum* cvs Bintje and Stirling (Figure 5.3.2). However, later quantification of *PDS* silencing in a variety of hosts was shown not to correlate with extent of photobleaching (Figure 5.3.4b). Quantification of the *PDS* transcript in PVX::*PDS*_{as} and PVX.*PDS*_{hp} *N. benthamiana* and potato was not performed during these early experiments. It is therefore possible that the hairpin may have silenced *PDS* transcript to a comparable level with the antisense construct. Nevertheless, subcloning of hairpin constructs was also time-consuming providing an additional reason for not pursuing their use during this project. Although previous experimentation using hairpins in other viral systems have successfully utilised hairpins of 40-60 bp (Lacomme *et al.*, 2003) it is possible that the hairpin *PDS* fragment was not sufficiently long to induce effective silencing. Although hairpins ranging from 98-853 bp have been reported to induce effective silencing by Wesley *et al.*, (2001), it is common opinion that hairpins over 100 bp are unstable in viral vectors and would most probably fail to replicate adequately. It is possible that the same hairpin vector in a more permissive host could have induced gene silencing (Lacomme *et al.*, 2003).

The PVX::*PDS*_{as} induced varying extents of foliar photobleaching in different host species. The photobleaching was significantly more extensive in diploids *N.*

benthamiana and *S. bulbocastanum* (Figure 5.3.4b). This could also be correlated with a greater accumulation of PVX CP in these diploids through western blot analysis (Figure 5.3.3). However, when *PDS* transcript levels in PVX::*PDS*_{as} plants were normalised relative to PVX::*GFP* controls, the reduction of the *PDS* transcript was consistently found to be around 20 % (Figure 5.3.4). Therefore, there is little correlation between observed foliar phenotypes and the degree of silencing of the transcript. Although tetraploid potatoes are less permissive to PVX replication than *N. benthamiana* and diploid potato cvs, the extent of silencing was comparable between all species tested. *S. tuberosum* could therefore possess a more efficient silencing machinery to promote comparable gene silencing to *N. benthamiana* with less virus accumulating or possibly the virus is less selected against by the machinery allowing a more prolonged silencing response. The later hypothesis is supported by Figure 5.3.4a, in which potatoes of 70 dpi still possessed a comparable extent of *PDS* silencing as those 21 dpi. Also, *N. benthamiana* inoculated with the same PVX::*PDS*_{as} construct had almost completely recovered from PVX infection. The hypothesis that tetraploid potato cvs possess a more efficient silencing response to PVX in comparison with *N. benthamiana* and diploid *Solanum* species cannot be confirmed from the data presented here and would require further experimentation. In addition, the photobleaching phenotype is a secondary response to silencing of *PDS* and is dependent on the amount of photo-protective carotenoids present in the leaves of each species and the strength of light the plant is exposed to. Therefore the lesser extent of photobleaching in potato cultivars may also be due to additional photo-protective pigments derived from a *PDS*-independent pathway. Nevertheless, the finding that PVX could efficiently induce *PDS* silencing in cultivated potatoes was the first published example of a successful VIGS system for tetraploid *S. tuberosum* (Faivre-Rampant *et al.*, 2004).

Following *PDS* silencing in *S. tuberosum* cultivars Bintje and Stirling, partial cDNA fragments of candidate HR-related genes were cloned in antisense into PVX vector pGR106. Three weeks post-inoculation, PVX::*SGT1*_{as} and PVX::*CathB*_{as} Stirling and Bintje had developed distinct morphological phenotypes. Silencing of *SGT1* resulted in a

branched and stunted phenotype that has been previously described in *SGT1* silenced *N. benthamiana* by Peart *et al.*, (2002). This gave an encouraging indication that the silencing of *SGT1* had been triggered to a similar extent in these potato cultivars. However, in addition PVX::*SGT1_{as}* inoculated potatoes also developed a lobed leaf morphology and yellowing of the leaf edges which has not been previously reported. Similar leaf morphologies have been described in response to over-expression of cytokinin synthesising or meristematic homeodomain proteins (Miltos Tsiantis, personal communication). Unfortunately, within the constraints of this project there was no time to investigate this phenomenon further. Nevertheless, the lobing phenotype suggests that *SGT1*, in addition to its role in defence, may play a role in the regulation of leaf shape during development. Interestingly, PVX::*CathB_{as}* potatoes developed a sparse and elongated stature, almost the complete converse of PVX::*Sgt1_{as}* plants. In addition, the foliage appeared to be chlorotic in comparison with PVX::*GFP*. Again, this was an encouraging indication of *CathB* silencing as one of the *Arabidopsis CathB* KO mutants (At4g01610) also displayed an elongated and chlorotic phenotype. The other PVX vectors did not produce any distinguishable morphological phenotypes at 21 dpi.

At 21 dpi, the PVX-inoculated cultivars Bintje and Stirling were evenly sprayed with 2 different races of *P. infestans* that should trigger a HR in Stirling either through triggering an *R* gene or through activating quantitative resistance. The only clear trend from these experiments was that PVX::*CYP1_{as}* inoculated Stirling did show a small but significant increase in disease symptoms in response to race 99/23. It seems likely that the fine spray of spores over the entire plant in combination with possibly patchy foliar silencing may not be an optimal system to test for loss of resistance. If time had allowed, a detached leaf assay would have been performed as described in Vleeshouwers *et al.* (1999) and/or inoculation with a defined concentration of non-host bacterium such as *E. amylovora* as in Chapter 4. However, early results indicate that PVX-induced silencing of HR-related genes could be a useful tool in the dissection of genes involved in potato disease resistance.

5.6. *Conclusions*

The main findings of this chapter are published in Faivre-Rampant *et al.* (2004), which concludes that PVX expression vector, pGR106 silences *PDS* transcript levels effectively in potato in comparison with other published reports of VIGS. The presence of the *PDS* transcript was reduced by 80 % of control plants in *N. benthamiana*, wild diploid potato *S. bulbocastanum* and cultivated tetraploid *S. tuberosum* cv Bintje, Stirling and Desiree. In addition, PVX-induced silencing of HR-related genes reproduced gene-silencing or gene knockout phenotypes published or observed in other plant species indicating that the silencing of genes other than *PDS* may be sufficient to study gene function (Peart *et al.*, 2002; and Chapter 4 of this thesis). Development of a functional screen for the HR-related role of genes silenced in Stirling and Bintje, using *P. infestans*, was still in progress at the end of this project. Nevertheless, initial results gave an encouraging indication that if more time was available to investigate other screening methods, PVX-based silencing could be used for the investigation of disease resistance gene functions in potato.

6. General Conclusions and Future Work

This project was a collaboration between the *Arabidopsis* community and the crop-based community through which the best resources available to both could be utilised to study aspects of disease resistance. It was hypothesised that candidate genes of interest from both models and crops could be studied simultaneously and the most interesting candidate genes investigated as far as possible in all systems.

The sequenced model *Arabidopsis* is most amenable for forward genetics-based approaches to gene discovery through large-scale mutagenesis and functional screening. Classical mutant screens have been used extensively as a means of dissecting genetic pathways and have traditionally utilised loss-of-function mutants. However, loss-of-function mutagenesis rarely identifies functionally redundant or essential genes. For example, screens for loss-of-function mutants in race-specific disease resistance pathways have predominantly identified mutant alleles of the cognate *R* gene e.g. *RPM1* (Bisgrove *et al.*, 1994). Similarly, loss-of-function screens undertaken in a number of laboratories for insensitivity to SA have uncovered alleles (*npr1/nim/sai1/eds16*) at the same gene locus (Cao *et al.*, 1997; Ryals *et al.*, 1997; Shah *et al.*, 1997). The activation tagging approach was used in this project to circumvent lethality and functional redundancy problems associated with classical mutagenesis systems. Activation tagging should therefore generate gain-of-function mutations using the strong activating properties of the CaMV 35S gene enhancer and the random integration of T-DNA (Tani *et al.*, 2004; Weigel *et al.*, 2000). Activation tagging has led to the previously identified *ADR1* gene (Grant *et al.*, 2003) and two *ads* mutants during this project. Identification of genes responsible for the susceptibility phenotype in the *ads* mutants cannot be confirmed at this stage and subsequently whether these mutations are gain- or loss-of-function. However, the regions in which the pSKI015 insertion lies in the *ads1* and *ads2* genomes have been identified, facilitating sequence-dependent techniques such as silencing or genetic reconstitution for further investigation.

In addition to the relatively novel approach of activation tagging to generate gain-of-function *Arabidopsis* mutants, few increased susceptibility screens have been developed to identify genes that could be involved in response to virulent pathogens (Volko *et al.*, 1998). The majority of mutant screens have focused on genes required for disease resistance induced by avirulent, non-host or necrotrophic pathogens (Eckardt, 2002). In this project it was hypothesised that by identifying and studying mutants susceptible to virulent *Pst* DC3000, components of inducible defence responses manipulated by the pathogen or required by the host plant could be identified. For example, *Pst* DC3000 has been shown to synthesis coronatine, a jasmonic acid analogue, stimulating the JA-dependent pathway, using the plant's own wound signalling to suppress the SA-dependent pathway. The *Arabidopsis* mutant *coil*, which cannot sense JA, has recently been shown to be more resistant to virulent *Pst* as its defence signalling cannot be manipulated through coronatine production (Cui *et al.*, 2005).

Isolating and studying the *ads1* mutant may have highlighted a region of genomic DNA that contains an element involved in activating genes in the JA-dependent or suppressing genes in the SA-dependent pathway. The gene(s) affected by the activation tag in *ads1* led to the constitutive production of high levels of H₂O₂, a response associated with induction of the HR and resistance to avirulent pathogens; it appears to be susceptible to avirulent *Pst* DC3000 (*avrB*). Currently, H₂O₂ is thought to induce the HR and defence gene expression leading to SAR that should subsequently lead to resistance to both virulent and avirulent pathogens (Wu *et al.*, 1995). However, it is possible that constitutive production of H₂O₂ causes a high degree of cellular stress and/or constitutive activation of H₂O₂-detoxifying mechanisms, perturbing basal defence responses to virulent or avirulent *Pst* DC3000. Furthermore, it appears that treating *ads1* with BTH could induce a level of resistance to virulent *Pst* DC3000, comparable with untreated Col-0. This indicates that *ads1* can induce SAR, once an analogue of SA is applied and that SA-dependent pathway is perturbed before, rather than after, SA production. Analysing the expression of genes associated with the HR (*GST1*), SAR (*PR-1*) and the JA-dependent pathway (*PDF1.2*), before and after *Pst* DC3000 inoculation, would provide further insights into whether an active JA pathway or a perturbed SA pathway is

responsible for increased susceptibility to *Pst* DC3000. If data presented here is correct, the insertion site of pSKI015 in *ads1* lies in a region where no genes have been annotated within 10 kb. Interestingly, this may lead towards identification of an unknown regulatory element or novel gene that again verifies the use of activation tagging for investigating complex phenotypes such as disease susceptibility.

The *ads2* mutant accumulated the stress-associated pigment anthocyanin which is associated with production of other antimicrobials such as phytoalexins and could be caused by over-expression of a stress-related protein (VanEtten *et al.*, 1994). The accumulation of anthocyanin could be indicative of an active stress-induced pathway which could involve both up-regulation of At1g05480, causing increased N-myristoylation/RAD9 activity by over-expression of At1g05500, which has been demonstrated to be responsive to abiotic stress. However, it could be predicted that *ads2* would possess a greater resistance to virulent pathogens due to the presence of phytoalexins or constitutive induction of abiotic and biotic stress pathways. It would be informative to analyse the expression of genes associated with HR, SAR and JA-dependent pathways before and after *Pst* DC3000 inoculation. Investigation of *ads2* resistance response induced through other R proteins in *Arabidopsis* may also prove to be helpful. These experiments would allow detection of which major inducible defence pathways are perturbed in *ads2* mutant.

It is possible that At3g05500, encoding a stress-related protein, also known as a rubber elongation factor (*REF*), is responsible for increased susceptibility (Kim *et al.*, 2003). *REF* is up-regulated in response to wounding in *Ficus carica*, in response to other abiotic stresses and peroxidase activity has been associated with *REF* activity (Kim *et al.*, 2003). Tomato and *Arabidopsis* metacaspases are referred to as latex-abundant proteins and there could be a connection between metacaspases and *REF*. Interestingly, the gene(s) overexpressed in *ads2* enhanced the resistance response to avirulent pathogens suggesting that induction of defence responses through a gene-for-gene interaction could overcome the apparently perturbed basal defence responses. Augmented resistance to avirulent *Pst* DC3000 could also be explained by elevated

peroxidase activity associated with an increase in REF activity. However, DAB staining could not reveal any significant difference in H₂O₂ production in *ads2* mutants compared with Col-0 demonstrating that peroxidase activity appeared unaffected by activation tag.

The gene up-regulated at the At3g05480 locus, in the *ads2* mutant was a RAD9-like protein with an N-myristoylation transferase domain. RAD9 is a cell cycle protein associated with caspase-3 cleavage during apoptosis and the N-myristoylation of proteins which promotes their affinity with membranes (de Jonge *et al.*, 2000; Qi *et al.*, 2000; Li *et al.*, 2003). Increased expression and protein activity of a RAD9-like protein could be responsible for the enhanced resistance to *Pst* DC3000 (*avrB*) by amplifying the activation of the proteolytic cascade as part of the HR. Measuring and comparing any caspase-3 like activities in *ads2* and Col-0 plants, using colorimetric substrates and inhibitors, may confirm whether At3g05480 is responsible for both increased *RPS2*-dependent resistance and decreased basal resistance.

Therefore, it is impossible to predict at present whether up-regulation of both genes together or one gene alone is responsible for the observed *ads2* mutant phenotypes associated. Both genes could be cloned behind a 35S promoter and transformed into Col-0 plants or T-DNA insertion mutants could be ordered to investigate the phenotypic effects on resistance. Analysing the expression of key marker genes in both biotic and abiotic pathways would also be useful to investigate whether JA-dependent or stress-related pathways are constitutively active in *ads2*. Ultimately, overexpression of genes at loci At3g05480 and At3g05500, could be responsible for susceptibility to *Pst* DC3000 in *ads2*, neither of which have been functionally analysed in the *Arabidopsis* literature to date, validating the use of activation tagging for identifying possible functions of novel genes in disease resistance.

Genetic screens in *Arabidopsis* have facilitated the discovery of many components of core defence signalling networks and the research community as a whole has generated highly accessible databases of detailed sequence and functional information. The availability and range of information in these databases has undoubtedly promoted the

transfer of genetic information from *Arabidopsis* to complement functional analysis of genes in crop species.

Resistance to invading avirulent biotrophic pathogens in crop species, such as barley, rice and potato, has been shown to require the HR. Therefore, signalling components and key regulators of the HR can be investigated in model plants and crop plants to compare and contrast the similarities. Fortunately, homologues of genes identified from *Arabidopsis* can be identified in crop species, using data from the sequencing projects under way on many members of the Solanaceae, such as EST sequencing of *Nicotiana benthamiana*, *N. tabacum* and *N. rustica*, a full genome project for tomato and anticipated potato genome project. Nevertheless, methods of identifying novel genes involved in plant disease resistance in crop plants have been successfully established and provide alternative routes to gene discovery since reverse genetic type screens are not economical with crop plants. One approach used for gene discovery in potato was used prior to the start of this project, known as suppression subtraction hybridisation (SSH) to isolate genes up-regulated early during an HR. The HR is of particular importance because it appears to be fundamental to every type of resistance against oomycetes e.g. *Phytophthora infestans* on potato and *P. parasitica* on *Arabidopsis* (Birch *et al.*, 1999; Birch and Whisson, 2001).

Classes of genes identified in the SSH library were similar to those found in a variety of other cell signalling pathways e.g. transcription factors, protein kinases and cysteine proteases (Birch *et al.*, 1999; Avrova *et al.*, 1999; Solomon *et al.*, 1999). Cysteine protease genes from the SSH (*CYP1* and *CathB*) and a gene involved in resistance to *P. parasitica* in *Arabidopsis* (*ADR1*) (Grant *et al.*, 2003) were selected for further analysis. A gene required for the powdery mildew-induced HR in barley (*SGT1*) which has been successfully studied in other plant species was selected as a control (Peart *et al.*, 2002). Expression analysis of these genes in potato during an incompatible reaction with *P. infestans* revealed that *CYP1* and *CathB* were up-regulated preceding and during the HR but only provided an indication that *ADR1* and *SGT1* are expressed preceding the HR. However, the role of these genes could not initially be investigated in *S. tuberosum* due

to the lack of a successful method of inducing gene silencing. Therefore, an established VIGS system was used to investigate the roles of equivalent genes in *N. benthamiana*, a close relative of potato and an experimental model of the HR (Burton *et al.*, 2000; MacFarlane and Popovich, 2000). As with *Arabidopsis* mutants, functional characterisation of VIGS phenotypes cause a bottle-neck in the speed of analysis and therefore one gene had to be prioritised for focused functional analysis. Cathepsin B was selected for further investigation because of the number of proteases already shown to be necessary for the HR and evidence of cathepsin B activity during PCD from studies in mammalian models. For example, cathepsin B deficient mice show increased resistance to apoptosis (Mathiasen and Jäättelä, 2002; Foghsgaard *et al.*, 200). It was therefore hypothesised that cathepsin B could be a component of the protease cascade regulating or effecting the HR.

In Chapter 4, it was shown that silencing cathepsin B in *N. benthamiana* plants caused *NbCathB* expression to be reduced by approximately 80 % , preventing a peak of protein activity at 6 hpi in comparison with TRV::GFP plants and consequently allowing 10-fold greater recovery of viable *Eam* and *Pst* DC3000 (*avrB*). In order to confirm the gene-silencing experiments, one general cathepsin inhibitor and three cathepsin B-specific inhibitors were used to confirm the gene silencing experiments. Two cathepsin B protein inhibitors prevented the peak of cathepsin B activity at 6 hpi, also suppressing the HR and allowing 10-fold greater bacteria to be recovered from inoculated area. This suggests that *N. benthamiana* cathepsin B has similar active sites as mammalian cathepsin B and that blocking this activity suppresses the HR. Cathepsin B may also play a role in PCD by cleaving PARP which is a commonly used marker of apoptosis although it would be useful to investigate other markers such as DNA laddering and cytochrome C release from the mitochondria. Preliminary data investigating the effect of cathepsin inhibitors on the cleavage of VirD2 during PCD *in planta* suggests that cathepsin B may be upstream of or required for caspase-3-like activity.

It is tempting to speculate that plant proteases involved in the execution of the HR are arranged in a proteolytic cascade, similar to that observed during mammalian PCD,

including cysteine, serine and aspartic proteases, some of which may possess caspase-like activities. For example, an animal analogue of the plant vacuolar processing enzyme (VPE) processes cathepsin B (Shirahama-Noda *et al.*, 2003; Hatsugai *et al.*, 2004). In turn cathepsin B has been demonstrated to act both independently or directly upstream of caspases, the major effectors and executioners of mammalian apoptosis (Vancompernelle *et al.*, 1998; Foghsgaard *et al.*, 2001; Kingham *et al.*, 2001). Interestingly, caspase-1 and -3 like activities have been measured during the HR, although no plant orthologues of caspase genes or proteins have been found (Chichkova *et al.*, 2004; Belenghi *et al.*, 2004; Hatsugai *et al.*, 2004). To investigate the manner in which cathepsin B may interact with other proteases activated during the HR, the effect of caspase-1 and -3 inhibitors (DEVD; YVAD; TATD) and VPE inhibitor (ESEN) on *in planta* cathepsin B activity could be investigated. In addition, purification of *N. benthamiana*, *S. tuberosum* and *Arabidopsis* cathepsin B proteins would allow the range of cellular substrates to be investigated using substrates of other proteases and the other proteases themselves as controls. Furthermore, purification of plant cathepsin B would allow repetition of experiments included in this thesis, such as PARP1 cleavage westerns and the effect of inhibitors during the cathepsin B colorimetric assay.

Attempts to engineer durable resistance in crop plants have mainly failed due to the complexity of disease resistance pathways and the diversity of pathogen systems (Stuiver and Custers, 2001). Highly specific interactions occur between a diverse range of pathogens and *AVR* gene products with the host *R* gene products and components responsible for orchestrating the HR. While novel genes are constantly being discovered in the model plant *Arabidopsis*, it must be considered that it has no specialised organs of economic value and small genome in comparison with other crop plants. Ultimately, *Arabidopsis* cannot mimic potato. In addition, although *N. benthamiana* is a close relative of potato it cannot mimic potato either and is not a host of *P. infestans*. Therefore, it was important that a system for functionally analysing candidate defence genes during potato-*P. infestans* interactions could be developed. On top of this, it was important that the HR-related genes identified from potato-*P. infestans* SSHs could be analysed in potato where it would be of greater relevance to their resistance role in this

pathosystem. The most successful method of gene silencing in other crop species has been VIGS triggered by cytoplasmic, autonomously replicating viruses have been successfully utilised to modify a range of metabolic pathways (Kumagai *et al.*, 1995), including defence responses (Romeis *et al.*, 2001; Ekengren *et al.*, 2003; Yoshioka *et al.*, 2003). Therefore, an important aim of this project was also to develop a VIGS system for cultivated, tetraploid potatoes used for the original SSH libraries.

VIGS has been successfully employed using a small selection of viruses, e.g. TRV, in a restricted number of hosts, primarily *Nicotiana* species (Ratcliff *et al.*, 2001). However, recent advances on other plant species such as tomato have indicated that TRV may have potential for developing gene silencing systems in other dicots (Dinesh-Kumar *et al.*, 2003; Liu *et al.*, 2002). Therefore TRV was initially tested on micropropagated tetraploid potato cultivars as described in MacFarlane (1996). RT-PCR of the CP on the RNA 2 genome showed that it was not replicating in any of the potato cultivars investigated. Therefore, PVX was tested because it should accumulate to appropriate levels within potato which triggers silencing defence mechanisms without extinguishing viral replication altogether (Ratcliff *et al.*, 1997). PVX expression vector pGR106 was demonstrated to silence *PDS* transcript levels effectively in potato in comparison with other published reports of VIGS. The presence of the *PDS* transcript was reduced by 80 % of control plants in *N. benthamiana*, wild diploid potato *S. bulbocastanum* and cultivated tetraploid *S. tuberosum* cv Bintje, Stirling and Desiree (Faivre-Rampant *et al.* 2004). In addition, PVX-induced silencing of HR-related genes reproduced gene-silencing or gene knockout phenotypes published or observed in other plant species indicating that the silencing of genes other than *PDS* may be sufficient to study gene function. Following *PDS* silencing in *S. tuberosum* cultivars Bintje and Stirling, partial cDNA fragments of candidate HR-related genes were cloned in antisense into PVX vector. Silencing of *SGT1* resulted in a branched and stunted phenotype that has been previously described in *SGT1* silenced *N. benthamiana* by Peart *et al.* (2002). PVX::*CathB*_{as} potatoes developed a sparse and elongated stature, almost the complete converse of PVX::*Sgt1*_{as} plants and phenotype was mirrored in one of the *Arabidopsis CathB* KO mutants (At4g01610). However, after spraying *P. infestans* oospores over

these silenced plants a significant effect on disease phenotypes was not measured. Future work would involve a detached leaf assay that would be performed as described in Vleeshouwers *et al.*, (2000), and/or inoculation of a defined concentration of non-host bacterium such as *E. amylovora* as in Chapter 4 in *N. benthamiana*.

This project allowed the focused functional characterisation of genes of interest in model systems using forward and reverse genetics approaches. Two novel *Arabidopsis* mutants are partially characterised and further research will generate fascinating insights into activated disease susceptibility. The SSH identified many appealing genes that could have been investigated in more detail by developing a high throughput VIGS in *N. benthamiana*. However, in depth analyses of the most interesting genes requires focusing of efforts and resources. Therefore, cathepsin B was found to be the most interesting defence-related gene identified and was investigated successfully in all plant host systems utilised within this collaboration. The requirement for cathepsin B in the HR revealed a structurally conserved cysteine protease in an ancient PCD mechanism shared by plants and animals.

7. References

- Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis*. *Proc Natl Acad Sci U S A*, **95**, 10306-10311.
- Able, A.J. (2003) Role of reactive oxygen species in the response of barley to necrotrophic pathogens. *Protoplasma*, **221**, 137-143.
- Abraham, K.J., Pierce, M.L. and Essenberg, M. (1999) The phytoalexins desoxyhemigossypol and hemigossypol are elicited by *Xanthomonas* in *Gossypium* cotyledons. *Phytochemistry*, **52**, 829-836
- Abraham, M.C. and Shaham, S. (2004) Death without caspases, caspases without death. *Trends Cell Biol*, **14**, 184-193.
- Abramovitch, R.B., Kim, Y.J., Chen, S., Dickman, M.B. and Martin, G.B. (2003) *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *Embo J*, **22**, 60-69.
- Abramovitch, R.B. and Martin, G.B. (2004) Strategies used by bacterial pathogens to suppress plant defenses. *Curr Opin Plant Biol*, **7**, 356-364.
- AGI. (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796-815.
- Alamillo, J.M. and Garcia-Olmedo, F. (2001) Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. *Plant J*, **25**, 529-540.
- Aligue, R., Akhavan-Niak, H. and Russell, P. (1994) A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. *Embo J*, **13**, 6099-6106.
- Allan, A.C. and Fluhr, R. (1997) Two Distinct Sources of Elicited Reactive Oxygen Species in Tobacco Epidermal Cells. *Plant Cell*, **9**, 1559-1572.
- Alonso, J.M., Stepanova, A.N., Lisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmermann, J., Barajas, P., Cheuk, R., Gadriab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, M., Weigel, D., Carter, D.E., Marchand, T., Risseuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C and Ecker, J.R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653-657.
- Alvarez, M.E. (2000) Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant Mol Biol*, **44**, 429-442.
- Angell, S.M. and Baulcombe, D.C. (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *Embo J*, **16**, 3675-3684.
- Asada, K. (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Annual Reviews of Plant Physiology and Plant Molecular Biology*, **50**, 601-639.

- Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J. (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature*, **415**, 977-983.
- Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D. and Parker, J.E. (2002) Regulatory role of SGT1 in early R gene-mediated plant defenses. *Science*, **295**, 2077-2080.
- Avrova, A., Taleb, N., Rokka, V.M., Heilbronn, J., Campbell, E., Hein, I., Gilroy, E.M., Cardle, L., Bradshaw, J.E., Stewart, H.E., Fakim, Y.M., Loake, G and Birch, P.R.J. (2004) Potato oxysterol binding protein and cathepsin B are rapidly up-regulated in defence pathways that distinguish R gene-mediated and field resistances to *Phytophthora infestans*. *Mol Plant Pathol*, **5**, 45-56.
- Avrova, A.O., Stewart, H.E., De Jong, W.D., Heilbronn, J., Lyon, G.D. and Birch, P.R. (1999) A cysteine protease gene is expressed early in resistant potato interactions with *Phytophthora infestans*. *Mol Plant Microbe Interact*, **12**, 1114-1119.
- Avrova, A.O., Venter, E., Birch, P.R. and Whisson, S.C. (2003) Profiling and quantifying differential gene transcription in *Phytophthora infestans* prior to and during the early stages of potato infection. *Fungal Genet Biol*, **40**, 4-14.
- Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K. and Schulze-Lefert, P. (2002) The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. *Science*, **295**, 2073-2076.
- Baker, B., Zambryski, P., Staskawicz, B. and Dinesh-Kumar, S.P. (1997) Signaling in plant-microbe interactions. *Science*, **276**, 726-733.
- Ballvora, A., Ercolano, M.R., Weiss, J., Meksem, K., Bormann, C.A., Oberhagemann, P., Salamini, F. and Gebhardt, C. (2002) The R1 gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *Plant J*, **30**, 361-371.
- Bateman, A. and Bennett, H.P. (1998) Granulins: the structure and function of an emerging family of growth factors. *J Endocrinol*, **158**, 145-151.
- Baulcombe, D. (1999a) Viruses and gene silencing in plants. *Arch Virol Suppl*, **15**, 189-201.
- Baulcombe, D.C. (1999b) Fast forward genetics based on virus-induced gene silencing. *Curr Opin Plant Biol*, **2**, 109-113.
- Baulcombe, D.C. (1999c) Gene silencing: RNA makes RNA makes no protein. *Curr Biol*, **9**, R599-601.
- Baulcombe, D.C., Chapman, S. and Santa Cruz, S. (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J*, **7**, 1045-1053.
- Baunsgaard, L., Fuglsang, A.T., Jahn, T., Korthout, H.A., de Boer, A.H. and Palmgren, M.G. (1998) The 14-3-3 proteins associate with the plant plasma membrane H(+)-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. *Plant J*, **13**, 661-671.
- Baxter-Burrell, A., Yang, Z., Springer, P.S. and Bailey-Serres, J. (2002) RopGAP4-dependent Rop GTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. *Science*, **296**, 2026-2028.
- Beclin, C., Berthome, R., Palauqui, J.C., Tepfer, M. and Vaucheret, H. (1998) Infection of tobacco or *Arabidopsis* plants by CMV counteracts systemic post-transcriptional silencing of nonviral (trans)genes. *Virology*, **252**, 313-317.

- Beers, E.P., Jones, A.M. and Dickerman, A.W. (2004) The S8 serine, C1A cysteine and A1 aspartic protease families in *Arabidopsis*. *Phytochemistry*, **65**, 43-58.
- Beers, E.P. and McDowell, J.M. (2001) Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Curr Opin Plant Biol*, **4**, 561-567.
- Belenghi, B., Acconcia, F., Trovato, M., Perazzolli, M., Bocedi, A., Polticelli, F., Ascenzi, P. and Delledonne, M. (2003) AtCYS1, a cystatin from *Arabidopsis thaliana*, suppresses hypersensitive cell death. *Eur J Biochem*, **270**, 2593-2604.
- Belenghi, B., Salomon, M. and Levine, A. (2004) Caspase-like activity in the seedlings of *Pisum sativum* eliminates weaker shoots during early vegetative development by induction of cell death. *J Exp Bot*, **55**, 889-897.
- Beligni, M.V. and Lamattina, L. (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta*, **210**, 215-221.
- Beligni, M.V. and Lamattina, L. (2002) Nitric oxide interferes with plant photo-oxidative stress by detoxifying reactive oxygen species. *Plant, Cell and Envir*, **25**, 737-748.
- Belkhadir, Y., Subramaniam, R. and Dangl, J.L. (2004) Plant disease resistance protein signaling: NBS-LRR proteins and their partners. *Curr Opin Plant Biol*, **7**, 391-399.
- Bell, K.S., Sebahia, M., Pritchard, L., Holden, M.T., Hyman, L.J., Holeva, M.C., Thomson, N.R., Bentley, S.D., Churcher, L.J., Mungall, K., Atkin, R., Bason, N., Brooks, K., Chillingworth, T., Clark, K., Doggett, J., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Norbertczak, H., Ormond, D., Price, C., Quail, M.A., Sanders, M., Walker, D., Whitehead, S., Salmond, G.P., Birch, P.R., Parkhill, J. and Toth, I.K. (2004) Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *Proc Natl Acad Sci U S A*, **101**, 11105-11110.
- Bendahmane, A., Kohn, B.A., Dedi, C. and Baulcombe, D.C. (1995) The coat protein of potato virus X is a strain-specific elicitor of Rx1-mediated virus resistance in potato. *Plant J*, **8**, 933-941.
- Bent, A.F., Innes, R.W., Ecker, J.R. and Staskawicz, B.J. (1992) Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. *Mol Plant Microbe Interact*, **5**, 372-378.
- Bevan, M., Bennetzen, J.L. and Martienssen, R. (1998) Genome studies and molecular evolution. Commonalities, contrasts, continuity and change in plant genomes. *Curr Opin Plant Biol*, **1**, 101-102.
- Birch, P.R.J. and Whisson, S.C. (2001) *Phytophthora infestans* enters the genomics era. *Mol Plant Pathol*, **2**, 257-263.
- Birch, P.R.J., Avrova, A.O., Armstrong, M., Venter, E., Taleb, N., Gilroy, E.M., Phillips, M.S. and Whisson, S.C. (2003) The potato - *Phytophthora infestans* interaction transcriptome. *Can J Plant Pathol*, **25**, 226-231.
- Birch, P.R.J., Avrova, A.O., Duncan, J.M., Lyon, G.D., and Toth, R.L. (1999) Isolation of potato genes that are induced during an early stage of the hypersensitive response to *Phytophthora infestans*. *Mol Plant Microbe Interact*, **12**, 356-361.

- Bisgrove, S.R., Simonich, M.T., Smith, N.M., Sattler, A. and Innes, R.W. (1994) A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. *Plant Cell*, **6**, 927-933.
- Bittner-Eddy, P.D. and Beynon, J.L. (2001) The *Arabidopsis* downy mildew resistance gene, RPP13-Nd, functions independently of NDR1 and EDS1 and does not require the accumulation of salicylic acid. *Mol Plant Microbe Interact*, **14**, 416-421.
- Blatt, M.R., Grabov, A., Brearley, J., Hammond-Kosack, K. and Jones, J.D. (1999) K⁺ channels of Cf-9 transgenic tobacco guard cells as targets for *Cladosporium fulvum* Avr9 elicitor-dependent signal transduction. *Plant J*, **19**, 453-462.
- Bleeker, A.B., Estelle, M.A., Somerville, S.C. and Kende, H. (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science*, **241**, 1086-1089.
- Boatright, K.M. and Salvesen, G.S. (2003) Mechanisms of caspase activation. *Curr Opin Cell Biol*, **15**, 725-731.
- Bogdan, C., Rollinghoff, M. and Diefenbach, A. (2000) The role of nitric oxide in innate immunity. *Immunol Rev*, **173**, 17-26.
- Bogdanove, A.J. (2002) Protein-protein interactions in pathogen recognition by plants. *Plant Mol Biol*, **50**, 981-989.
- Bogdanove, A.J. and Martin, G.B. (2000) AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. *Proc Natl Acad Sci U S A*, **97**, 8836-8840.
- Bolduc, N., Ouellet, M., Pitre, F. and Brisson, L.F. (2003) Molecular characterization of two plant BI-1 homologues which suppress Bax-induced apoptosis in human 293 cells. *Planta*, **216**, 377-386.
- Bolwell, G.P., Bindschedler, L.V., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., Gerrish, C. and Minibayeva, F. (2002) The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *J Exp Bot*, **53**, 1367-1376.
- Bonas, U. and Lahaye, T. (2002) Plant disease resistance triggered by pathogen-derived molecules: refined models of specific recognition. *Curr Opin Microbiol*, **5**, 44-50.
- Bortner, C.D., Hughes, F.M., Jr. and Cidlowski, J.A. (1997) A primary role for K⁺ and Na⁺ efflux in the activation of apoptosis. *J Biol Chem*, **272**, 32436-32442.
- Botella, M.A., Parker, J.E., Frost, L.N., Bittner-Eddy, P.D., Beynon, J.L., Daniels, M.J., Holub, E.B. and Jones, J.D. (1998) Three genes of the *Arabidopsis* RPP1 complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell*, **10**, 1847-1860.
- Boter, M., Ruiz-Rivero, O., Abdeen, A. and Prat, S. (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. *Genes Dev*, **18**, 1577-1591.
- Bouarab, K., Melton, R., Peart, J., Baulcombe, D. and Osbourn, A. (2002) A saponin-detoxifying enzyme mediates suppression of plant defences. *Nature*, **418**, 889-892.
- Boucher, C., Gobeil, S., Samejima, K., Earnshaw, W.C. and Poirier, G.G. (2001) Identification and analysis of caspase substrates: proteolytic cleavage of poly(ADP-ribose)polymerase and DNA fragmentation factor 45. *Methods Cell Biol*, **66**, 289-306.

- Bowler, C. Van Montagu, M. and Inzé, D. (1992) Superoxide dismutases and stress tolerance. *Ann Rev of Plant Physiol and Plant Mol Biol*, **43**, 83-116.
- Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F. and Dong, X. (1994) A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *Plant Cell*, **6**, 1845-1857.
- Bozhkov, P.V., Filonova, L.H., Suarez, M.F., Helmersson, A., Smertenko, A.P., Zhivotovsky, B. and von Arnold, S. (2004) VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation. *Cell Death Differ*, **11**, 175-182.
- Brigneti, G., Martin-Hernandez, A.M., Jin, H., Chen, J., Baulcombe, D.C., Baker, B. and Jones, J.D. (2004) Virus-induced gene silencing in *Solanum* species. *Plant J*, **39**, 264-272.
- Brookes, P.S., Salinas, E.P., Darley-Usmar, K., Eiserich, J.P., Freeman, B.A., Darley-Usmar, V.M. and Anderson, P.G. (2000) Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome c release. *J Biol Chem*, **275**, 20474-20479.
- Brown, J.K. (2002) Yield penalties of disease resistance in crops. *Curr Opin Plant Biol*, **5**, 339-344.
- Bryan, G.T., Wu, K.S., Farrall, L., Jia, Y., Hershey, H.P., McAdams, S.A., Faulk, K.N., Donaldson, G.K., Tarchini, R. and Valent, B. (2000) A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene Pi-ta. *Plant Cell*, **12**, 2033-2046.
- Buell, C.R. (1998) *Arabidopsis*: A weed leading the field of plant-pathogen interactions. *Plant Phys Biochem*, **36**, 177-186.
- Burton, R.A., Gibeaut, D.M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D.C. and Fincher, G.B. (2000) Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell*, **12**, 691-706.
- Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994) Characterisation of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell*, **6**, 1583-1592.
- Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X. (1997) The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell*, **88**, 57-63.
- Cardinale, F., Meskiene, I., Ouaked, F. and Hirt, H. (2002) Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. *Plant Cell*, **14**, 703-711.
- Cecconi, F. (1999) Apaf1 and the apoptotic machinery. *Cell Death Differ*, **6**, 1087-1098.
- Century, K.S., Holub, E.B. and Staskawicz, B.J. (1995) *NDR1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc Natl Acad Sci USA*, **92**, 6597-6601.
- Cessna, S.G. and Low, P.S. (2001) Activation of the oxidative burst in aequorin-transformed *Nicotiana tabacum* cells is mediated by protein kinase- and anion channel-dependent release of Ca²⁺ from internal stores. *Planta*, **214**, 126-134.
- Chae, H.S., Faure, F. and Kieber, J.J. (2003) The *eto1*, *eto2*, and *eto3* mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. *Plant*

Cell, **15**, 545-559.

- Chalhoub, B., Belcram, H. and Caboche M. (2004) Efficient cloning of plant genomes into bacterial artificial chromosome (BAC) libraries with larger and more uniform insert size. *Plant Biotechnol. J*, **2**, 181-188.
- Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H., Jr., Van Montagu, M., Inze, D. and Van Camp, W. (1998) Defense activation and enhanced pathogen tolerance induced by H₂O₂ in transgenic tobacco. *Proc Natl Acad Sci U S A*, **95**, 5818-5823.
- Champion, A., Picaud, A. and Henry, Y. (2004) Reassessing the MAP3K and MAP4K relationships. *Trends Plant Sci*, **9**, 123-129.
- Chandok, M.R., Ytterberg, A.J., van Wijk, K.J. and Klessig, D.F. (2003) The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. *Cell*, **113**, 469-482.
- Chang, H.Y. and Yang, X. (2000) Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev*, **64**, 821-846.
- Chapman, S., Kavanagh, T. and Baulcombe, D. (1992) Potato virus X as a vector for gene expression in plants. *Plant J*, **2**, 549-557.
- Chen, Z., Kloek, A.P., Boch, J., Katagiri, F. and Kunkel, B.N. (2000) The *Pseudomonas syringae* avrRpt2 gene product promotes pathogen virulence from inside plant cells. *Mol Plant Microbe Interact*, **13**, 1312-1321.
- Chen, Z., Kloek, A.P., Cuzick, A., Moeder, W., Tang, D., Innes, R.W., Klessig, D.F., McDowell, J.M. and Kunkel, B.N. (2004) The *Pseudomonas syringae* type III effector AvrRpt2 functions downstream or independently of SA to promote virulence on *Arabidopsis thaliana*. *Plant J*, **37**, 494-504.
- Chichkova, N.V., Kim, S.H., Titova, E.S., Kalkum, M., Morozov, V.S., Rubtsov, Y.P., Kalinina, N.O., Taliansky, M.E. and Vartapetian, A.B. (2004) A plant caspase-like protease activated during the hypersensitive response. *Plant Cell*, **16**, 157-171.
- Chini, A., Grant, J.J., Seki, M., Shinozaki, K. and Loake, G.J. (2004) Drought tolerance established by enhanced expression of the CC-NBS-LRR gene, ADR1, requires salicylic acid, EDS1 and ABI1. *Plant J*, **38**, 810-822.
- Clarke, A., Desikan, R., Hurst, R.D., Hancock, J.T. and Neill, S.J. (2000) NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J*, **24**, 667-677.
- Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*, **16**, 735-743.
- Clough, S.J., Fessler, K.A., Yu, I.C., Lippok, B., Smith, R.K., Jr. and Bent, A.F. (2000) The *Arabidopsis* dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. *Proc Natl Acad Sci U S A*, **97**, 9323-9328.
- Coffeen, W.C. and Wolpert, T.J. (2004) Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*. *Plant Cell*, **16**, 857-873.

- Cogoni, C. and Macino, G. (2000) Post-transcriptional gene silencing across kingdoms. *Curr Opin Genet Dev*, **10**, 638-643.
- Conklin, P.L., Williams, E.H. and Last, R.L. (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. *Proc Natl Acad Sci U S A*, **93**, 9970-9974.
- Cook, R.J. (1998) Toward a successful multinational crop plant genome initiative. *Proc Natl Acad Sci U S A*, **95**, 1993-1995.
- Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G. and Brasier, G.M. (2000) A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genet and Biol*, **30**, 17-32.
- Corpas, F.J., Barroso, J.B. and del Rio, L.A. (2001) Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends Plant Sci*, **6**, 145-150.
- Cote, F. and Hahn, M.G. (1994) Oligosaccharins: structures and signal transduction. *Plant Mol Biol*, **26**, 1379-1411.
- Creelman, R.A. and Mullet, J.E. (1997) Biosynthesis and Action of Jasmonates in Plants. *Annu Rev Plant Physiol Plant Mol Biol*, **48**, 355-381.
- Creissen, G., Firmin, J., Fryer, M., Kular, B., Leyland, N., Reynolds, H., Pastori, G., Wellburn, F., Baker, N., Wellburn, A. and Mullineaux, P. (1999) Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *Plant Cell*, **11**, 1277-1292.
- Crute, I.R. and Pink, D. (1996) Genetics and Utilization of Pathogen Resistance in Plants. *Plant Cell*, **8**, 1747-1755.
- Cui, J., Bahrami, A.K., Pringle, E.G., Hernandez-Guzman, G., Bender, C.L., Pierce, N.E. and Ausubel, F.M. (2005) *Pseudomonas syringae* manipulates systemic plant defences against pathogens and herbivores. *Proc Natl Acad Sci USA*, **102**, 1791-1796.
- Czempinski, K., Frachisse, J.M., Maurel, C., Barbier-Brygoo, H. and Mueller-Roeber, B. (2002) Vacuolar membrane localization of the *Arabidopsis* 'two-pore' K⁺ channel KCO1. *Plant J*, **29**, 809-820.
- D'Amours, D., Desnoyers, S., D'Silva, I. and Poirier, G.G. (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J*, **342** (Pt 2), 249-268.
- D'Silva, I., Poirier, G.G. and Heath, M.C. (1998) Activation of cysteine proteases in cowpea plants during the hypersensitive response--a form of programmed cell death. *Exp Cell Res*, **245**, 389-399.
- Dale, P.J., Clarke, B. and Fontes, E.M. (2002) Potential for the environmental impact of transgenic crops. *Nat Biotechnol*, **20**, 567-574.
- Dangl, J.L. and Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826-833.
- Daniell, H. (2002) Molecular strategies for gene containment in transgenic crops. *Nat Biotechnol*, **20**, 581-586.
- Dat, J.F., Pellinen, R., Beeckman, T., Van De Cotte, B., Langebartels, C., Kangasjarvi, J., Inze, D. and Van Breusegem, F. (2003) Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. *Plant J*, **33**, 621-632.

- Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*, **51**, 987-1000.
- Dean, J.V. and Harper, J.E. (1988) The conversion of nitrite to nitrogen oxide(s) by the constitutive NAD(P)H- nitrate reductase enzyme from soybean. *Plant Physiology*, **88**, 389-395.
- del Pozo, O. and Lam, E. (1998) Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Curr Biol*, **8**, 1129-1132.
- del Pozo, O., Pedley, K.F. and Martin, G.B. (2004) MAPKKK α is a positive regulator of cell death associated with both plant immunity and disease. *EMBO J*, **23**, 3072-3082.
- del Rio, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M., Gomez, M. and Barroso, J.B. (2002) Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *J Exp Bot*, **53**, 1255-1272.
- Delaney, T.P. (1997) Genetic dissection of acquired resistance to disease. *Plant Physiol*, **113**, 5-12.
- Delaney, T.P. (2000) New mutants provide clues into regulation of systemic acquired resistance. *Trends Plant Sci*, **5**, 49-51.
- Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gutrella, M., Kessmann, H., Ward, E. and Ryals, J. (1994) A Central Role of Salicylic Acid in Plant Disease Resistance. *Science*, **266**, 1247-1249.
- Delledonne, M., Polverari, A. and Murgia, I. (2003) The functions of nitric oxide-mediated signaling and changes in gene expression during the hypersensitive response. *Antioxid Redox Signal*, **5**, 33-41.
- Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature*, **394**, 585-588.
- Delledonne, M., Zeier, J., Marocco, A. and Lamb, C. (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc Natl Acad Sci U S A*, **98**, 13454-13459.
- Dempsey DA, Shah, J. and Klessig D.F. (1999) Salicylic acid and disease resistance in plants. *Crit Rev Plant Sci*, **18**, 547-575.
- Dennis, C. and Surridge, C. (2000) *Arabidopsis thaliana* genome. Introduction. *Nature*, **408**, 791.
- Desikan, R., Clarke, A., Atherfold, P., Hancock, J.T. and Neill, S.J. (1999) Harpin induces mitogen-activated protein kinase activity during defence responses in *Arabidopsis thaliana* suspension cultures. *Planta*, **210**, 97-103.
- Devlin, P.F., Somers, D.E., Quail, P.H. and Whitelam, G.C. (1997) The Brassica rapa elongated internode (EIN) gene encodes phytochrome B. *Plant Mol Biol*, **34**, 537-547.
- Dickman, M.B., Park, Y.K., Oltersdorf, T., Li, W., Clemente, T. and French, R. (2001) Abrogation of disease development in plants expressing animal antiapoptotic genes. *Proc Natl Acad Sci U S A*, **98**, 6957-6962.
- Diekmann, D., Abo, A., Johnston, C., Segal, A.W. and Hall, A. (1994) Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. *Science*, **265**, 531-533.

- Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A. and Dangl, J.L. (1994) *Arabidopsis* mutants simulating disease resistance response. *Cell*, **77**, 565-577.
- Dill, A. and Sun, T. Synergistic derepression of gibberellin signaling by removing RGA and GAI function in *Arabidopsis thaliana*. *Genetics*, **159**, 777-785.
- Dinesh-Kumar, S.P., Anandalakshmi, R., Marathe, R., Schiff, M. and Liu, Y. (2003) Virus-induced gene silencing. *Methods Mol Biol*, **236**, 287-294.
- Dixon, M.S., Hatzixanthis, K., Jones, D.A., Harrison, K. and Jones, J.D. (1998) The tomato Cf-5 disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. *Plant Cell*, **10**, 1915-1925.
- Doares, S.H., Narvaez-Vasquez, J., Conconi, A. and Ryan, C.A. (1995) Salicylic Acid Inhibits Synthesis of Proteinase Inhibitors in Tomato Leaves Induced by Systemin and Jasmonic Acid. *Plant Physiol*, **108**, 1741-1746.
- Doke, N. (1975) Prevention of the hypersensitive reaction of potato cells to infection with an incompatible race of *Phytophthora infestans* by constituents of the zoospores. *Physiol Plant Pathol*, **7**, 1-7.
- Doke, N. (1983) Involvement of superoxide anion generation in hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to hyphal wall components. *Physiol Plant Pathol*, **23**, 345-357.
- Doke, N., Miura, Y., Sanchez, L.M., Park, H.J., Noritake, T., Yoshioka, H. and Kawakita, K. (1996) The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defence--a review. *Gene*, **179**, 45-51.
- Dong, X. (1998) SA, JA, ethylene, and disease resistance in plants. *Curr Opin Plant Biol*, **1**, 316-323.
- Dong, X. (2004) NPR1, all things considered. *Curr Opin Plant Biol*, **7**, 547-552.
- Doyle, J.J. and Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13-15.
- Draper, J. (1997) Salicylate, superoxide synthesis and cell suicide in plant defence. *Trends Plant Sci*, **2**, 162-165.
- Drapier, J.C., Wietzerbin, J. and Hibbs, J.B., Jr. (1988) Interferon-gamma and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. *Eur J Immunol*, **18**, 1587-1592.
- Drenth, A., Janssen, E.M. and Govers F. (1995) Formation and Survival of oospores of *Phytophthora infestans* Under Natural Conditions. *Plant Pathol*, **44**, 86-94.
- Du, L. and Poovaiah, B.W. (2004) A novel family of Ca²⁺/calmodulin-binding proteins involved in transcriptional regulation: interaction with fsh/Ring3 class transcription activators. *Plant Mol Biol*, **54**, 549-569.
- Duncan, J.M. *Phytophthora*-an abiding threat to our crops. *Microbiology Today*, **26**, 114-116.
- Duriez, P.J. and Shah, G.M. (1997) Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem Cell Biol*, **75**, 337-349.
- Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide,

cyclic GMP, and cyclic ADP-ribose. *Proc Natl Acad Sci U S A*, **95**, 10328-10333.

- Dyer, J.H., Zheng, S. and Wang, X. (1996) Structural heterogeneity of phospholipase D in 10 dicots. *Biochem. Biophys. Res. Commun.*, **221**, 31-36.
- Earnshaw, W.C., Martins, L.M. and Kaufmann, S.H. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem*, **68**, 383-424.
- Ebél, J. (1998) Oligoglucoside elicitor-mediated activation of plant defense. *Bioessays*, **20**, 569-576.
- Eckardt, N.A. (2002) Plant disease susceptibility genes? *Plant Cell*, **14**, 1983-1986.
- Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P. and Martin, G.B. (2003) Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. *Plant J*, **36**, 905-917.
- Ellis, J., Dodds, P. and Pryor, T. (2000) Structure, function and evolution of plant disease resistance genes. *Curr Opin Plant Biol*, **3**, 278-284.
- Faivre-Rampant, O., Gilroy, E.M., Hrubikova, K., Hein, I., Millam, S., Loake, G.J., Birch, P.R., Taylor, M. and Lacomme, C. (2004) Potato virus X-induced gene silencing in leaves and tubers of potato. *Plant Physiol*, **134**, 1308-1316.
- Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J. and Parker, J.E. (1999) EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proc Natl Acad Sci U S A*, **96**, 3292-3297.
- Fankhauser, C. and Casal, J.J. (2004) Phenotypic characterisation of a photomorphogenic mutant. *Plant J*, **39**, 747-760.
- Feys, B., Benedetti, C.E., Penfold, C.N. and Turner, J.G. (1994) *Arabidopsis* Mutants Selected for Resistance to the Phytotoxin Coronatine are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. *Plant Cell*, **6**, 751-759.
- Feys, B.J., Moisan, L.J., Newman, M.A. and Parker, J.E. (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. *Embo J*, **20**, 5400-5411.
- Feys, B.J. and Parker, J.E. (2000) Interplay of signaling pathways in plant disease resistance. *Trends Genet*, **16**, 449-455.
- Flor, H.H. (1956) Host-parasite interaction in flax rust-Its genetics and other implications. *Phytopathology*, **45**, 680-685.
- Flor, H.H. (1971) Current status of the gene-for-gene concept. *Ann Rev of Phytopathol*, **9**, 275-296.
- Föluhr, R. (2001) Sentinels of disease. Plant resistance genes. *Plant Physiol*, **127**, 1367-1374.
- Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M. and Jaattela, M. (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol*, **153**, 999-1010.
- Förster, H., and Coffey, M. D. (1990) Mating-Behavior of *Phytophthora-Parasitica* - Evidence for Sexual Recombination in Oospores Using DNA Restriction Fragment Length Polymorphisms as Genetic Markers. *Exp Mycol*, **14**, 351-359.

- Freialdenhoven, A., Scherag, B., Hollricher, K., Collinge, D.B., Thordal-Christensen, H. and Schulze-Lefert, P. (1994) Nar-1 and Nar-2, Two Loci Required for Mla12-Specified Race-Specific Resistance to Powdery Mildew in Barley. *Plant Cell*, **6**, 983-994.
- Friedrich, L., Vernooij, B., Gaffney, T., Morse, A. and Ryals, J. (1995) Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Mol Biol*, **29**, 959-968.
- Fry, W.E., Drenth, A., Spielman, L.J., Mantel, B.C., Davidse, L.C. and Goodwin, S.B. (1991) Population genetic structure of *Phytophthora infestans* in the Netherlands. *Phytopathology*, **81**, 1330-1336.
- Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J., Milgroom, M.G. and Drenth, A. (1992) Population genetics and intercontinental migrations of *Phytophthora infestans*. *Ann Rev in Phytopathol*, **30**, 107-129.
- Fry, W.E., Goodwin, S.B., Dyer A.T., Matuszak J.M., Drenth A, Tooley P.W., Sujkowski L.S., Koh Y.J., Cohen B.A., Spielman L.J., Deahl K.L., Inglis D.A. and Sandlan K.P. (1993) Historical and Recent Migrations of *Phytophthora infestans* - Chronology, Pathways, and Implications. *Plant Disease*, **77**, 653-661.
- Fry, W.E. and Goodwin, S.B. (1997) Resurgence of the Irish potato famine fungus. *Bioscience*, **47**, 373-370.
- Frye, C.A. and Innes, R.W. (1998) An *Arabidopsis* mutant with enhanced resistance to powdery mildew. *Plant Cell*, **10**, 947-956.
- Frye, C.A., Tang, D. and Innes, R.W. (2001) Negative regulation of defense responses in plants by a conserved MAPKK kinase. *Proc Natl Acad Sci U S A*, **98**, 373-378.
- Fu, H., Doelling, J.H., Rubin, D.M. and Vierstra, R.D. (1999) Structural and functional analysis of the six regulatory particle triple-A ATPase subunits from the *Arabidopsis* 26S proteasome. *Plant J*, **18**, 529-539.
- Garrido-Ramirez, E.R., Sudarshana, M.R., Lucas, W.J. and Gilbertson, R.L. (2000) Bean dwarf mosaic virus BV1 protein is a determinant of the hypersensitive response and avirulence in *Phaseolus vulgaris*. *Mol Plant Microbe Interact*, **13**, 1184-1194.
- Gebhardt, C. and Valkonen, J.P. (2001) Organization of genes controlling disease resistance in the potato genome. *Annu Rev Phytopathol*, **39**, 79-102.
- Gelli, A. and Blumwald, E. (1997) Hyperpolarization-activated Ca²⁺-permeable channels in the plasma membrane of tomato cells. *J Membr Biol*, **155**, 35-45.
- Glazebrook, J. (2001) Genes controlling expression of defense responses in *Arabidopsis*--2001 status. *Curr Opin Plant Biol*, **4**, 301-308.
- Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T. and Katagiri, F. (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J*, **34**, 217-228.
- Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A. and Finley, D. (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell*, **94**, 615-623.

- Gobeil, S., Boucher, C.C., Nadeau, D. and Poirier, G.G. (2001) Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Differ*, **8**, 588-594.
- Gobel, C., Feussner, I., Schmidt, A., Scheel, D., Sanchez-Serrano, J., Hamberg, M. and Rosahl, S. (2001) Oxylinin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. *J Biol Chem*, **276**, 6267-6273.
- Gomez-Cambronero, J. and Keire, P. (1998) Phospholipase D: a novel major player in signal transduction. *Cell Signal*, **10**, 387-397.
- Goodwin, S.B., Cohen, B.A. and Fry, W.E. (1994) Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. *Proc Natl Acad Sci U S A*, **91**, 11591-11595.
- Görg, R., Hollricher, K., Schulze-Lefert, P. (1993) Functional analysis and RFLP-mediated mapping of the *Mlg* resistance locus in barley. *Plant J.*, **3**, 857-866
- Gorren, A.C., Schrammel, A., Schmidt, K. and Mayer, B. (1996) Decomposition of S-nitrosoglutathione in the presence of copper ions and glutathione. *Arch Biochem Biophys*, **330**, 219-228.
- Govrin, E.M. and Levine, A. (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr Biol*, **10**, 751-757.
- Grant, J.J., Chini, A., Basu, D. and Loake, G.J. (2003) Targeted activation tagging of the *Arabidopsis* NBS-LRR gene, ADR1, conveys resistance to virulent pathogens. *Mol Plant Microbe Interact*, **16**, 669-680.
- Grant, J.J. and Loake, G.J. (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol*, **124**, 21-29.
- Grant, J.J., Yun, B.W. and Loake, G.J. (2000) Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *Plant J*, **24**, 569-582.
- Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A. and Mansfield, J. (2000) The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *Plant J*, **23**, 441-450.
- Grant, M. and Mansfield, J. (1999) Early events in host-pathogen interactions. *Curr Opin Plant Biol*, **2**, 312-319.
- Greenberg, J.T. and Yao, N. (2004) The role and regulation of programmed cell death in plant-pathogen interactions. *Cell Microbiol*, **6**, 201-211.
- Greenberg, J.T. (2005) Degrade or die: a dual function for autophagy in the plant immune response. *Dev Cell*, **8**, 799-801
- Guicciardi, M.E., Miyoshi, H., Bronk, S.F. and Gores, G.J. (2001) Cathepsin B knockout mice are resistant to tumour necrosis factor-alpha-mediated hepatocyte apoptosis and liver injury: implications for therapeutic applications. *Am J Pathol*. **59**, 2045-2054.
- Guo, F.Q., Okamoto, M. and Crawford, N.M. (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science*, **302**, 100-103.

- Guo, H and Ecker, J.R. (2004) The ethylene signalling pathway: new insights. *Curr Opin Plant Biol*, **7**, 40-49.
- Gupta, R. and Luan, S. (2003) Redox control of protein tyrosine phosphatases and mitogen-activated protein kinases in plants. *Plant Physiol*, **132**, 1149-1152.
- Guzman, P. and Ecker, J.R. (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell*, **2**, 513-523.
- Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, **286**, 950-952.
- Hammond-Kosack, K.E. and Jones, J.D. (1996) Resistance gene-dependent plant defense responses. *Plant Cell*, **8**, 1773-1791.
- Hammond-Kosack, K.E. and Jones, J.D. (1997) Plant Disease Resistance Genes. *Annu Rev Plant Physiol Plant Mol Biol*, **48**, 575-607.
- Hammond-Kosack, K.E., Tang, S., Harrison, K. and Jones, J.D. (1998) The tomato Cf-9 disease resistance gene functions in tobacco and potato to confer responsiveness to the fungal avirulence gene product avr 9. *Plant Cell*, **10**, 1251-1266.
- Hass, C., Lohrmann, J., Albrecht, V., Sweere, U., Hummel, F., Yoo, S.D., Hwang, I., Zhu, T., Schafer, E., Kudla, J. and Harter, K. (2004) The response regulator 2 mediates ethylene signalling and hormone signal integration in *Arabidopsis*. *Embo J*, **23**, 3290-3302.
- Hatsugai, N., Kuroyanagi, M., Yamada, K., Meshi, T., Tsuda, S., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (2004) A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science*, **305**, 855-858.
- Hauck, P., Thilmony, R. and He, S.Y. (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc Natl Acad Sci U S A*, **100**, 8577-8582.
- Hayashi, Y., Yamada, K., Shimada, T., Matsushima, R., Nishizawa, N.K., Nishimura, M. and Hara-Nishimura, I. (2001) A proteinase-storing body that prepares for cell death or stresses in the epidermal cells of *Arabidopsis*. *Plant Cell Physiol*, **42**, 894-899.
- Heath, M.C. (2000a) Hypersensitive response-related death. *Plant Mol Biol*, **44**, 321-334.
- Heath, M.C. (2000b) Nonhost resistance and nonspecific plant defenses. *Curr Opin Plant Biol*, **3**, 315-319.
- Heil, M. and Baldwin, I.T. (2002) Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends Plant Sci*, **7**, 61-67.
- Heil, M. and Bostock, R.M. (2002) Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Ann Bot (Lond)*, **89**, 503-512.
- Hein, I., Williamson, S., Russell, J. and Powell, W. (2005) Isolation of high molecular weight DNA suitable for BAC library construction from woody perennial soft-fruit species. *BioTech*, **38**, 69-71

- Hein, I., Campbell, E.I., Woodhead, M., Hedley, P.E., Young, V., Morris, W.L., Ramsay, L., Stockhaus, J., Lyon, G.D., Newton, A.C. and Birch, P.R. (2004) Characterisation of early transcriptional changes involving multiple signalling pathways in the Mla13 barley interaction with powdery mildew (*Blumeria graminis* f. sp. *hordei*). *Planta*, **218**, 803-813.
- Hellens, R.P., Edwards, A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol Bio*, **42**, 819-832.
- Henzler, T. and Steudle, E. (2000) Transport and metabolic degradation of hydrogen peroxide in *Chara corallina*: model calculations and measurements with the pressure probe suggest transport of H₂O₂ across water channels. *J Exp Bot*, **51**, 2053-2066.
- Hoeberichts, F.A., ten Have, A. and Woltering, E.J. (2003) A tomato metacaspase gene is upregulated during programmed cell death in *Botrytis cinerea*-infected leaves. *Planta*, **217**, 517-522.
- Hoisington, D., Khairallah, M., Reeves, T., Ribaut, J.M., Skovmand, B., Taba, S. and Warburton, M. (1999) Plant genetic resources: what can they contribute toward increased crop productivity? *Proc Natl Acad Sci U S A*, **96**, 5937-5943.
- Holub, E.B. (2001) The arms race is ancient history in *Arabidopsis*, the wildflower. *Nat Rev Genet*, **2**, 516-527.
- Huang, C., Ni, Y., Wang, T., Gao, Y., Haudenschild, C.C. and Zhan, X. (1997) Down-regulation of the filamentous actin cross-linking activity of cortactin by Src-mediated tyrosine phosphorylation. *J Biol Chem*, **272**, 13911-13915.
- Hubert, D.A., Tornero, P., Belkhadir, Y., Krishna, P., Takahashi, A., Shirasu, K. and Dangl, J.L. (2003) Cytosolic HSP90 associates with and modulates the *Arabidopsis* RPM1 disease resistance protein. *Embo J*, **22**, 5679-5689.
- Huckelhoven, R., Dechert, C. and Kogel, K.H. (2003) Overexpression of barley BAX inhibitor 1 induces breakdown of mlo-mediated penetration resistance to *Blumeria graminis*. *Proc Natl Acad Sci U S A*, **100**, 5555-5560.
- Hwang, J.U., Suh, S., Yi, H., Kim, J. and Lee, Y. (1997) Actin Filaments Modulate Both Stomatal Opening and Inward K⁺-Channel Activities in Guard Cells of *Vicia faba* L. *Plant Physiol*, **115**, 335-342.
- Ichikawa, T., Nakazawa, M., Kawashima, M., Muto, S., Gohda, K., Suzuki, K., Ishikawa, A., Kobayashi, H., Yoshizumi, T., Tsumoto, Y., Tsumura, Y., Iizumi, H., Goto, Y. and Matsui, M. (2003) Sequence database of 1172 T-DNA insertion sites in *Arabidopsis* activation-tagging lines that showed phenotypes in T1 generation. *Plant J*, **36**, 421-429.
- Ishitani, M., Liu, J., Halfter, U., Kim, C.S., Shi, W. and Zhu, J.K. (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. *Plant Cell*, **12**, 1667-1678.
- Jabs, T., Dietrich, R.A. and Dangl, J.L. (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science*, **273**, 1853-1856.
- Jabs, T., Tschope, M., Colling, C., Hahlbrock, K. and Scheel, D. (1997) Elicitor-stimulated ion fluxes and O₂⁻ from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Natl Acad Sci U S A*, **94**, 4800-4805.

- Jabs, T. (1999) Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem. Pharmacol.*, **57**, 231-245.
- Jensen, A.B., Raventos, D. and Mundy, J. (2002) Fusion genetic analysis of jasmonate-signalling mutants in *Arabidopsis*. *Plant J*, **29**, 595-606.
- Jeong, D.H., An, S., Kang, H.G., Moon, S., Han, J.J., Park, S., Lee, H.S., An, K. and An, G. (2002) T-DNA insertional mutagenesis for activation tagging in rice. *Plant Physiol*, **130**, 1636-1644.
- Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M. and Glazebrook, J. (1999) *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc Natl Acad Sci U S A*, **96**, 13583-13588.
- Johnson, G.V. and Guttman, R.P. (1997) Calpains: intact and active? *Bioessays*, **19**, 1011-1018.
- Johnson, P.R. and Ecker, J.R. (1998) The ethylene gas signal transduction pathway: a molecular perspective. *Annu Rev Genet*, **32**, 227-254.
- Jonak, C., Okresz, L., Bogre, L. and Hirt, H. (2002) Complexity, cross talk and integration of plant MAP kinase signalling. *Curr Opin Plant Biol*, **5**, 415-424.
- Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J. and Jones, J.D. (1994) Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science*, **266**, 789-793.
- Jones, J.D. (1994) Plant pathology. Resistance crumbles? *Curr Biol*, **4**, 67-69.
- Jones, J.D. (2001) Putting knowledge of plant disease resistance genes to work. *Curr Opin Plant Biol*, **4**, 281-287.
- Jones, L., Hamilton, A.J., Voinnet, O., Thomas, C.L., Maule, A.J. and Baulcombe, D.C. (1999) RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell*, **11**, 2291-2301.
- Joza, N., Kroemer, G. and Penninger, J.M. (2002) Genetic analysis of the mammalian cell death machinery. *Trends Genet*, **18**, 142-149.
- Kadota, Y., Goh, T., Tomatsu, H., Tamauchi, R., Higashi, K., Muto, S. and Kuchitsu, K. (2004) Cryptogein-induced initial events in tobacco BY-2 cells: pharmacological characterization of molecular relationship among cytosolic Ca²⁺ transients, anion efflux and production of reactive oxygen species. *Plant Cell Physiol*, **45**, 160-170.
- Kamoun, S. (2001) Nonhost resistance to *Phytophthora*: novel prospects for a classical problem. *Curr Opin Plant Biol*, **4**, 295-300.
- Kamoun, S., Huitema, E. and Vleeshouwers, V.G. (1999) Resistance to oomycetes: a general role for the hypersensitive response? *Trends Plant Sci*, **4**, 196-200.
- Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. and Bredesen, D.E. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, **262**, 1274-1277.
- Kaufmann, S.H., Desnoyers, S., Ottaviano, Y., Davidson, N.E. and Poirier, G.G. (1993) Specific cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. *Cancer*

Res, **53**, 3976-3985.

- Kawai-Yamada, M., Jin, L., Yoshinaga, K., Hirata, A. and Uchimiya, H. (2001) Mammalian Bax-induced plant cell death can be down-regulated by overexpression of *Arabidopsis* Bax Inhibitor-1 (AtBI-1). *Proc Natl Acad Sci U S A*, **98**, 12295-12300.
- Kawai-Yamada, M., Ohori, Y. and Uchimiya, H. (2004) Dissection of *Arabidopsis* Bax inhibitor-1 suppressing Bax-, hydrogen peroxide-, and salicylic acid-induced cell death. *Plant Cell*, **16**, 21-32.
- Kawano, T. (2003) Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Rep*, **21**, 829-837.
- Kearney, B. and Staskawicz, B.J. (1990) Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*. *Nature*, **346**, 385-386.
- Keen, N.T. (1975) Specific elicitors of plant phytoalexin production- Determination of race specificity in pathogens. *Science*, **187**, 74-75.
- Keen, N.T. (1992) The molecular biology of disease resistance. *Plant Mol Biol*, **19**, 109-122.
- Keller, H., Pamboukdjian, N., Ponchet, M., Poupet, A., Delon, R., Verrier, J.L., Roby, D. and Ricci, P. (1999) Pathogen-induced elicitor production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. *Plant Cell*, **11**, 223-235.
- Keller, T., Damude, H.G., Werner, D., Doerner, P., Dixon, R.A. and Lamb, C. (1998) A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca²⁺ binding motifs. *Plant Cell*, **10**, 255-266.
- Kim, C.Y. and Zhang, S. (2004) Activation of a mitogen-activated protein kinase cascade induces WRKY family of transcription factors and defense genes in tobacco. *Plant J*, **38**, 142-151.
- Kim, J.S., Kim, Y.O., Ryu, H.J., Kwak, Y.S., Lee, J.Y. and Kang, H. (2003) Isolation of stress-related genes of rubber particles and latex in fig tree (*Ficus carica*) and their expressions by abiotic stress or plant hormone treatments. *Plant Cell Physiol*, **44**, 412-414.
- Kim, Y.M., Talanian, R.V. and Billiar, T.R. (1997) Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J Biol Chem*, **272**, 31138-31148.
- King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med*, **44**, 301-307.
- Kingham, P.J. and Pocock, J.M. (2001) Microglial secreted cathepsin B induces neuronal apoptosis. *J Neurochem*, **76**, 1475-1484.
- Kinoshita, T., Yamada, K., Hiraiwa, N., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (1999) Vacuolar processing enzyme is up-regulated in the lytic vacuoles of vegetative tissues during senescence and under various stressed conditions. *Plant J*, **19**, 43-53.
- Kitagawa, K., Skowyra, D., Elledge, S.J., Harper, J.W. and Hieter, P. (1999) SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. *Mol Cell*, **4**, 21-33.
- Kliebenstein, D.J., Dietrich, R.A., Martin, A.C., Last, R.L. and Dangl, J.L. (1999) LSD1 regulates

salicylic acid induction of copper zinc superoxide dismutase in *Arabidopsis thaliana*. *Mol Plant Microbe Interact*, **12**, 1022-1026.

- Knight, H. and Knight, M.R. (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci*, **6**, 262-267.
- Kong, J., Chia, L., Goh, N., Chia, T. and Brouillard, R. (2003) Analysis and biological activities of anthocyanins. *Phytochemistry*, **64**, 923-933
- Kroj, T., Rudd, J.J., Nurnberger, T., Gabler, Y., Lee, J. and Scheel, D. (2003) Mitogen-activated protein kinases play an essential role in oxidative burst-independent expression of pathogenesis-related genes in parsley. *J Biol Chem*, **278**, 2256-2264.
- Krüger, J., Thomas, C.M., Golstein, C., Dixon, M.S., Smoker, M., Tang, S., Mulder, L. and Jones, J.D. (2002) A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. *Science*, **296**, 744-747.
- Kumagai, M.H., Donson, J., della-Cioppa, G., Harvey, D., Hanley, K. and Grill, L.K. (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc Natl Acad Sci U S A*, **92**, 1679-1683.
- Kunkel, B.N. and Brooks, D.M. (2002) Cross talk between signaling pathways in pathogen defense. *Curr Opin Plant Biol*, **5**, 325-331.
- Kurihara, Y. and Watanabe, Y. (2004) *Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc Natl Acad Sci U S A*, **101**, 12753-12758.
- Lacomme, C., Hrubikova, K. and Hein, I. (2003) Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *Plant J*, **34**, 543-553.
- Lacomme, C. and Santa Cruz, S. (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proc Natl Acad Sci U S A*, **96**, 7956-7961.
- Lahaye, T. (2002) The *Arabidopsis* RRS1-R disease resistance gene--uncovering the plant's nucleus as the new battlefield of plant defense? *Trends Plant Sci*, **7**, 425-427.
- Lahaye, T. and Bonas, U. (2001) Molecular secrets of bacterial type III effector proteins. *Trends Plant Sci*, **6**, 479-485.
- Lal, A.S., Parker, P.J. and Segal, A.W. (1999) Characterization and partial purification of a novel neutrophil membrane-associated kinase capable of phosphorylating the respiratory burst component p47^{phox}. *Biochem J*, **338** (Pt 2), 359-366.
- Laloi, C., Apel, K. and Danon, A. (2004) Reactive oxygen signalling: the latest news. *Curr Opin Plant Biol*, **7**, 323-328.
- Lam, E. and del Pozo, O. (2000) Caspase-like protease involvement in the control of plant cell death. *Plant Mol Biol*, **44**, 417-428.
- Lam, E., Kato, N. and Lawton, M. (2001) Programmed cell death, mitochondria and the plant hypersensitive response. *Nature*, **411**, 848-853.
- Lam, E., Pontier, D. and del Pozo, O. (1999) Die and let live - programmed cell death in plants. *Curr Opin Plant Biol*, **2**, 502-507.

- Lamb, C. and Dixon, R.A. (1997) The Oxidative Burst In Plant Disease Resistance. *Annu Rev Plant Physiol Plant Mol Biol*, **48**, 251-275.
- Lauge, R. and De Wit, P.J. (1998) Fungal avirulence genes: structure and possible functions. *Fungal Genet Biol*, **24**, 285-297.
- Lawrence, R.J. and Pikaard, C.S. (2003) Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. *Plant J*, **36**, 114-121.
- Lawton, K., Weymann, K., Friedrich, L., Vermooij, B., Uknes, S. and Ryals, J. (1995) Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol Plant Microbe Interact*, **8**, 863-870.
- Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J*, **10**, 71-82.
- Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J. and Ward, E. (1998) Functional analysis of regulatory sequences controlling *PR-1* gene expression in *Arabidopsis*. *Plant J*, **16**, 223-233.
- Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R. and Pugin, A. (2002) Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell*, **14**, 2627-2641.
- Lee, J. and Rudd, J.J. (2002) Calcium-dependent protein kinases: versatile plant signalling components necessary for pathogen defence. *Trends Plant Sci*, **7**, 97-98.
- Lee, M. (1998) Genome projects and gene pools: new germplasm for plant breeding? *Proc Natl Acad Sci U S A*, **95**, 2001-2004.
- Lee, M.W., Hirai, I. and Wang, H.G. (2003) Caspase-3-mediated cleavage of Rad9 during apoptosis. *Oncogene*, **22**, 6340-6346.
- Lehmann, F.G. (1970) Molecular weight determination by gel chromatography on sepharose 6B. *Clin Chim Acta*, **28**, 335-339.
- Leist, M. and Jäättelä, M. (2001) Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol*, **2**, 589-598.
- Leon, J. S.-S.J. (1999) Molecular biology of jasmonic acid biosynthesis in plants. *Plant Physiol Biochem*, **37**, 373-380.
- Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, **79**, 583-593.
- Li, J., Billiar, T.R., Talanian, R.V. and Kim, Y.M. (1997b) Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem Biophys Res Commun*, **240**, 419-424.
- Li, J., Brader, G. and Palva, E.T. (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell*, **16**, 319-331.
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997a) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an

apoptotic protease cascade. *Cell*, **91**, 479-489.

- Li, Q., Lawrence, C.B., Xing, H.Y., Babbitt, R.A., Bass, W.T., Maiti, I.B. and Everett, N.P. (2001) Enhanced disease resistance conferred by expression of an antimicrobial magainin analogue in transgenic tobacco. *Planta*, **212**, 635-639.
- Lim, M.T. and Kunkel, B.N. (2004) Mutations in the *Pseudomonas syringae* avrRpt2 gene that dissociate its virulence and avirulence activities lead to decreased efficiency in AvrRpt2-induced disappearance of RIN4. *Mol Plant Microbe Interact*, **17**, 313-321.
- Lin, J.Y., Seguin, R., Keller, K. and Chadee, K. (1995) Transforming growth factor-beta 1 primes macrophages for enhanced expression of the nitric oxide synthase gene for nitric oxide-dependent cytotoxicity against *Entamoeba histolytica*. *Immunology*, **85**, 400-407.
- Lindbo, J.A., Silva-Rosales, L., Proebsting, W.M. and Dougherty, W.G. (1993) Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. *Plant Cell*, **5**, 1749-1759.
- Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J. and Stamler, J.S. (2001) A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature*, **410**, 490-494.
- Liu, L., Yan, Y., Zeng, M., Zhang, J., Hanes, M.A., Ahearn, G., McMahon, T.J., Dickfeld, T., Marshall, H.E., Que, L.G. and Stamler, J.S. (2004a) Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell*, **116**, 617-628.
- Liu, Y., Burch-Smith, T., Schiff, M., Feng, S. and Dinesh-Kumar, S.P. (2004b) Molecular chaperone Hsp90 associates with resistance protein N and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. *J Biol Chem*, **279**, 2101-2108.
- Liu, Y., Schiff, M. and Dinesh-Kumar, S.P. (2002) Virus-induced gene silencing in tomato. *Plant J*, **31**, 777-786.
- Liu, Y., Schiff, M., Marathe, R. and Dinesh-Kumar, S.P. (2002) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J*, **30**, 415-429.
- Liu, Y.G., Mitsukawa, N., Oosumi, T. and Whittier, R.F. (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J*, **8**, 457-463.
- Liu, Y.G. and Whittier, R.F. (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics*, **25**, 674-681.
- Lo, S.C. and Nicholson, R.L (1998) Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls. *Plant Physiol*, **116**, 979-989.
- Loake, G. (2001) Plant cell death: unmasking the gatekeepers. *Curr Biol*, **11**, R1028-1031.
- Lorrain, S., Vaillau, F., Balague, C. and Roby, D. (2003) Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends Plant Sci*, **8**, 263-271.
- Lu, R., Malcuit, I., Moffett, P., Ruiz, M.T., Peart, J., Wu, A.J., Rathjen, J.P., Bendahmane, A., Day, L. and Baulcombe, D.C. (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *Embo J*, **22**, 5690-5699.

- Ludwig, A.A., Romeis, T. and Jones, J.D. (2004) CDPK-mediated signalling pathways: specificity and cross-talk. *J Exp Bot*, **55**, 181-188.
- Lund, S.T., Stall, R.E. and Klee, H.J. (1998) Ethylene regulates the susceptible response to pathogen infection in tomato. *Plant Cell*, **10**, 371-382.
- MacFarlane, S.A. (1996) Rapid cloning of uncharacterised tobacco rattle virus isolates using long template (LT) PCR. *J Virol Methods*, **56**, 91-98.
- MacFarlane, S.A. and Popovich, A.H. (2000) Efficient expression of foreign proteins in roots from tobavirus vectors. *Virology*, **267**, 29-35.
- Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L. (2003) *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell*, **112**, 379-389.
- Mackey, D., Holt, B.F., Wiig, A. and Dangl, J.L. (2002) RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell*, **108**, 743-754.
- Maleck, K., Neuenschwander, U., Cade, R.M., Dietrich, R.A., Dangl, J.L. and Ryals, J.A. (2002) Isolation and characterization of broad-spectrum disease-resistant *Arabidopsis* mutants. *Genetics*, **160**, 1661-1671.
- Marathe, R. and Dinesh-Kumar, S.P. (2003) Plant defense: one post, multiple guards? *Mol Cell*, **11**, 284-286.
- Marathe, R., Smith, T.H., Anandalakshmi, R., Bowman, L.H., Fagard, M., Mourrain, P., Vaucheret, H. and Vance, V.B. (2000) Plant viral suppressors of post-transcriptional silencing do not suppress transcriptional silencing. *Plant J*, **22**, 51-59.
- Martin, G.B. (1999) Functional analysis of plant disease resistance genes and their downstream effectors. *Curr Opin Plant Biol*, **2**, 273-279.
- Martin, M., Leon, J., Dammann, C., Albar, J.P., Griffiths, G. and Sanchez-Serrano, J.J. (1999) Antisense-mediated depletion of potato omega3 fatty acid desaturase lowers linolenic acid content and reduces gene activation in response to wounding. *Eur. J. Biochem.* **262**, 283-90.
- Martinon, F. and Tschopp, J. (2004) Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell*, **117**, 561-574
- Mathiasen, I.S. and Jaattela, M. (2002) Triggering caspase-independent cell death to combat cancer. *Trends Mol Med*, **8**, 212-220.
- Mayer, B. and Andrew, P. (1998) Nitric oxide synthases: catalytic function and progress towards selective inhibition. *Naunyn Schmiedebergs Arch Pharmacol*, **358**, 127-133.
- Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. and Koornneef, M. (1998) *Arabidopsis thaliana*: a model plant for genome analysis. *Science*, **282**, 662, 679-682.
- Menke, F.L., Champion, A., Kijne, J.W. and Memelink, J. (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *Embo J*, **18**, 4455-

4463.

- Messing, J. and Llaca, V. (1998) Importance of anchor genomes for any plant genome project. *Proc Natl Acad Sci U S A*, **95**, 2017-2020.
- Messmer, U.K., Lapetina, E.G. and Brune, B. (1995) Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds. *Mol Pharmacol*, **47**, 757-765.
- Messmer, U.K., Reed, U.K. and Brune, B. (1996) Bcl-2 protects macrophages from nitric oxide-induced apoptosis. *J Biol Chem*, **271**, 20192-20197.
- Mestre, P., Brigneti, G., Durrant, M.C. and Baulcombe, D.C. (2003) Potato virus Y NIa protease activity is not sufficient for elicitation of Ry-mediated disease resistance in potato. *Plant J*, **36**, 755-761.
- Mittler, R., Shulaev, V. and Lam, E. (1995) Coordinated Activation of Programmed Cell Death and Defense Mechanisms in Transgenic Tobacco Plants Expressing a Bacterial Proton Pump. *Plant Cell*, **7**, 29-42.
- Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*, **7**, 405-410
- Moffett, P., Farnham, G., Peart, J. and Baulcombe, D.C. (2002) Interaction between domains of a plant NBS-LRR protein in disease resistance-related cell death. *Embo J*, **21**, 4511-4519.
- Mukalazi, J., Adipala, E., Sengooba, T., Hakiza, J.J., Olanya, M. and Kidanemariam, H.M. (2000) Metalaxyl resistance, mating type and pathogenicity of *Phytophthora infestans* in Uganda. *Crop Protection*, **20**, 379-388.
- Munnik, T., Meijer, H.J., Ter Riet, B., Hirt, H., Frank, W., Bartels, D. and Musgrave, A. (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. *Plant J*, **22**, 147-154.
- Muntener, K., Zwicky, R., Csucs, G., Rohrer, J. and Baici, A. (2004) Exon skipping of cathepsin B: mitochondrial targeting of a lysosomal peptidase provokes cell death. *J Biol Chem*, **279**, 41012-41017.
- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant*, **15**, 473-497.
- Murray, S.L., Thomson, C., Chini, A., Read, N.D. and Loake, G.J. (2002) Characterization of a novel, defense-related *Arabidopsis* mutant, *cir1*, isolated by luciferase imaging. *Mol Plant Microbe Interact*, **15**, 557-566.
- Muskett, P.R., Kahn, K., Austin, M.J., Moisan, L.J., Sadanandom, A., Shirasu, K., Jones, J.D. and Parker, J.E. (2002) *Arabidopsis* RAR1 exerts rate-limiting control of R gene-mediated defenses against multiple pathogens. *Plant Cell*, **14**, 979-992.
- Mysore, K.S. and Ryu, C.M. (2004) Nonhost resistance: how much do we know? *Trends Plant Sci*, **9**, 97-104.
- Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a Chimeric Chalcone Synthase Gene into *Petunia* Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell*, **2**, 279-289.

- Nathan, C. and Xie, Q.W. (1994) Nitric oxide synthases: roles, tolls, and controls. *Cell*, **78**, 915-918.
- Neill, S., Desikan, R. and Hancock, J. (2002) Hydrogen peroxide signalling. *Curr Opin Plant Biol*, **5**, 388-395.
- Newton, K. and Strasser, A. (1998) The Bcl-2 family and cell death regulation. *Curr Opin Genet Dev*, **8**, 68-75.
- Niggeweg, R., Thurrow, C., Weigel, R., Pfitzner, U. and Gatz, C. (2000) Tobacco TGA factors differ with respect to interaction with NPR1, activation potential and DNA-binding properties. *Plant Mol Biol*, **42**, 775-788.
- Nimchuk, Z., Rohmer, L., Chang, J.H. and Dangl, J.L. (2001) Knowing the dancer from the dance: *R*-gene products and their interactions with other proteins from host and pathogen. *Curr Opin Plant Biol*, **4**, 288-294.
- Nowotny, M., Spiechowicz, M., Jastrzebska, B., Filipek, A., Kitagawa, K. and Kuznicki, J. (2003) Calcium-regulated interaction of Sgt1 with S100A6 (calcyclin) and other S100 proteins. *J Biol Chem*, **278**, 26923-26928.
- Numberger, T. and Scheel, D. (2001) Signal transmission in the plant immune response. *Trends Plant Sci*, **6**, 372-379.
- Olsen, A.N., Mundy, J. and Skriver, K. (2002) Peptomics, identification of novel cationic *Arabidopsis* peptides with conserved sequence motifs. *In Silico Biol*, **2**, 441-451.
- Osterlund, M.T. and Paterson, A.H. (2002) Applied plant genomics: the secret is integration. *Curr Opin Plant Biol*, **5**, 141-145.
- Ouaked, F., Rozhon, W., Lecourieux, D. and Hirt, H. (2003) A MAPK pathway mediates ethylene signaling in plants. *Embo J*, **22**, 1282-1288.
- Overmyer, K., Brosche, M. and Kangasjarvi, J. (2003) Reactive oxygen species and hormonal control of cell death. *Trends Plant Sci*, **8**, 335-342.
- Parker, J.E., Coleman, M.J., Szabo, V., Frost, L.N., Schmidt, R., van der Biezen, E.A., Moores, T., Dean, C., Daniels, M.J. and Jones, J.D. (1997) The *Arabidopsis* downy mildew resistance gene RPP5 shares similarity to the toll and interleukin-1 receptors with N and L6. *Plant Cell*, **9**, 879-894.
- Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., Daniels, M.J. (1996) Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. *Plant Cell*, **8**, 2033-2046.
- Pearce, G., Moura, D.S., Stratmann, J. and Ryan, C.A., Jr. (2001) RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development. *Proc Natl Acad Sci U S A*, **98**, 12843-12847.
- Peart, J.R., Lu, R., Sadanandom, A., Malcuit, I., Moffett, P., Brice, D.C., Schauser, L., Jaggard, D.A., Xiao, S., Coleman, M.J., Dow, M., Jones, J.D., Shirasu, K. and Baulcombe, D.C. (2002) Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. *Proc Natl Acad Sci U S A*, **99**, 10865-10869.
- Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E. and Schroeder, J.I. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*, **406**, 731-734.

- Peng, Z., Grimberg, E. and Sagi-Eisenberg, R. (2002) Suppression of Synaptotagmin II restrains phorbol ester-induced downregulation of protein kinase Calpha by diverting the kinase from a degradative pathway to the recycling endocytic compartment. *J Cell Sci*, **115**, 3083-3092.
- Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Mettraux, J.P., Manners, J.M. and Broekaert, W.F. (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell*, **8**, 2309-2323.
- Peterhansel, C., Freialdenhoven, A., Kurth, J., Kolsch, R. and Schulze-Lefert, P. (1997) Interaction Analyses of Genes Required for Resistance Responses to Powdery Mildew in Barley Reveal Distinct Pathways Leading to Leaf Cell Death. *Plant Cell*, **9**, 1397-1409.
- Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B. and Mundy, J. (2000) *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell*, **103**, 1111-1120.
- Petnicki-Ocwieja, T., Schneider, D.J., Tam, V.C., Chancey, S.T., Shan, L., Jamir, Y., Schechter, L.M., Janes, M.D., Buell, C.R., Tang, X., Collmer, A. and Alfano, J.R. (2002) Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. *Proc Natl Acad Sci U S A*, **99**, 7652-7657.
- Pfyffer, G., Borashi-Gaia, E., Weber, B., Hoesh, L., Orpin, GC. and Rast, D. (1990) A further report on the occurrence of acyclic sugar alcohols in fungi. *Mycol Res*, **92**, 219-222.
- Piatelli, M.J., Doughty, C. and Chiles, T.C. (2002) Requirement for a hsp90 chaperone-dependent MEK1/2-ERK pathway for B cell antigen receptor-induced cyclin D2 expression in mature lymphocytes. *J. Biol. Chem.*, **277**, 12144-12150
- Pilloff, R.K., Devadas, S.K., Enyedi, A. and Raina, R. (2002) The *Arabidopsis* gain-of-function mutant *dll1* spontaneously develops lesions mimicking cell death associated with disease. *Plant J*, **30**, 61-70.
- Pontier, D., Mittler, R. and Lam, E. (2002) Mechanism of cell death and disease resistance induction by transgenic expression of bacterio-opsin. *Plant J*, **30**, 499-509.
- Pratt, W.B., Galigniana, M.D., Harrell, J.M. and DeFranco, D.B. (2004) Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. *Cell Signal*, **16**, 857-872.
- Qi, Q., Rajala, R.V.S., Anderson, W., Jiang, C., Rozwadoeski, K., Selvaraj, G., Sharma, R. and Datla, R. (2000) Molecular Cloning, Genomic Organization, and Biochemical Characterization of Myristoyl-CoA: Protein N-Myristoyltransferase from *Arabidopsis thaliana*. *J. Biol Chem*, **275**, 9673-9683.
- Qutob, D., Hraber, P.T., Sobral, B.W. and Gijzen, M. (2000) Comparative analysis of expressed sequences in *Phytophthora sojae*. *Plant Physiol*, **123**, 243-254.
- Rairdan GJ, D.N., Delaney TP. (OCT 2001) Salicylic acid and NIM1/NPR1-independent gene induction by incompatible *Peronospora parasitica* in *Arabidopsis*. *Mol Plant Microbe Interact*, **14**, 1235-1246.
- Ratcliff, F., Harrison, Bryan D., Baulcombe, David C., A and. (1997) Similarity Between Viral Defense

and Gene Silencing in Plants. *Science*, **276**, 1558-1560.

- Ratcliff, F., Martin-Hernandez, A.M. and Baulcombe, D.C. (2001) Technical Advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J*, **25**, 237-245.
- Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M. and Chory, J. (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. *Plant Cell*, **5**, 147-157.
- Reglinski, T., Lyon, G.D. and Newton, A.C. (1994) Induction of resistance mechanisms in Barley by yeast-derived elicitors. *Annals of Applied Biology*, **124**, 509-517.
- Reignault, P., Frost, L.N., Richardson, H., Daniels, M.J., Jones, J.D. and Parker, J.E. (1996) Four Arabidopsis RPP loci controlling resistance to the Noco2 isolate of *Peronospora parasitica* map to regions known to contain other RPP recognition specificities. *Mol Plant Microbe Interact*, **9**, 464-473.
- Ren, D., Yang, H. and Zhang, S. (2002) Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. *J Biol Chem*, **277**, 559-565.
- Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. and Knight, M.R. (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. *Nature*, **427**, 858-861.
- Reuber, T.L., Plotnikova, J.M., Dewdney, J., Rogers, E.E., Wood, W. and Ausubel, F.M. (1998) Correlation of defense gene induction defects with powdery mildew susceptibility in Arabidopsis enhanced disease susceptibility mutants. *Plant J*, **16**, 473-485.
- Richberg, M.H., Aviv, D.H. and Dangl, J.L. (1998) Dead cells do tell tales. *Curr Opin Plant Biol*, **1**, 480-485.
- Roberts, M.R. (2000) Regulatory 14-3-3 protein-protein interactions in plant cells. *Curr Opin Plant Biol*, **3**, 400-405.
- Robertson, D., Davies, D.R., Gerrish, C., Jupe, S.C. and Bolwell, G.P. (1995) Rapid changes in oxidative metabolism as a consequence of elicitor treatment of suspension-cultured cells of French bean (*Phaseolus vulgaris* L.). *Plant Mol Biol*, **27**, 59-67.
- Robson, C.A. and Vanlerberghe, G.C. (2002) Transgenic plant cells lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. *Plant Physiol*, **129**, 1908-1920.
- Rogers, E.E. and Ausubel, F.M. (1997) Arabidopsis enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. *Plant Cell*, **9**, 305-316.
- Rogers, L.M., Kim, Y.K., Guo, W., Gonzalez-Candelas, L., Li, D. and Kolattukudy, P.E. (2000) Requirement for either a host- or pectin-induced pectate lyase for infection of *Pisum sativum* by *Nectria hematococca*. *Proc Natl Acad Sci U S A*, **97**, 9813-9818.
- Rojo, E., Martin, R., Carter, C., Zouhar, J., Pan, S., Plotnikova, J., Jin, H., Paneque, M., Sanchez-Serrano, J.J., Baker, B., Ausubel, F.M. and Raikhel, N.V. (2004) VPEgamma Exhibits a Caspase-like Activity that Contributes to Defense against Pathogens. *Curr Biol*, **14**, 1897-1906.

- Romeis, T. (2001) Protein kinases in the plant defence response. *Curr Opin Plant Biol*, **4**, 407-414.
- Romeis, T., Ludwig, A.A., Martin, R. and Jones, J.D. (2001) Calcium-dependent protein kinases play an essential role in a plant defence response. *Embo J*, **20**, 5556-5567.
- Romeis, T., Piedras, P. and Jones, J.D. (2000) Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *Plant Cell*, **12**, 803-816.
- Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H. and Jones, J.D. (1999) Rapid Avr9- and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. *Plant Cell*, **11**, 273-287.
- Rommens, C.M. and Kishore, G.M. (2000) Exploiting the full potential of disease-resistance genes for agricultural use. *Curr Opin Biotechnol*, **11**, 120-125.
- Rooney, H.C., van't Klooster, J.W., van der Hoorn, R.A., Joosten, M.H., Jones, J.D. and de Wit, P.J. (2005) *Cladosporium* Avr2 inhibits tomato Rcr3 protease required for Cf-2-dependent disease resistance. *Science*, Epub ahead of print.
- Rossier, O., Wengelnik, K., Hahn, K. and Bonas, U. (1999) The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian bacterial pathogens. *Proc Natl Acad Sci U S A*, **96**, 9368-9373.
- Rossig, L., Fichtlscherer, B., Breitschopf, K., Haendeler, J., Zeiher, A.M., Mulsch, A. and Dimmeler, S. (1999) Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. *J Biol Chem*, **274**, 6823-6826.
- Rouis, S., Traincard, F., Gargouri, R., Darteville, S., Jeannequin, O., Mazie, J.C. and Ayadi, H. (2001) Inhibition of potato virus Y NIa activity: preparation of monoclonal antibody directed against PVY NI protein that inhibits cleavage of PVY polyprotein. *Arch Virol*, **146**, 1297-1306.
- Rouster, J., Leah, R., Mundy, J. and Cameron-Mills, V. (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. *Plant J*, **11**, 513-523.
- Royo, J., Leon, J., Vancanneyt, G., Albar, J.P., Rosahl, S., Ortego, F., Castanera P. and Sanchez-Serrano, J.J. (1999) Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 1146-1151.
- Rustérucci, C., Aviv, D.H., Holt, B.F., 3rd, Dangl, J.L. and Parker, J.E. (2001) The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in *Arabidopsis*. *Plant Cell*, **13**, 2211-2224.
- Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P. and Uknes, S. (1997) The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell*, **9**, 425-439.
- Ryu, C.M., Anand, A., Kang, L. and Mysore, K.S. (2004) Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. *Plant J*, **40**, 322-331.
- Sambrook, J., Fritsch, E.F. and Maniatis, T.; Hrsg. (1989) *Molecular Cloning - A Laboratory Manual*, 2nd Edition. Cold Spring Harbour Laboratory Press, New York.

- Sanchez, P., de Torres Zabala, M. and Grant, M. (2000) AtBI-1, a plant homologue of Bax inhibitor-1, suppresses Bax-induced cell death in yeast and is rapidly upregulated during wounding and pathogen challenge. *Plant J*, **21**, 393-399.
- Sang, Y., Cui, D. and Wang, X. (2001) Phospholipase D and phosphatidic acid-mediated generation of superoxide in *Arabidopsis*. *Plant Physiol*, **126**, 1449-1458.
- Sanmartín, M., Jaroszewski, L., Raikhel, N.V. and Rojo, E. (2005) Caspases. Regulating death since origin of life. *Plant Physiol*; **137**, 841-847.
- Santamaria, M., Thomson, C.J., Read, N.D. and Loake, G.J. (2001) The promoter of a basic *PR1*-like gene, AtPRB1, from *Arabidopsis* establishes an organ-specific expression pattern and responsiveness to ethylene and methyl jasmonate. *Plant Mol Biol*, **47**, 641-652.
- Sato, N. (1979) Effects of soil-temperature on the field infection of potato-tubers by *Phytophthora infestans*. *Phytopathol*, **69**, 989-993.
- Scheel, D. (1998) Resistance response physiology and signal transduction. *Curr Opin Plant Biol*, **1**, 305-310.
- Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C. and Manners, J.M. (2000) Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proc Natl Acad Sci U S A*, **97**, 11655-11660.
- Schomburg, F.M., Bizzell, C.M., Lee, D.J., Zeevaart, J.A. and Amasino, R.M. (2003) Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. *Plant Cell*, **15**, 151-163.
- Schraudner, M., Moeder, W, Wiese, C, Van Camp, W, Inzé, D, Langebartels, C, and Sandermann, H Jr. (1998) Ozone-induced oxidative burst in the ozone biomonitor plant tobacco Bel W3. *Plant Journal*, **16**, 235-245.
- Schwinn, F.J. and Morton, H.V. (1990) Antiresistance strategies: design and implementation on practice. In *managing Resistance to Agrochemicals*, Green, M.B., LeBaron, H.M. and Moberg, W. Keds. American Chemical Society, Washington DC, 170-183.
- Shah, J., Tsui, F. and Klessig, D.F. (1997) Characterization of a salicylic acid-insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Mol Plant Microbe Interact*, **10**, 69-78.
- Shan, W., Cao, M., Leung, D. and Tyler, B.M. (2004) The *Avr1b* locus of *Phytophthora sojae* encodes an elicitor and regulator for avirulence on soybean carrying *Rps1b*. *Mol Plant Microbe Interact*, **17**, 394-403
- Shirahama-Noda, K., Yamamoto, A., Sugihara, K., Hashimoto, N., Asano, M., Nishimura, M. and Hara-Nishimura, I. (2003) Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice. *J Biol Chem*, **278**, 33194-33199.
- Shirasu, K., Lahaye, T., Tan, M.W., Zhou, F., Azevedo, C. and Schulze-Lefert, P. (1999) A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. *Cell*, **99**, 355-366.

- Shirasu, K., Nakajima, H., Rajasekhar, V.K., Dixon, R.A. and Lamb, C. (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell*, **9**, 261-270.
- Shirasu, K. and Schulze-Lefert, P. (2000) Regulators of cell death in disease resistance. *Plant Mol Biol*, **44**, 371-385.
- Shirasu, K. and Schulze-Lefert, P. (2003) Complex formation, promiscuity and multi-functionality: protein interactions in disease-resistance pathways. *Trends Plant Sci*, **8**, 252-258.
- Shrivastav, A., Pasha, M.K., Selvakumar, P., Gowda, S., Olson, D.J.H., Ross, A.R.S., Dimmock, J.R. and Sharma, R.K. (2003) Potent inhibitor of *N*-myristoylation: a novel molecular target for cancer. *Cancer Res*, **63**, 7975-7978.
- Slusarenko, A.J., Longland, A. (1986) Changes in gene activity during expression of the hypersensitive response in *Phaseolus vulgaris* cv red mexican to an avirulent race-1 isolate of *Pseudomonas syringae* pv *phaseolicola*. *Physiol Mol Plant Pathol*, **29**, 79-94.
- Smith, C.J., Watson, C.F., Bird, C.R., Ray, J., Schuch, W. and Grierson, D. (1990) Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Mol Gen Genet*, **224**, 477-481.
- Smyth, S., Khachatourians, G.G. and Phillips, P.W. (2002) Liabilities and economics of transgenic crops. *Nat Biotechnol*, **20**, 537-541.
- Solano, R. and Ecker, J.R. (1998) Ethylene gas: perception, signaling and response. *Curr Opin Plant Biol*, **1**, 393-398.
- Solomon, M., Belenghi, B., Delledonne, M., Menachem, E. and Levine, A. (1999) The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell*, **11**, 431-444.
- Sreedhar, A.S., Mihaly, K., Pato, B., Schnaider, T., Stetak, A., Kis-Petik, K., Fidy, J., Simonics, T., Maraz, A. and Csermely, P. (2003) Hsp90 inhibition accelerates cell lysis. Anti-Hsp90 ribozyme reveals a complex mechanism of Hsp90 inhibitors involving both superoxide- and Hsp90-dependent events. *J Biol Chem*, **278**, 35231-35240.
- Sreedhar, A.S., Soti, C. and Csermely, P. (2004) Inhibition of Hsp90: a new strategy for inhibiting protein kinases. *Biochim Biophys Acta*, **1697**, 233-242.
- Stahl, E.A., Dwyer, G., Mauricio, R., Kreitman, M. and Bergelson, J. (1999) Dynamics of disease resistance polymorphism at the *Rpm1* locus of *Arabidopsis*. *Nature*, **400**, 667-671.
- Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G. and Jones, J.D. (1995) Molecular genetics of plant disease resistance. *Science*, **268**, 661-667.
- Staswick, P.E., Tiryaki, I. and Rowe, M.L. (2002) Jasmonate response locus JAR1 and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. *Plant Cell*, **14**, 1405-1415.
- Steensgaard, P., Garre, M., Muradore, I., Transidico, P., Nigg, E.A., Kitagawa, K., Earnshaw, W.C., Faretta, M. and Musacchio, A. (2004) Sgt1 is required for human kinetochore assembly. *EMBO Rep*, **5**, 626-631.

- Stennicke, H.R., Deveraux, Q.L., Humke, E.W., Reed, J.C., Dixit, V.M. and Salvesen, G.S. (1999) Caspase-9 can be activated without proteolytic processing. *J Biol Chem*, **274**, 8359-8362.
- Stoka, V., Turk, B., Schendel, S.L., Kim, T.H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M., Bromme, D., Krajewski, S., Reed, J.C., Yin, X.M., Turk, V. and Salvesen, G.S. (2001) Lysosomal protease pathways to apoptosis. Cleavage of bid, not procaspases, is the most likely route. *J Biol Chem*, **276**, 3149-3157.
- Stuiver, M.H. and Custers, J.H. (2001) Engineering disease resistance in plants. *Nature*, **411**, 865-868.
- Sujkowski, L.S., Goodwin, S.B., Dyer, A.T. and Fry, W.E. (1994) Increased genotypic diversity via migration and possible occurrence of sexual reproduction of *Phytophthora infestans* in Poland. *Phytopathology*, **84**, 201-207.
- Susi, P., Hohkuri, M., Wahloors, T. and Kilby, N.J. (2004) Characteristics of RNA silencing in plants: similarities and differences across kingdoms. *Plant Mol Biol*, **54**, 157-174.
- Suzuki, H., Xia, Y., Cameron, R., Shadle, G., Blount, J., Lamb, C. and Dixon, R.A. (2004) Signals for local and systemic responses of plants to pathogen attack. *J Exp Bot*, **55**, 169-179.
- Takahashi, A., Casais, C., Ichimura, K. and Shirasu, K. (2003) HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in *Arabidopsis*. *Proc Natl Acad Sci U S A*, **100**, 11777-11782.
- Takahashi, M. and Asada, K. (1988) Superoxide production in aprotic interior of chloroplast thylakoids. *Arch Biochem Biophys*, **267**, 714-722.
- Takken, W., Luderer, R., Gabriels, S.H., Westerink, N., Lu, R., de Wit, P.J. and Joosten, M.H. (2000) A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. *Plant J*, **24**, 275-283.
- Tani, H., Chen, X., Nurmberg, P., Grant, J.J., SantaMaria, M., Chini, A., Gilroy, E., Birch, P.R. and Loake, G.J. (2004) Activation tagging in plants: a tool for gene discovery. *Funct Integr Genomics*, **4**, 258-266.
- Thomas, C.L., Jones, L., Baulcombe, D.C. and Maule, A.J. (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *Plant J*, **25**, 417-425.
- Thomma, B.P., Eggemont, K., Tierens, K.F. and Broekaert, W.F. (1999) Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. *Plant Physiol*, **121**, 1093-1102.
- Thomma, B.P., Penninckx, I.A., Broekaert, W.F. and Cammue, B.P. (2001) The complexity of disease signaling in *Arabidopsis*. *Curr Opin Immunol*, **13**, 63-68.
- Thompson, G.A. and Okuyama, H. (2000) Lipid-linked proteins of plants. *Progr Lipid Res*, **39**, 19-39
- Thordal-Christensen, H., Zang, Z.G., Wei, Y.D. and Collinge, D.B. (1997) Subcellular localisation of H₂O₂ in plants. H₂O₂ accumulation in papille and hypersensitive response during the Barley Powdery Mildew Interaction. *Plant J*, **11**, 1187-1194.
- Tian, M., Huitema, E., Da Cunha, L., Torto-Alalibo, T. and Kamoun, S. (2004) A Kazal-like extracellular

serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. *J Biol Chem*, **279**, 26370-26377.

- Tiwari, B.S., Belenghi, B. and Levine, A. (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant Physiol*, **128**, 1271-1281.
- Ton, J., Van Pelt, J.A., Van Loon, L.C. and Pieterse, C.M.J. (2002) Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. *Mol Plant Microbe Interact*, **15**, 27-34.
- Tör, M., Gordon, P., Cuzick, A., Eulgem, T., Sinapidou, E., Mert-Turk, F., Can, C., Dangl, J.L. and Holub, E.B. (2002) *Arabidopsis* SGT1b is required for defense signaling conferred by several downy mildew resistance genes. *Plant Cell*, **14**, 993-1003.
- Tornero, P., Conejero, V. and Vera, P. (1997) Identification of a new pathogen-induced member of the subtilisin-like processing protease family from plants. *J Biol Chem*, **272**, 14412-14419.
- Tornero, P., Merritt, P., Sadanandom, A., Shirasu, K., Innes, R.W. and Dangl, J.L. (2002) RAR1 and NDR1 contribute quantitatively to disease resistance in *Arabidopsis*, and their relative contributions are dependent on the R gene assayed. *Plant Cell*, **14**, 1005-1015.
- Torres, M.A., Dangl, J.L. and Jones, J.D. (2002) *Arabidopsis* gp91^{phox} homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc Natl Acad Sci U S A*, **99**, 517-522.
- Toth, I.K., Bell, K.S., Holeva, M.C. and Birch, P.R.J. (2003) Soft rot erwiniae: from genes to genomes. *Mol Plant Microbe Interact*, **4**, 1364-3703.
- Tyler, B.M. (2002) Molecular basis of recognition between *Phytophthora* pathogens and their hosts. *Annu Rev Phytopathol*, **40**, 137-167.
- Tyler, B.M. (2001) Genetics and genomics of the oomycete host interface. *Trends Genet*, **17**, 611-614.
- Valentine, T., Shaw, J., Blok, V.C., Philips, M.S., Oparka, K.J. and Lacomme, C. (2004) Efficient virus-induced gene silencing in roots using a modified tobacco rattle virus vector. *Plant Physiol*, **136**, 3999-4009.
- Van Camp W, Van Montagu, M. and Inze D. (1998) H₂O₂ and NO: redox signals in disease resistance. *Trends Plant Sci*, **3**, 330-334.
- van der Biezen, E.A., Freddie, C.T., Kahn, K., Parker, J.E. and Jones, J.D. (2002) *Arabidopsis* RPP4 is a member of the RPP5 multigene family of TIR-NB-LRR genes and confers downy mildew resistance through multiple signalling components. *Plant J*, **29**, 439-451.
- van der Biezen, E.A., Sun, J., Coleman, M.J., Bibb, M.J. and Jones, J.D. (2000) *Arabidopsis* RelA/Spot homologs implicate (p)ppGpp in plant signaling. *Proc Natl Acad Sci U S A*, **97**, 3747-3752.
- VanEtten, H.D., Mansfield, J.W., Bailey, J.A. and Farmer, E.E. (1994) Two classes of plant antibiotic: phytoalexins versus "phytoanticipins". *Plant Cell*, **6**, 1191-1192
- van der Hoorn, R.A. and Jones, J.D. (2004) The plant proteolytic machinery and its role in defence. *Curr Opin Plant Biol*, **7**, 400-407.

- van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N. and Stuitje, A.R. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell*, **2**, 291-299.
- van der Vossen, E.A., van der Voort, J.N., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D.C., Bakker, J., Stiekema, W.J. and Klein-Lankhorst, R.M. (2000) Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. *Plant J*, **23**, 567-576.
- van Wees, S.C., de Swart, E.A., van Pelt, J.A., van Loon, L.C. and Pieterse, C.M. (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A*, **97**, 8711-8716.
- Vancompermolle, K., Van Herreweghe, F., Pynaert, G., Van de Craen, M., De Vos, K., Totty, N., Sterling, A., Fiers, W., Vandenabeele, P. and Grooten, J. (1998) Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. *FEBS Lett*, **438**, 150-158.
- Vandenabeele, S., Van Der Kelen, K., Dat, J., Gadjev, I., Boonefaes, T., Morsa, S., Rottiers, P., Slooten, L., Van Montagu, M., Zabeau, M., Inzé, D. and Van Breusegem, F. (2003) A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. *Proc Natl Acad Sci U S A*, **100**, 16113-16118.
- VanEtten, H.D., Mansfield, J.W., Bailey, J.A. and Farmer, E.E. (1994) Two Classes of Plant Antibiotics: Phytoalexins versus "Phytoanticipins". *Plant Cell*, **6**, 1191-1192.
- Vaucheret, H., Vazquez, F., Crete, P. and Bartel, D.P. (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev*, **18**, 1187-1197.
- Verhaegen, S., McGowan, A.J., Brophy, A.R., Fernandes, R.S. and Cotter, T.G. (1995) Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. *Biochem. Pharmacol.*, **50**, 1021-1029
- Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994) Salicylic Acid Is Not the Translocated Signal Responsible for Inducing Systemic Acquired Resistance but Is Required in Signal Transduction. *Plant Cell*, **6**, 959-965.
- Visser, P.B. and Bol, J.F. (1999) Nonstructural proteins of Tobacco rattle virus which have a role in nematode-transmission: expression pattern and interaction with viral coat protein. *J Gen Virol*, **80 (Pt 12)**, 3273-3280.
- Vleeshouwers, V.G., van Dooijeweert, W., Govers, F., Kamoun, S. and Colon, L.T. (2000) The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. *Planta*, **210**, 853-864.
- Voinnet, O. (2001) RNA silencing as a plant immune system against viruses. *Trends Genet*, **17**, 449-459.
- Voinnet, O., Pinto, Y.M. and Baulcombe, D.C. (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc Natl Acad Sci U S A*, **96**, 14147-14152.
- Volko, S.M., Boller, T. and Ausubel, F.M. (1998) Isolation of new *Arabidopsis* mutants with enhanced disease susceptibility to *Pseudomonas syringae* by direct screening. *Genetics*, **149**, 537-548.
- Walbot, V. (2000) *Arabidopsis thaliana* genome. A green chapter in the book of life. *Nature*, **408**, 794-

795.

- Wang, J. and Mezza, G. (2002) Effects of anthocyanins and other phenolic compounds on the production of tumour necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. *J Agric Food Chem*, **50**, 4183-4189.
- Wang, K.L., Li, H. and Ecker, J.R. (2002) Ethylene biosynthesis and signaling networks. *Plant Cell*, **14 Suppl**, S131-151.
- Wang, M.B. and Waterhouse, P.M. (2002) Application of gene silencing in plants. *Curr Opin Plant Biol*, **5**, 146-150.
- Waugh, M., Hrabec, P., Weller, J., Wu, Y., Chen, G., Inman, J., Kiphart, D. and Sobral, B. (2000) The phytophthora genome initiative database: informatics and analysis for distributed pathogenomic research. *Nucleic Acids Res*, **28**, 87-90.
- Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., Nguyen, J.T., Sato, S., Wang, Z.Y., Xia, Y., Dixon, R.A., Harrison, M.J., Lamb, C.J., Yanofsky, M.F. and Chory, J. (2000) Activation tagging in *Arabidopsis*. *Plant Physiol*, **122**, 1003-1013.
- Wen, C. and Chang, C. (2002) *Arabidopsis RGL1* encodes a negative regulator of gibberellin responses. *Plant Cell*, **14**, 87-100.
- Wendehenne, D., Pugin, A., Klessig, D.F. and Durner, J. (2001) Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci*, **6**, 177-183.
- Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J*, **27**, 581-590.
- Weymann, K., Hunt, M., Uknes, S., Neuenschwander, U., Lawton, K., Steiner, H.Y. and Ryals, J. (1995) Suppression and Restoration of Lesion Formation in *Arabidopsis lsd* Mutants. *Plant Cell*, **7**, 2013-2022.
- Whalen, M., Innes, R., Bent, A. and Staskawicz, B. (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis thaliana* and a bacterial gene determining avirulence on both *Arabidopsis* and soybean. *Plant Cell*, **3**, 49-59.
- White, F.F., Yang, B. and Johnson, L.B. (2000) Prospects for understanding avirulence gene function. *Curr Opin Plant Biol*, **3**, 291-298.
- Wichmann, G. and Bergelson, J. (2004) Effector genes of *Xanthomonas axonopodis* pv. *vesicatoria* promote transmission and enhance other fitness traits in the field. *Genetics*, **166**, 693-706.
- Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*, **414**, 562-565.
- Wohlgemuth, H., Mittelstrass, K., Kschieschan, S., Bender, J., Weigel, H.-J., Overmyer, K., Kangasjarvi, J., Sandermann, H. and Langebartels, C. (2002) Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. *Plant Cell Environ.*, **25**, 717-726
- Wojtaszek, P. (2000) Nitric oxide in plants. To NO or not to NO. *Phytochemistry*, **54**, 1-4.

- Woltering, E.J. (2004) Death proteases come alive. *Trends Plant Sci*, **9**, 469-472.
- Wu, G., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C. and Shah, D.M. (1995) Disease resistance conferred by expression of a gene encoding H₂O₂-generating glucose oxidase in transgenic potato plants. *Plant Cell*, **7**, 1357-1368.
- Xia, Y., Suzuki, H., Borevitz, J., Blount, J., Guo, Z., Patel, K., Dixon, R.A. and Lamb, C. (2004) An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. *Embo J*, **23**, 980-988.
- Xing, T., Ouellet, T. and Miki, B.L. (2002) Towards genomic and proteomic studies of protein phosphorylation in plant-pathogen interactions. *Trends Plant Sci*, **7**, 224-230.
- Yamada, K., Matsushima, R., Nishimura, M. and Hara-Nishimura, I. (2001) A slow maturation of a cysteine protease with a granulin domain in the vacuoles of senescing *Arabidopsis* leaves. *Plant Physiology*, **127**, 1626-1634.
- Yang, Z.W. and Yang, F.Y. (1997) Sensitivity of Ca²⁺ transport of mitochondria to reactive oxygen species. *Biosci Rep*, **17**, 557-567.
- Yoon, G.M., Cho, H.S., Ha, H.J., Liu, J.R. and Lee, H.S. (1999) Characterization of NtCDPK1, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein. *Plant Mol Biol*, **39**, 991-1001.
- Yoshioka, H., Numata, N., Nakajima, K., Katou, S., Kawakita, K., Rowland, O., Jones, J.D. and Doke, N. (2003) *Nicotiana benthamiana* gp91^{phox} homologs NbrbohA and NbrbohB participate in H₂O₂ accumulation and resistance to *Phytophthora infestans*. *Plant Cell*, **15**, 706-718.
- Yu, I.C., Parker, J. and Bent, A.F. (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis* dnd1 mutant. *Proc Natl Acad Sci U S A*, **95**, 7819-7824.
- Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993) The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell*, **75**, 641-652.
- Yuanxin, Y., Chengcai, A., Li, L., Jiayu, G., Guihong, T. and Zhangliang, C. (2003) T-linker-specific ligation PCR (T-linker PCR): an advanced PCR technique for chromosome walking or for isolation of tagged DNA ends. *Nucleic Acids Res*, **31**, e68.
- Yun, B.W., Atkinson, H.A., Gaborit, C., Greenland, A., Read, N.D., Pallas, J.A. and Loake, G.J. (2003) Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in *Arabidopsis* against wheat powdery mildew. *Plant J*, **34**, 768-777.
- Zha, J., Weiler, S., Oh, K.J., Wei, M.C. and Korsmeyer, S.J. (2000) Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science*, **290**, 1761-1765.
- Zhang, C., Czymmek, K.J. and Shapiro, A.D. (2003) Nitric oxide does not trigger early programmed cell death events but may contribute to cell-to-cell signaling governing progression of the *Arabidopsis* hypersensitive response. *Mol Plant Microbe Interact*, **16**, 962-972.
- Zhang, C., Gutsche, A.T. and Shapiro, A.D. (2004) Feedback control of the *Arabidopsis* hypersensitive response. *Mol Plant Microbe Interact*, **17**, 357-365.

- Zhang, S., Du, H. and Klessig, D.F. (1998) Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitors from *Phytophthora* spp. *Plant Cell*, **10**, 435-450.
- Zhang, S. and Klessig, D.F. (1998) Resistance gene N-mediated de novo synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. *Proc Natl Acad Sci USA*, **95**, 7433-7438.
- Zhou, F., Andersen, C.H., Burhenne, K., Fischer, P.H., Collinge, D.B. and Thordal-Christensen, H. (2000) Proton extrusion is an essential signalling component in the HR of epidermal single cells in the barley-powdery mildew interaction. *Plant J*, **23**, 245-254.
- Zeiss, C.J. (2003) The apoptosis-necrosis continuum: insights from genetically altered mice. *Vet Pathol*, **40**, 481-495.
- Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G. and Boller, T. (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature*, **428**, 764-767.
- Zottini, M., Formentin, E., Scattolin, M., Carimi, F., Lo Schiavo, F. and Terzi, M. (2002) Nitric oxide affects plant mitochondrial functionality in vivo. *FEBS Lett*, **515**, 75-78.

Appendix 1: Cathepsin B Alignments

A. cDNA alignments: BG590588, *S. tuberosum*; X81995, *N. rustica*; AF359422, *N. tabacum*

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1
BG590588 .....GC TTTGAA.... ..GTCTTTAA TAACTCCTTT GCTTTTGGGT
X81995.1 GGCACGAGGC CAAATATGGC GTTGAATCAC ATGTCCTTGA CCACTCTCTT TCTTTTGATT
AF359422 .....AA GCT ..ACT

100
BG590588 GCTTCTTTA TCCTGATCTT ACAGGTTGCT GCAGAGAAGC CAATATCTGA AGCTAAACTT
X81995.1 GGTGGCTCTA TCATTGTTTT ACAGGTTGTT GCAGAACAAAC CAATATCCCA AGCTAAAGCG
AF359422 GAACCTCCGA TTCTCAAATT TCACGGTG.. ..AGATTTC ATAAAGCTG. ...TATATTT

150
BG590588 GAATCTGCAA TCCTTCAAGA CTCGATCGTC AAACAAGTCA ATGAGAATGC CGAAGCTGGA
X81995.1 GAATCTGCAA TCCTTCAAGA CTCGATCGTT AAACAAGTCA ATGAAAATGA AAAAGCTGGA
AF359422 TGTTTGGACA AACTGCGTTG CACTTTCTTT TGTGCAAT.G ATACATCTCT CACCGCTCTT

200
BG590588 TGGAAAGCTG CATTCAACCC TCAATTATCG AATTTACAGG TTTCCCAATT TAAGCGCCTT
X81995.1 TGGAAAGCTG CACTGAACCC TCGATTCTCA AATTTACAGG TTTCCCAATT TAAGCGCCTT
AF359422 TTCATGAATA TGTT..ACCT TTCATTGACA .....GG TTTCCCAATT TAAGCGCCTT

250
BG590588 CTTGGAGTTA AGCCTGCAAG AGAAGGTGAT TTGGAGGGA TTCCTGTTCT AACTCATCCA
X81995.1 CTTGGAGTTA AGCCTGCAAG AAAGGGTGAT TTAAAGGGCA TTCCTATTTT AACTCATCCA
AF359422 CTTGGAGTTA AGCCCAACAG AAAGGTGAT TTAAGGGCA TTCCTATTTT AACTCATCCA

301
BG590588 ARACTTAAGG AGTTGCCAAA AGAGTTTGAT GCACGAAAGG CTTGGCCTCA ATGCAGCACA
X81995.1 ARACTTTTGG AGTTGCCACA AGAGTTTGAT GCACGAGTGG CTTGGTCTAA CTGTAGCACT
AF359422 ARACTTTTGG AGTTGCCACA AGAGTTTGAT GCACGAGTGG CTTGGCCTAA CTGTAGCACT

401
BG590588 ATTGGA AAAA TTCTGCAATCA GGGACATTGC GGTTCCTGTT TGCTGTTGAG TCGCTGTCTG
X81995.1 ATTGGGACAA TTCTTCAATCA GGGACACTGT GGTTCCTGTT TGCTGTTGAG TCACTGTCCG
AF359422 ATTGGGACAA TTCTTGTTTA GT.....T TATTATTTT GTGCTT.... .GATTTAGTG

450
BG590588 ATCGTTTCTG TATCCATTAT AATTTGAGTA TCTCTCTGTC TGTAATGAT CTGTAGCGT
X81995.1 ATCGTTTCTG TATTCATTAT GGCTTGAATA TCTCTCTGTC AGCAAATGAT CTCTATGAT
AF359422 ATAGTG...G GGTGC..TAAT G..... .CTTCCATC TTTTGATG.T C...ATGCA.

500
BG590588 GCTGTGGGTT TTTATGCGGG AGTGTGTTGT ATGGTGGATA TCCTATAGCA GCATGGCGAT
X81995.1 GCTGTGGGTT TTTATGTTGG GATGTTGTTG ATGGTGGATA TCCGTTACAA GCTTGAAGT
AF359422 .CCGGTACTA TTT..TCGGC GCACAT.... CAGGTGAAT .....A TCTTGG...T

551
BG590588 ACTTTAAACG TAGGGGAGTG GTCACAGAAE AGTGTGATCC TTACTTCGAC ACTACTGGAT
X81995.1 ACTTTGTCCG CAAGGGTGTG GTCACAGATG AGTGTGACCC TTACTTTGAT AACGAGGGAT
AF359422 TCTAGTATCA ..... ..CTCCTATG TATTCTCC TAAATTGCA AAT.....

651
BG590588 GTTCCACCC TGGTTGTGAA CCGCTCTACC CCACTCCAAA ATGTCATAGG AAAGTGTGTC
X81995.1 GTTCCACCC TGGATGTGAA CCTGCATATC CCACCCAAA GTGTCACAGG AA.GTGC GTT
AF359422 .TCTCTTCC AGGT..... ..ACTCTAA. ...TAACTG ATCGTTTGT

701
BG590588 AAGGGGAAC GAGTTATGGA GGAAATCAAA GCATTACGGT GTCAATGCAT ACAGGGTCAG
X81995.1 AAACAGAATT TA..CTCTGGA GTAGGTCCAA GCATTTTGGC GTCAATGCAT ACATGATCAG
AF359422 TTTACGGGAC A....CTGTG GTTCTTGTG GCTTTTGGT GCTGTGAAT .CATTTGCC

751
BG590588 CATGATCCC CAGAGTATCA TGGCGGAAGT TTACCAAAT GGACCAGTTG AGGT.....
X81995.1 CTCCGATCCA CACAGTATCA TGACAGAAGT GTACAAGAAT GGACCAGTTG AGGTCTCTTT
AF359422 ATCGTTTCTG TA...TTCA TTATGGCTG GTAAATTCAT T.TCTTAATG TGAATACTTG

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      800                                     850
BG590588 .....
X81995.1 TACTGTTTAC GAGGATTTTCG CTCACTACAA GTCTGGAGTT TACAAGCATG TAACTGGTGA
AF359422 CAAGGTTTAT TCTAGTTATT GTCACTG.AT GATTCAAATT TGA...CGTG AGGCAGAATA

      8 51                                     900
BG590588 .....
X81995.1 TATAATGGGA GGCCATGCTG TTAAACTCAT CCGATGGGGA ACCTCCGAGG ATGGAGAGGA
AF359422 TCTCTCTGTC AGCAAA.... TGATCTCTT AGCATGCTGT GGCTTTTAT GTGGGGATGG

      901                                     950
X81995.1 CTATTGGCTT CT TGCCAATC AGTGGAAACAG AGGCTGGGGT GATGATGGTT ACT TTAAGAT

      1 051
X81995.1 CAGAAGAGGA AC AAACGAAT GTGAAA TCGA AGATGAAGT G GTTGCAGGAT TG CCTTCAGC

      1101
X81995.1 CAGAAATCTG AA CGTGGAAC TTGATG TCTC TGATGCTTTC CTTGACGCCG CAA TGTGAAT

      1151
X81995.1 GCTAATTCTT CA ACCAAACG CTAAT AGAA TGCAAGTTTC TGAAGACTGG GGA ACAATAA

      1201
X81995.1 CATATTAAGT TT ATTATTCT TCCCCT CTTA AGTTGTCTGC AATTGTTCCTA CTA TCTTATT

      1251                                     130 1
X81995.1 ATCAGTGAAG TG CTTTCCTT ATCTGT TATG TATATCTTCA CAAGTACTTT GTC AATCTTG

      1351
X81995.1 GCACTGTTGT AA CATTAATA TGAATT TTAC TTAACATTA T CAATGAACTG TA TTATTGTG
    
```

B. Protein alignment (Boxed region represents peptidase domain)

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                                     ↓
N.benthamiana 1 -----MAMNHMSLTLFLLIGASIVLVQVVARQPIISQAKAESAILQDSIYKQVNEREKA
N.rustica      1 -----MAMNHMSLTLFLLIGASIVLVQVVARQPIISQAKAESAILQDSIYKQVNEREKA
S.tuberosum   1 -----MYLTLKSLTLPFLLLGAFPIILIQVVAENPISRAKESAILQDSIYKQVNEREKA
A.thaliana    1 MADNCIRLLHSAIVFFCLGLLISLPHLLQGHAEHESKQLTSEWILQNEIYKQVNEREKA
H.sapiens     1 -----MWQLWASLCCLLVEAN---ARSRPSFHPFLSDREIVNYVVKR-NT

N.benthamiana 55 GWEKALNPRFSNFTYSQPERLLGVKPTRREGDLKGIPILETPLLELFPQEPDARVAWPCCS
N.rustica     55 GWEKALNPRFSNFTYSQPERLLGVKPTRREGDLKGIPILETPLLELFPQEPDARVAWPCCS
S.tuberosum  55 GWEKALNPRFSNFTYSQPERLLGVKPTRREGDLKGIPILETPLLELFPQEPDARVAWPCCS
A.thaliana   61 GWEKASPNDRFANATVAEFPKRLLVKPTPETEPLGVPIVSDISLLELFPDARTANSCQ
H.sapiens    40 TWCAGHN--DYSVDYSLYLERLCC--TFPLGGPFPQRVMPTEDLLLEASPDAREQWPCCP

N.benthamiana 115 TIGRIIDQGCSCGSCWAFGAVRSLSDRFICRYG--LNISLSANDLLACCGFLCGDGCDDGGY
N.rustica     115 TIGRIIDQGCSCGSCWAFGAVRSLSDRFICRYG--LNISLSANDLLACCGFLCGDGCDDGGY
S.tuberosum  115 TIGRIIDQGCSCGSCWAFGAVRSLSDRFICRYG--LSISLVNDLLACCGFLCGDGCDDGGY
A.thaliana   121 SIGRIIDQGCSCGSCWAFGAVRSLSDRFICRYG--MNVSLVNDLLACCGFLCGDGCDDGGY
H.sapiens    95 TIREIFDQGCSCGSCWAFGAVRSLSDRFICRYG--MNVSLVNDLLACCGFLCGDGCDDGGY

N.benthamiana 173 PLCAWYFVYRKGVYV-----ECDFPY-----FDEEGCSHPGCEPAYTPFKCHRECVRQ
N.rustica     173 PLCAWYFVYRKGVYV-----ECDFPY-----FDEEGCSHPGCEPAYTPFKCHRECVRQ
S.tuberosum  173 PLCAWYFVYRKGVYV-----ECDFPY-----FDEEGCSHPGCEPAYTPFKCHRECVRQ
A.thaliana   179 PLCAWYFVYRKGVYV-----ECDFPY-----FDEEGCSHPGCEPAYTPFKCHRECVRQ
H.sapiens    155 PABAANFVYRKGVYVGGGLYESHVGCSPYSIPPCRHVNGRERFCTGEGDTPFKCSKICRPG

N.benthamiana 221 NL-LVSRSKKFGVNAVMISSDPHSIMTEITNGPVVVSFTVYEDFARIKSGVIKVTCDV
N.rustica     221 NL-LVSRSKKFGVNAVMISSDPHSIMTEITNGPVVVSFTVYEDFARIKSGVIKVTCDI
S.tuberosum  221 NV-LVSRSKKFGVNAVMISSDPHSIMAEVYKNGPVVVSFTVYEDFARIKSGVIKVTCDI
A.thaliana   227 NQ-LVSRSKKFGVNAVMISSDPHSIMAEVYKNGPVVVSFTVYEDFARIKSGVIKVTCDI
H.sapiens    215 YSPTYKQDKNYCVKSGVSVNSKLDIMAEVYKNGPVVVSFTVYEDFARIKSGVIKVTCDI

N.benthamiana 280 MGGHAVELIGWTSKEDGEDYNLLANQWNRGWDGDDGIFKIRRGTDKCEIIEDEYVAGLPSAR
N.rustica     280 MGGHAVELIGWTSKEDGEDYNLLANQWNRGWDGDDGIFKIRRGTDKCEIIEDEYVAGLPSAR
S.tuberosum  280 MGGHAVELIGWTSKEDGEDYNLLANQWNRGWDGDDGIFKIRRGTDKCEIIEDEYVAGLPSAR
A.thaliana   286 MGGHAVELIGWTSKEDGEDYNLLANQWNRGWDGDDGIFKIRRGTDKCEIIEDEYVAGLPSAR
H.sapiens    275 MGGHAVELIGWTSKEDGEDYNLLANQWNRGWDGDDGIFKIRRGTDKCEIIEDEYVAGLPSAR

N.benthamiana 340 NLEWELDVSDAPLDAAM
N.rustica     340 NLEWELDVSDAPLDAAM
S.tuberosum  340 NLEWELDVSDAPLDAAM
A.thaliana   346 NVVEGTTTSEDLLVSSP
H.sapiens    334 QYWEK-----
    
```

Appendix 11

S. tuberosum PDS gene and fragment cloned into PVX

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>gi|42495021|gb|AY484445.1| Solanum tuberosum phytoene desaturase (pds)
mRNA, partial cds; nuclear gene for chromoplast product
TTTCAGTAAAATGCCTCAAATTGGACTTGTTCCTGCTGTTAATTTGAGAGTCCAAGGTAGTTCAGCTTAT
CTTTGGAGCTCGAGGTCGTCTTCTTTGGGAACTGAAAGTCGAGATGGTTGCTTGCAAAGGAATTCGTTAT
GTTTTGCTGGTAGCGAATCAATGGGTCATAAGTTAAAGATTTCGTGCCACGACCAGAAGATTGGTTAAGGA
CTTGGGGCCTTTAAAGGTAGTATGCATGATTATCCAAGACCAGAGCTAGACAATACAGTTAACTATTTG
GAGGCTGCATTCTTATCATCAACATTCCGTGCTTCTCCGCGCCCACTAAACCATTGGAGATTGTTATTG
CTGGTGCAGGTTTTGGTGGTTTGTCTACAGCAAAATATTTGGCAGATGCTGGTCAAAAACCGATACTACT
GGAGGCAAGGGATGTTCTAGGTGGAAAGGTAGCTGCATGGAAAGATGATGATGGAGATTGGTACGAGACT
GGTTTGCATATATTCTTTGGGGCTTACCCAAATATTCAGAACCTGTTTGGAGAATTAGGGATTAATGATC
GATTGCAGTGGAAAGAACATTCAATGATATTTGCAATGCCAAGTAAGCCAGGAGAATTTAGCCGCTTTGA
TTTCCCCGAAGCTTTACCCGCTCCTTTAAATGGAGTTTGGCCATCCTAAAGAACAATGAAATGCTTACA
TGGCCAGAGAAAGTCAAATTTGCAATTGGA CTCTTGCCAGCAATGCTTGAGGGCAATCTTATGTTGAAG
CTCAAGACGGGATAAGTGTTAAGGACTGGATGAGAAAGCAAGGTGTGCCGATAGGGTGACAGATGAAGT
GTTTCATCGCCATGTCAAAGGCACCTTAACTTTATAAACCCCTGACGAACTGTCAATGCAGTGCATCTTGATC
GCATTGAACAGGTTTCTCCAGGAGAAAATGGTTCAAAAAATGGCCTTTTTAGATGGTAATCCTCCTGAGA
GACTTGCATGCCGATTGTTGAACACATCGAGTCAAAGGTGGCCAAGTCAGATTGAACTCACGAATAAA
AAAGATTGAGTTGAAATGAGGATGGGAGTGTCAAGTGTTTTATACTGAATGACGGTAGTACAGTTGAGGGC
GATGCTTTTGTGTTTGCCACTCCAGTGGATATTTTCAAGCTGCTTTTGCCTGAAGACTGGAAAGAGATTC
CATATTTCCAAAAGTTGGAGAAGTTAGTCGGAGTACCTGTTATAAATGTACATATATGGTTCGACAGAAA
ACTGAAGAACACATATGATCATTGCTCTTCAGCAGAAGCTCACTGCTCAGTGTGTATGCTGACATGTCT
GTCA CATGTAAGGAATATTACAACCCCAATCAGTCTATGTTGGAATTGGT TTTTGCACCTGCAGAAGAGT
GGATATCTCGCAGCGACTCAGAAATAATTGATGCTACGATGAAGGAACTAGCAACACTTTTCTGATGA
AATTTCAGCAGATCAAAGCAAAGCAAAAATATTTAAAGTATCATGTTGTCAAAAACCTCAAAGGTCTGTTTAT
AAAACAGTGCCAGGTTGTGAACCTTGTCCGCCATTGCAAAGATCCCTATAGAGGGGTTTTATTTAGCCG
GTGA

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Grey = Pds_{as} fragment, Black = PDS_{hp}

Appendix 1II

Potato VIGS paper from Chapter 5

Potato Virus X-Induced Gene Silencing in Leaves and Tubers of Potato¹

Odile Faivre-Rampant², Eleanor M. Gilroy², Katarina Hrubikova, Ingo Hein, Steve Millam, Gary J. Loake, Paul Birch, Mark Taylor, and Christophe Lacomme*

Programmes of Cell-to-Cell Communication (E.M.G., K.H., C.L.), Quality, Health and Nutrition (O.F.-R., M.T.), Genome Dynamics (I.H.), Gene Expression (S.M.), and Plant-Pathogen Interaction (E.M.G., P.B.), Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom; and Institute of Cellular and Molecular Biology, Edinburgh University, Edinburgh EH9 3JR, United Kingdom (E.M.G., G.J.L.)

Virus induced gene silencing (VIGS) is increasingly used to generate transient loss-of-function assays and has potential as a powerful reverse-genetics tool in functional genomic programs as a more rapid alternative to stable transformation. A previously described potato virus X (PVX) VIGS vector has been shown to trigger silencing in the permissive host *Nicotiana benthamiana*. This paper demonstrates that a PVX-based VIGS vector is also effective in triggering a VIGS response in both diploid and cultivated tetraploid *Solanum* species. We show that systemic silencing of a phytoene desaturase gene is observed and maintained throughout the foliar tissues of potato plants and was also observed in tubers. Here we report that VIGS can be triggered and sustained on in vitro micropropagated tetraploid potato for several cycles and on in vitro generated microtubers. This approach will facilitate large-scale functional analysis of potato expressed sequence tags and provide a noninvasive reverse-genetic approach to study mechanisms involved in tuber and microtuber development.

The recent completion of the Arabidopsis genome (Arabidopsis Genome Initiative, 2000; Yamada et al., 2003) has provided a platform to significantly expedite the rate of knowledge acquisition. To fully exploit this sequence information, however, high throughput screens are required to accurately ascribe gene function. In model species such as Arabidopsis, efficient tools for forward and reverse genetics have been developed (Krysan et al., 1999; Springer, 2000; Tani et al., 2004). Furthermore, insertional mutagenesis programs based on T-DNA or transposon tagging have provided valuable resources for the analysis of gene function (Sessions et al., 2002; Pan et al., 2003).

In Arabidopsis and especially other model plant systems such programs suffer from a number of possible limitations, including: lack of genome-wide coverage, gene target bias, lethality, and functional redundancy (Jeong et al. 2002; Gidoni et al., 2003; Kumar and Fladung, 2003). Additionally, for polyploid crop species such as potato (*Solanum tuberosum*), mutagenesis programs are complicated and resource-

intensive. Other approaches, such as dsRNA-mediated suppression of gene expression, have also proved successful in Arabidopsis, although these approaches rely on the generation of large numbers of transgenic lines (Chuang and Meyerowitz, 2000; Smith et al., 2000; Wesley et al., 2001; Harmon and Kay, 2003; Zhao et al., 2003). For many crop species the efficiency of transformation is low and prevents high throughput analysis using this approach.

Virus induced gene silencing (VIGS) is increasingly being used to generate transient loss-of-function assays to assess gene function, as a more rapid alternative to stable transformation (Baulcombe, 1999; Lu et al., 2003a). VIGS triggers an RNA-mediated defense mechanism directly targeting the integrity of the invading viral genome. This sequence-specific phenomenon lowers the titer of the invading virus through an endogenous RNase-inducible mechanism leading to viral RNA degradation (Baulcombe, 1999; Ratcliff et al., 1999; Goldbach et al., 2003). By introducing host cDNA fragments within the viral genome, it is possible to redirect this mechanism to corresponding endogenous host mRNAs, therefore providing a means to down-regulate host gene expression.

VIGS vectors such as potato virus X (PVX), tobacco rattle virus (TRV) or tomato golden mosaic virus (TGMV) have been optimized in the permissive host *Nicotiana benthamiana* (Kjemtrup et al., 1998; Ratcliff et al., 2001). Recent examples show that VIGS can be extended to model plants such as Arabidopsis using the bipartite geminivirus cabbage leaf curl virus (CbLCV; Turnage et al., 2002), crop species such as tomato using a previously characterized TRV VIGS

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vector (Liu et al., 2002), or developed for monocot species such as barley (*Hordeum vulgare*), using barley stripe mosaic virus (BSMV; Holzberg et al., 2002).

The effectiveness of a VIGS vector relies firstly on the ability of the virus to replicate and accumulate to sufficient levels in the host plant to generate dsRNA molecules that initiate silencing, as recent studies have shown that dsRNA generation is a limiting factor in VIGS (Lacomme et al., 2003). Secondly, the virus must be devoid of strong genome-based, posttranscriptional gene silencing-suppressors such as HC-Pro (potyviruses) or 2b (cucumoviruses; Anandalakshmi et al., 1998; Brigneti et al., 1998) that protect the virus against this RNA-mediated resistance mechanism. Given these conditions, it is likely that a plant virus that is able to trigger a significant VIGS response in the host *N. benthamiana*, has the potential to be used for VIGS in a distinct susceptible host.

As some crop species develop unique organs like tubers or edible fruits, model plants such as *Arabidopsis* or *Nicotiana* are often of limited relevance. Potato, which is a member of the Solanaceae family, is the world's fourth largest crop. Furthermore, global production is increasing by 4.5% annually (<http://www.cipotato.org/potato/POTstats.htm>). The availability of a VIGS vector for potato would significantly accelerate the discovery of genes integral to tuber quality or resistance to potato-specific pathogens, for example.

This paper demonstrates that a PVX-based VIGS vector is effective in triggering a VIGS response in both wild diploid and cultivated tetraploid Solanum species. We show that systemic silencing of a phytoene desaturase (*pds*) gene is observed and maintained throughout the foliar tissues of potato plants and is also extended to tubers and in vitro generated microtubers. This offers the potential for large-scale functional screens to identify genes involved in tuber development, metabolism, and foliar or tuber resistance to commercially important pathogens.

RESULTS AND DISCUSSION

PVX Infects Both Diploid and Tetraploid Solanum Species

A susceptible host is a prerequisite for the development of an efficient VIGS system, as viral replication and in planta accumulation of the virus condition the generation of dsRNA molecules that initiate silencing (Voinnet, 2001). Some plant viruses such as PVX have a relatively broad host-range, including several Solanaceae species (Brunt et al., 1996). In this study, a previously described binary PVX-expression vector (Jones et al., 1999; Lu et al., 2003b) was tested for its capacity to infect both wild diploid and cultivated tetraploid Solanum species. Cultivars were selected due either to their ability to be stably transformed and propagated in vitro (*Solanum*

tuberosum L. cv Desiree), or the differential interactions occurring between either susceptible or resistant cultivars (*S. tuberosum* L. cvs Bintje and Stirling, respectively; Birch et al., 1999) to *Phytophthora infestans*, or as a potential source of novel resistance genes to *P. infestans* (*Solanum bulbocastanum*; Song et al., 2003).

Infectivity of PVX in these Solanum hosts was investigated using a PVX construct carrying a GFP insert (PVX.GFP construct, Fig. 1a). Following agro-inoculation of young source leaves of potato plantlets, both infiltrated and systemic upper-uninoculated leaves were harvested. Virus accumulation was monitored by immunodetection of viral PVX coat protein (CP) by western blotting (Fig. 1b).

On the basis of semiquantitative western analysis, more PVX CP was detected in both inoculated and upper-uninoculated (systemic) leaves in *S. bulbocastanum* than in *S. tuberosum* L. cvs Desiree, Stirling, or Bintje (Fig. 1b, lower and upper sections). In *S. bulbocastanum* PVX accumulation was comparable to that observed in *N. benthamiana* at the same time postinoculation on both inoculated and systemic leaves (Fig. 1b). In all Solanum species and cultivars that were tested, PVX-CP was also detected in systemic leaves by 14 dpi (Fig. 1b, upper section). Therefore, all plants tested tolerate substantial PVX accumulation.

The PVX Vector Triggers VIGS of Endogenous *pds* in Foliar Tissues in Solanum Species

The silencing effectiveness of the binary PVX vector was assessed by its ability to silence an endogenous *pds* gene in these different Solanum species. Down-regulation of endogenous *pds* gene expression leads to a characteristic photobleaching phenotype, therefore providing an indication of gene silencing (Kumagai et al., 1995; Ratcliff et al., 2001). As RNA silencing is homology-dependant, a potato *pds* cDNA fragment was subcloned into PVX. The cDNA fragment selected was a region showing sequence identity of 91% with an *N. benthamiana* *pds* cDNA (including stretches of 24, 26, 33 and 47 nucleotides of 100% identity between both cDNAs, Fig. 1c). This would allow silencing of the corresponding genes in both species to compare the relative VIGS. The cDNA region was subcloned in antisense orientation into the PVX vector (construct PVX.PDS_{AS}, Fig. 1a). Following challenge with PVX.PDS_{AS} photobleaching was observed on all *N. benthamiana* plants by 12 to 15 d postinoculation, suggestive of *pds* silencing (Fig. 2l). When the Solanum species and cultivars were infected with PVX.PDS_{AS} white patches of photobleached tissues were observed by 3 weeks post-inoculation in all infected plants (*S. bulbocastanum*, Fig. 2, d and j; *S. tuberosum* L. cvs Bintje, Fig. 2, a, b, and f; Stirling, Fig. 2g; and Desiree, Fig. 2h) as opposed to plants infected with PVX.GFP where no symptoms of PVX infection were visible (*S. bulbocastanum*, Fig. 2c and *S. tuberosum* L. cv Bintje, Fig. 2e). In silenced plants, the degree of photobleaching varied, however, from covering most of the leaf surface for

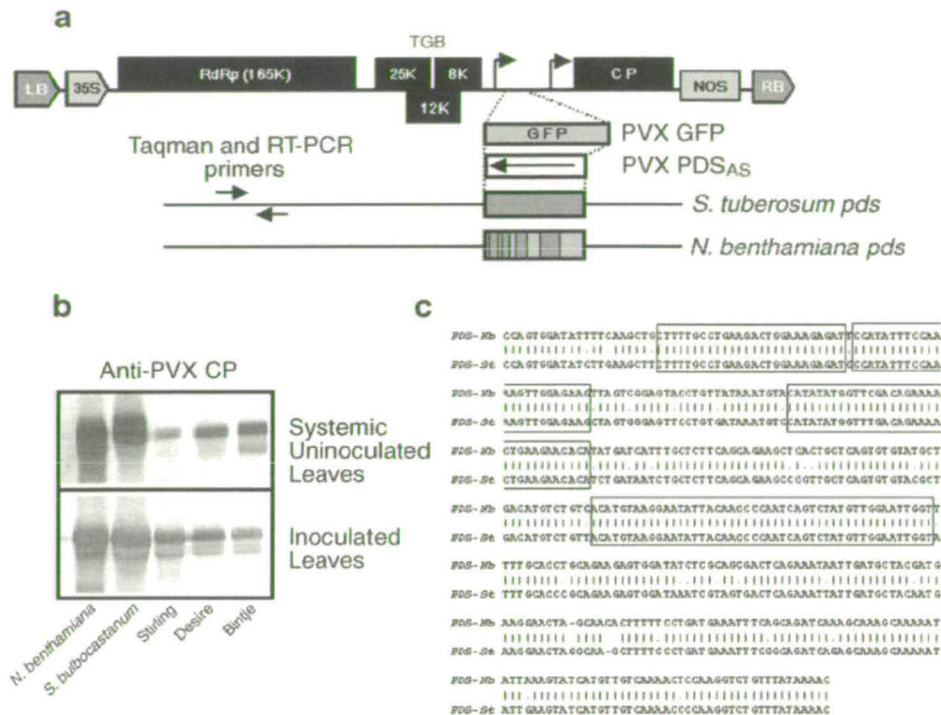


Figure 1. PVX VIGS vector accumulates in a range of Solanum species. **a**, Schematic representation of a PVX vector in the pGREEN0000 binary plasmid: 35S, 35S promoter of cauliflower mosaic virus; RdRp (165K), PVX 165K RNA-dependent RNA polymerase; TGB 25K, 8K, and 12K, PVX triple gene block movement proteins; CP, viral coat protein gene; NOS, nopaline synthase transcriptional terminator; LB and RB, left and right T-DNA border sequences. Schematic representation of *Solanum tuberosum* and *Nicotiana benthamiana* full-length *pds* cDNAs (black line). The *pds* cDNA region from *S. tuberosum* cloned into PVX is represented as a dark gray box. The corresponding region from *N. benthamiana* is boxed in light gray, sequence stretches of more than 21 nucleotides identical to *S. tuberosum* are represented as dark gray rectangles. Arrows indicate the position of the primers used for RT-PCR and real time RT-PCR. **b**, Western-blot analysis of PVX CP protein accumulation in *N. benthamiana*, *S. bulbocastanum*, and *S. tuberosum* cvs in inoculated and systemic upper uninoculated leaves at 15 dpi. **c**, Nucleotide alignment of *pds* cDNA region cloned into PVX from *S. tuberosum* (*PDS-St*) with *N. benthamiana* (*PDS-Nb*). Boxes indicate the identical sequences stretches of more than 21 nucleotides.

S. bulbocastanum (Fig. 2, d and j), to patches uniformly distributed on the leaf surface close to leaf veins for *S. tuberosum* cvs Bintje, Stirling, and Desiree (respectively Fig. 2, f, g, and h). This systemic photobleaching was sustained for the duration of the experiment (up to 3 months postchallenge with PVX.PDS_{AS}) as silenced leaves remained photobleached and newly developing leaves underwent photobleaching as observed in the earlier stages of the VIGS response (Fig. 2, and b; data not shown).

VIGS effectiveness was analyzed at the transcript level by monitoring *pds* mRNA accumulation by reverse transcription (RT)-PCR and real-time RT-PCR. Leaf samples were taken from three to six different plants challenged by either PVX.PDS_{AS} or PVX.GFP (the latter as a control of PVX infection). RT-PCR experiments detected a lower amount of *pds* PCR product in the silenced leaves than in the control samples (Fig. 3a). The levels of control ubiquitin RT-PCR product were similar in all samples tested (Fig. 3a). Real-time RT-PCR was then used to quantify the levels of normalized *pds* mRNA in both silenced and control leaf tissues. A decrease in normalized *pds* mRNA

levels ranging from 70% ± 17% to 84% ± 5% was detected in silenced tissues of *S. tuberosum* cv L. Desiree and *S. bulbocastanum* (Fig. 3b) when compared with PVX.GFP-infected control leaves. A comparable decrease in normalized *pds* mRNA was detected between leaves of diploid *S. bulbocastanum* and tetraploid *S. tuberosum* L. cv Bintje, Stirling, and Desiree (respectively 84% ± 5%, 78% ± 17%, 84% ± 15%, and 70% ± 17%). This decrease in *pds* mRNA was similar to that observed during PVX.PDS_{AS} VIGS in *N. benthamiana* (78% ± 10%, Fig. 3b), although the extent of photobleaching appeared greater than that observed with Solanum leaves (Fig. 2l). This corroborates the fact that in these experiments and as previously reported (Ratcliff et al., 2001; Lacomme et al., 2003), the extent of photobleaching does not always correlate with differences in *pds* mRNA levels.

To further characterize the bleached phenotype in potato following *pds* silencing, the levels of phytoene were quantified by reverse-phase HPLC in extracts from both *N. benthamiana* and potato leaves challenged with either PVX.GFP or PVX.PDS_{AS} VIGS constructs. An increase in the level of phytoene, the substrate for

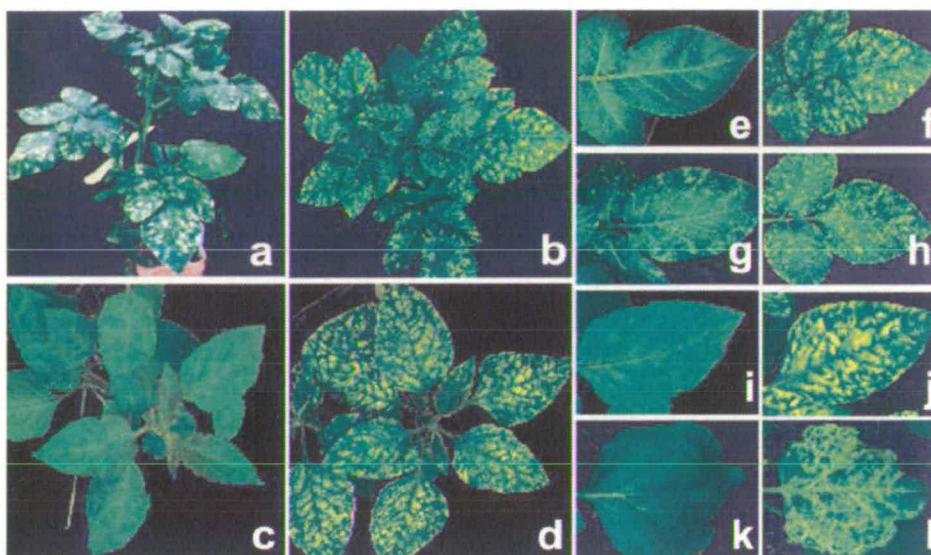


Figure 2. PVX.PDS_{AS} triggers VIGS in diploid and tetraploid *Solanum* species. Photobleaching phenotypes observed by 21 dpi on tetraploid *S. tuberosum* cv Bintje (a and b) and diploid *S. bulbocastanum* (d) and PVX.GFP control infected plant (c). Close-up on photobleached leaves of *S. tuberosum* cvs Bintje (f), Stirling (g), Desiree (h), *S. bulbocastanum* (j), *N. benthamiana* (l), and on symptomless PVX.GFP infected leaf of cv Bintje (e) and *S. bulbocastanum* (i). Uninfected *N. benthamiana* leaf (k).

PDS, is known to occur during VIGS of the *pds* gene (Kumagai et al., 1995). Phytoene also accumulates to high levels in leaves treated with the herbicide norflurazon, a chemical inhibitor of PDS activity (Kumagai et al., 1995). The peaks in the HPLC chromatogram corresponding to cis- and trans-phytoene were identified by comparison between norflurazon-treated and untreated plants and the characteristic absorption spectra of those peaks (Fraser et al., 2000; data not shown). Increase in phytoene accumulation was quantified as the number of area units under phytoene peaks on the HPLC chromatogram (Holzberg et al., 2002).

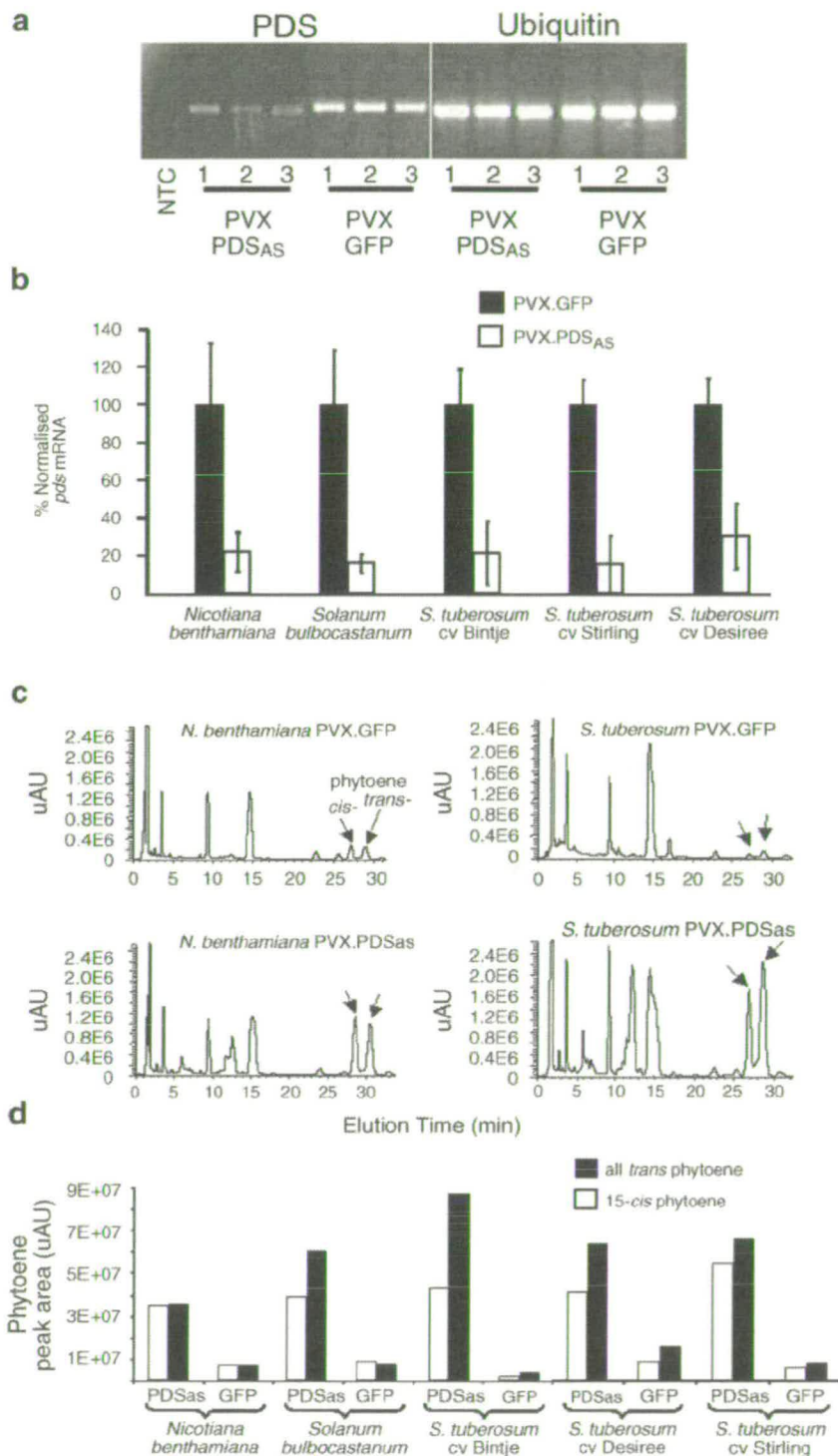
Typical HPLC chromatograms are presented in Figure 3c. In both silenced PVX.PDS_{AS} infected *N. benthamiana* and *S. tuberosum* plants an increase in 15 cis- and trans-phytoene levels was observed (Fig. 3c, lower left and right sections, respectively) in comparison to PVX.GFP control plants. (Fig. 3c, upper section). In silenced *N. benthamiana* leaves, phytoene levels increased by 5- to 10-fold in comparison to PVX.GFP control plants (Fig. 3d). A comparable result was obtained for all *Solanum* species and cultivars tested (Fig. 3d). Although *N. benthamiana* displayed the strongest photobleaching, the increase in phytoene accumulation was lower than that observed in *Solanum* species and cultivars. Moreover, although *S. bulbocastanum* displayed the strongest photobleaching phenotype among the *Solanum* genotypes, similar levels of phytoene accumulation were observed in *S. tuberosum* L cv Desiree and Stirling, and these were lower than that observed in cv Bintje (Fig. 3d). This indicates that the increase in phytoene level may vary from 5- to 10-fold in *Solanum* silenced leaf tissue

despite comparable decreases in *pds* mRNA levels. We thus propose that a similar, if not stronger, VIGS of *pds* in *Solanum* species and cultivars triggers a milder photobleaching phenotype than observed in *N. benthamiana* and this indicates that leaf photobleaching is not quantitatively coupled to the degree of *pds* silencing between different species.

Systemic VIGS of *pds* in Potato Tubers and In Vitro Generated Microtubers

We evaluated the potential of a VIGS-based approach for in vitro grown potato species by down-regulation of *pds* in *S. tuberosum* L cv Desiree micropropagated plants. In vitro material provides a means to substantially reduce glasshouse space requirements for high throughput functional studies. Stab-agroinoculation (Takken et al., 2000; Lu et al., 2003a) of leaves of in vitro grown plants with plated *Agrobacterium* transformed with either PVX.GFP or PVX.PDS_{AS} constructs was an effective method to generate reproducible PVX infections. By 4 weeks postinoculation, development of systemic photobleached areas on leaves was clearly visible on PVX.PDS_{AS} inoculated plants (Fig. 4a, middle and right section) in comparison with PVX.GFP controls (Fig. 4a, left section). Micropropagation of nodal cuttings from silenced plants led to regenerated potato plants displaying a comparable photobleaching phenotype within 3 to 4 weeks (Fig. 4b; data not shown). To determine whether this photobleached phenotype was maintained through several cycles of subculture, micropropagation of the original PVX.PDS_{AS} challenged plant was repeated, and a sustained photobleaching was still observed even after the

Figure 3. Molecular and biochemical characterization of *pds* VIGS in diploid and tetraploid *Solanum* species. a, RT-PCR of *S. tuberosum* cv Bintje *pds*-silenced and control plants in response to challenge with PVX constructs. Both RT-PCR products corresponding to endogenous *pds* and *ubiquitin* mRNAs have been assessed. PCR conditions ranging from 20 to 50 amplification cycles were tested in both cases. Presented here are 30 cycles corresponding to the log-linear phase of amplified PCR product in nonsilenced tissues (challenged PVX.GFP construct). NTC, nontemplate control; replicates are leaves from three different challenged plants. b, Real-time RT-PCR determination of normalized relative amounts of *pds* mRNA levels in silenced and control plants challenged with PVX.PDS_{AS} or PVX.GFP constructs (21 dpi). Sampled leaves were cut in half for either RNA extraction (real-time RT-PCR) or phytoene isolation (HPLC analysis). Values are expressed in percentage of normalized *pds* mRNA related to PVX.GFP control. Values represent the means of at least three leaves from different plants per construct per experiment ±se. For each sample, real-time RT-PCR was carried out in triplicate. c, HPLC analysis of phytoene accumulation induced by PVX.PDS_{AS} VIGS vector. Typical HPLC profile from *S. tuberosum* cv Bintje and *N. benthamiana* is presented here. As before, silenced leaves from plant challenged with PVX.PDS_{AS} and corresponding leaves from PVX.GFP plants were sampled at 21 dpi. Arrows indicate peaks for both 15 *cis*-phytoene and all *trans*-phytoene. d, Quantification of phytoene accumulation in silenced and control *Solanum* species and *N. benthamiana*. Values for 15 *cis*-phytoene and *trans*-phytoene are HPLC area units (arbitrary units) and represent the means of at least three leaves from different plants per construct per experiment (se < 20%).



fifth consecutive subculture (data not shown). The three first subcultures of in vitro grown plants challenged by either PVX.GFP or PVX.PDS_{AS} (subculture 1 and subculture 3) were analyzed using real time RT-PCR. A significant decrease in *pds* mRNA was observed in both subculture 1 and subculture 3 (Fig. 4c; respectively, 70% ± 7% and 63% ± 11%), the latter

representing 12 weeks of in vitro propagation. Phytoene accumulation was measured in these in vitro silenced and control plants. Norflurazon treated in vitro plants developed comparable photobleaching to that observed with glasshouse grown plants, and the chromatogram peak for phytoene was identified as before (data not shown). A similar increase in 15

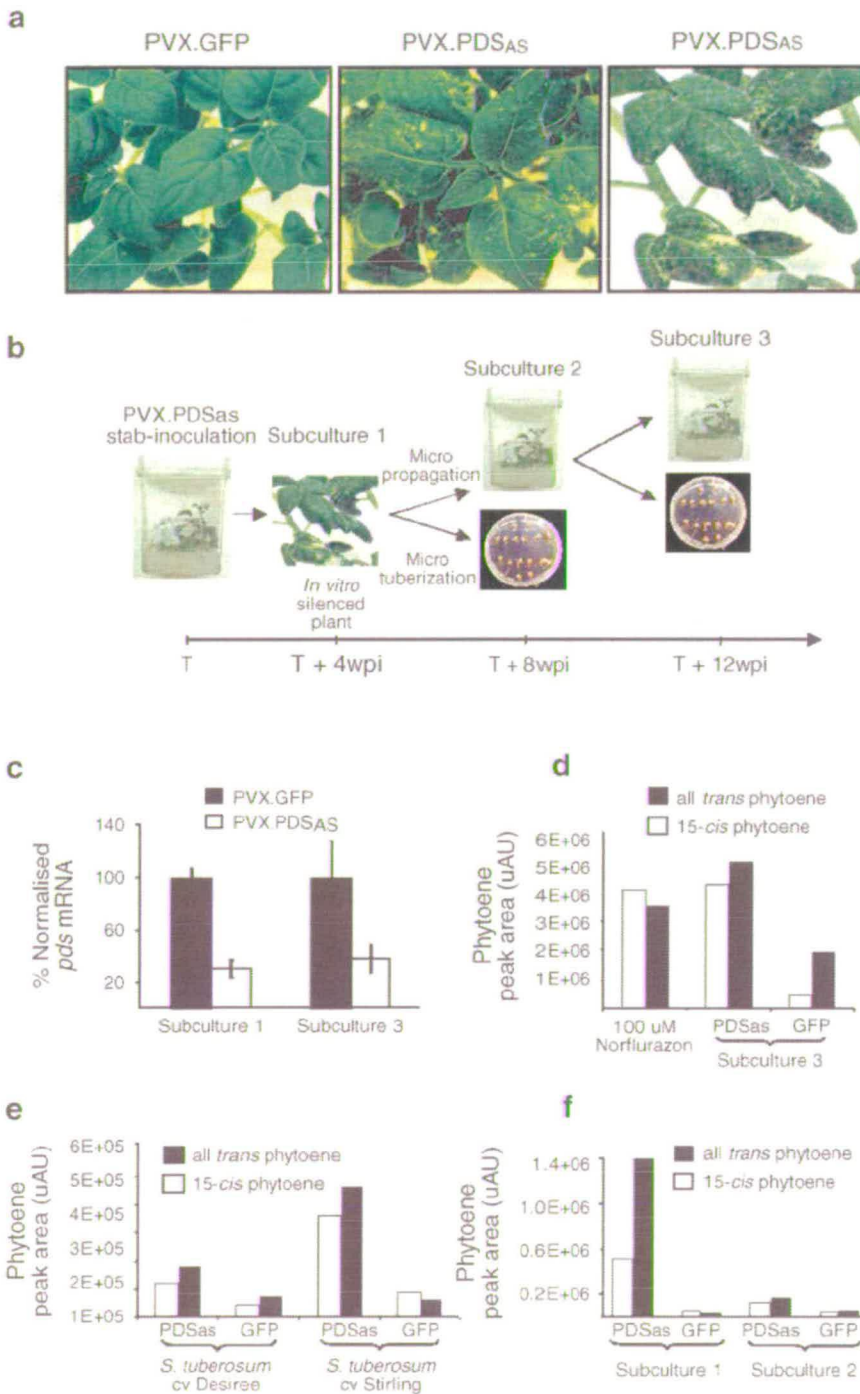


Figure 4. Systemic *pds* silencing in tubers and in vitro propagated plants and microtubers. **a**, Photobleaching phenotypes observed on in vitro propagated *S. tuberosum* cv Desiree after 3 subcultures (representing 12 weeks of in vitro propagation post-challenge with PVX.PDS_{AS}, middle section) or 1 subculture (4 weeks post-challenge with PVX.PDS_{AS}, right section). PVX.GFP control infected plant is shown on the left section. **b**, Schematic representation of in vitro culture and generation of in vitro grown microtubers. Time scale in week post-challenge with PVX construct is presented. **c**, Real-time RT-PCR determination of normalized relative amounts of *pds* mRNA levels in silenced and control leaves from in vitro grown *S. tuberosum* cv Desiree plants challenged with PVX.PDS_{AS} or PVX.GFP constructs. Leaves were harvested after 4 weeks post challenge (28 dpi, subculture 1) or after 12 weeks post challenge (more than 80 dpi, subculture 3). As before, sampled leaves were cut in half for either RNA extraction (real-time RT-PCR) or phytoene isolation (HPLC analysis). Values are expressed in percentage of normalized *pds* mRNA related to PVX.GFP control. Values represent the means of at least three leaves from five different in vitro plants per construct per experiment \pm se. For each sample, real-time RT-PCR was carried out in triplicate. **d**, Quantification of phytoene accumulation in leaves of in vitro *S. tuberosum* cv Desiree challenged with PVX.PDS_{AS} or PVX.GFP. Phytoene accumulation was monitored in norflurazon-treated in vitro plants. Values for 15 *cis*-phytoene and *trans*-phytoene are HPLC area units (arbitrary units) and represent the means of at least three leaves from different plants per construct per experiment ($se < 20\%$). **e**, Quantification of phytoene accumulation in tubers (approximately 15 weeks after challenge with PVX constructs) from silenced (PVX.PDS_{AS} challenged) and control (PVX.GFP challenged) *S. tuberosum* cv Desiree and Stirling. Values for 15 *cis*-phytoene and *trans*-phytoene are HPLC area units (arbitrary units) and represent at least ten tubers from at least 2 different plants per construct per experiment ($se < 20\%$). **f**, Quantification of phytoene accumulation in in vitro generated microtubers from silenced and control *S. tuberosum* cv Desiree, after 8 weeks (subculture 1) or 12 weeks (subculture 2) initial challenge with PVX constructs. Values for 15 *cis*-phytoene and *trans*-phytoene are HPLC area units (arbitrary units) and represent 15 to 20 microtubers generated from 4 to 6 different in vitro grown plants per construct per experiment ($se < 20\%$).

cis- and *trans*-phytoene was observed in both norflurazon-treated and PVX.PDS_{AS} silenced plants (Fig. 4d), ranging from 3-fold (*trans*-phytoene) to 8-fold (15 *cis*-phytoene) in comparison with control plants challenged with PVX.GFP (Fig. 4d) after 3 cycles of micropropagation. This indicates that *pds* VIGS characteristics are similar both in glasshouse and in vitro conditions and confirmed the relative stability of

the VIGS phenotype observed with in vitro grown plants.

Although VIGS proved effective in potato leaves, much research in potato is directed at investigating the tuber life-cycle, improving storage organ quality and resistance to phytopathogens. Therefore, it was important to determine whether gene silencing was observed in tubers. However, a major drawback is the

variability in tuberization time, and the glasshouse space required in making such reverse genetics approach. In vitro grown potato offers an interesting alternative, as in vitro microtuberization is synchronized and controlled (Fig. 4b; Xu et al., 1998). Indeed, fully developed microtubers were obtained by 9 weeks of culture ("Materials and Methods," Fig. 4b), whereas in glasshouse conditions, fully developed tubers were obtained by 12 to 15 weeks post-sowing.

The systemic nature of the VIGS phenotype in tuber tissues from both glasshouse-grown plants and in vitro generated microtubers was investigated. Fully developed tubers from glasshouse plants were obtained by 3 months post-challenge with either PVX.GFP or PVX.PDS_{AS}. Similarly, mature in vitro generated microtubers derived from in vitro control and silenced plants challenged by PVX.GFP or PVX.PDS_{AS} were collected and analyzed by HPLC to monitor the extent of accumulation of phytoene in these organs. HPLC phytoene profiles from tubers harvested from *S. tuberosum* L. cvs Desiree and Stirling plants challenged with PVX.PDS_{AS} indicated, respectively, up to a 2- to 5-fold increase in phytoene accumulation in comparison to control PVX.GFP infected plants (Fig. 4e). In contrast, PVX.PDS_{AS} challenged microtubers showed an accumulation of up to 20-fold more phytoene than control microtubers infected by PVX.GFP (Fig. 4f). A more marginal but significant difference (2-fold) in phytoene increase was still detected in the next generation of subcultured microtubers (subculture 2), indicating a less sustained VIGS phenotype in these tissues and experimental conditions (Fig. 4f) than observed in foliar tissues of in vitro micropropagated plants. These results indicate that systemic silencing of endogenous genes in potato, exemplified here by *pds*, does not only extend to foliar tissues but potentially spreads through the whole plant, including tubers. This silencing state can be transmitted and detected for several generations through vegetative propagation.

This study represents the first demonstration of VIGS-mediated down-regulation of gene expression in both diploid and tetraploid *Solanum* species. Here we report that the carotenoid biosynthetic pathway, where PDS is one of the early steps, can be manipulated by extending the use of a previously described PVX VIGS vector to a different plant host tolerating sufficient levels of PVX accumulation. In parallel, a previously described tobacco rattle virus (TRV, Ratcliff et al., 2001) vector was also tested. However, it did not reach detectable accumulation levels nor did it induce VIGS in the selected tetraploid *S. tuberosum* cultivars (data not shown). In contrast, recombinant PVX.PDS_{AS} vector was detected by RT-PCR in silenced microtubers and in all others silenced organs, including leaves and tubers (data not shown). This emphasizes the prerequisite of a threshold of virus and/or dsRNA accumulation to generate an effective VIGS response.

The microtuberization system, in conjunction with VIGS, has a number of potential benefits compared with analysis of tubers produced conventionally in

glasshouse conditions. Microtubers develop rapidly in a relatively synchronous manner under controlled tissue-culture conditions. This should enable easier identification of tuber phenotype alterations, making it more amenable for characterization of gene function.

The effectiveness of VIGS in potato tubers opens the way for high throughput analysis of gene function to identify genes involved in important traits such as tuber development, metabolism, and pathogen resistance. Furthermore, this reverse genetic VIGS-approach should be particularly powerful in combination with analyses of the transcriptome and metabolome.

MATERIALS AND METHODS

Construction of PVX-Derived Vectors

The PVX vector (pGR106, Jones et al., 1999; Lu et al., 2003b) used in this study was obtained from David Baulcombe (Sainsbury Laboratory, Norwich, UK). PVX.GFP was generated by cloning a PCR fragment amplified from a *gfp* cDNA template (GenBank accession number U62637; Cramer et al., 1996) using specific oligonucleotide primers incorporating *AscI* and *NotI* restriction sites respectively at the 5'- and 3'- termini for cloning into pGR106. The construct PVX.PDS_{AS} was generated by cloning in antisense orientation into pGR106 a *NotI-AscI* 412-bp *pds* cDNA fragment, corresponding to nucleotides 1,133 to 1,529 from *Solanum tuberosum pds* cDNA (GenBank accession number AY484445).

Agrobacterium Infection of Plants

Agrobacterium tumefaciens strain LB4404, carrying the helper plasmid pSoup (Hellens et al., 2000) was transformed with constructs PVX.GFP or PVX.PDS_{AS}. Agroinfiltration of *N. benthamiana* and *Solanum* species with PVX vector was performed as previously described (Lu et al., 2003a). For in vitro agroinoculation, plated individual agrobacteria transformed with PVX.GFP or PVX.PDS_{AS} constructs were picked with a sterile tip and punched onto a leaf of a 2-week old potato plant (Takken et al., 2000).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from frozen control and silenced leaves using the Qiagen RNeasy Plant Mini kit (Qiagen, Valencia, CA), following the manufacturer's instructions. DNaseI treatment and first strand cDNA synthesis were as previously described (Lacomme et al., 2003).

Immunoblot Analysis

Protein extraction and western-blot analysis were as previously described (Lacomme and Santa Cruz, 1999). Membranes were probed with rabbit polyclonal antiserum, raised against the PVX CP as previously described (Santa Cruz et al., 1996).

RT-PCR and SYBR Real-Time RT-PCR Experiments

For RT-PCR analysis, primers that anneal outside the region of the *pds* cDNA cloned into the virus vectors to trigger silencing (nucleotides 1,126–1,516) were used to ensure that only the endogenous *pds* mRNA is reverse-transcribed as indicated in Figure 1a. Potato *ubiquitin* cDNA (GenBank accession no. BQ045862) was used as an internal constitutively expressed control. First-strand cDNA was used as a template for PCR amplification through 20, 25, 30, 40, and 50 cycles. As 30 cycles of amplification was within the log-linear phase of *pds* PCR product amplification in the nonsilenced control samples (data not shown), these conditions were selected for comparison of relative accumulation of both *pds* and *ubiquitin* mRNAs in all samples. The following primers were used: RTPoPDS_{for} (5'-CTC GAG GTC TTC TTT GG-3'); RTPoPDS_{rev} (5'-GTT TAG TTG GGC GTG GAG AA-3'); RTPoUBI_{for} (5'-GCA GTT GGA

GGA CCG AC-3'); and RTPoUBIrev (5'-GGC CAT CTT CCA ACT GTT TC-3'). Similarly, for SYBR real-time RT-PCR (QuantiTect SYBR Green PCR kit, Qiagen, Crawley, UK) experiments, primer pairs were designed outside the region of the *S. tuberosum pds* cDNA targeted for silencing (Fig. 1a) and for the internal control *ubiquitin* cDNA using the Primer Express software supplied with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer's guideline for primer design. The following primers were used: StPDSfwd (5'-CCA AGA CCA GAG CTA GAC AAT ACA GT-3'); StPDSrev (5'-CCA CCC AAA CCT GCA-3'); StUBI1fwd (5'-ACA CCA TTG ATA ATG TCA AGG CTA AG-3'); and StUBI1rev (5'-GCC ATC CTC CAA TTG CTT TC-3'). The GenBank accession numbers for *N. benthamiana pds* and *ubiquitin* cDNA are as previously mentioned (Lacomme et al., 2003). Primer concentrations giving the lowest threshold cycle (C_t) value were selected for further analysis. Detection of real-time RT-PCR products, calculations and statistical analysis were performed as previously described (Lacomme et al., 2003).

Plant Material and Growth Conditions

All work involving virus-infected material was carried out in containment glasshouses under SEERAD license GM/180/2003. Potato cultivars Stirling, Bintje, and Desiree were micropropagated in sterile conditions by removing 5 cm of young potato stems from virus-tested potato plants from the Scottish Agricultural Science Agency (SASA, Edinburgh), removing leaves and dividing the stem into individual pieces, each containing a node with an axillary bud. Six stem pieces were cultivated per petri dish containing 20 mL of Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 20% Suc, 0.8% bacto agar (DIFCO Laboratories, Detroit) and adjusted to pH 5.8, sealed with Nescofilm (Bando Chemical, Kobe, Japan). Plants were then grown at 22°C with 16 h photoperiod and 110 $\mu\text{E m}^{-2} \text{s}^{-1}$. When potato plants were 3 to 4 weeks old, they were then transferred to soil in controlled environment chambers with a 16 h photoperiod (22°C, light intensity ranging from 400–1000 $\mu\text{E m}^{-2} \text{s}^{-1}$).

In Vitro Microtuberization

After 28 d culture, potato plants were divided into single nodes and placed on Murashige and Skoog medium supplemented with 60% Suc, 7 mM chlorocholine chloride (CCC), 8 μM benzylaminopurine (BAP), and 0.8% agar (DIFCO). CCC and BAP were added aseptically after autoclaving by filter sterilizing. Fifteen nodes were placed in each dish and the dishes were sealed with Nescofilm. The cultures were then placed in an incubator in an 8 h photoperiod at 80 $\mu\text{E m}^{-2} \text{s}^{-1}$ at 16°C for 7 d prior to a total darkness at the same temperature for a further 28 d. The microtubers were then harvested and frozen for further analyses.

Extraction and HPLC Analysis of Phytoene from Transfected Plants

Phytoene was extracted from infected leaves, tubers and microtubers. The method used was as described in Holzberg et al. (2002). A total of 50 mg of freeze-dried leaves (3 leaves from different plants), 100 mg of freeze-dried tubers (representing at least 10 mature tubers), or 100 mg of freeze-dried in vitro generated microtubers (representing 15–20 microtubers deriving from at least 4 independent in vitro grown plants) were extracted in 100% methanol. The samples were then centrifuged for 5 min at 4,000 rpm at 4°C. The supernatants were dried under a stream of nitrogen. Residues were redissolved in 500 μL of 100% methanol and 10 μL were separated by HPLC (Surveyor system, Thermo Finnigan, Bellefonte, PA) with a Phenomenex 2 \times 250 mm C-18 column using acetonitrile/methanol/2-propanol (85:10:5, v/v/v) at a flow rate of 300 $\mu\text{L} \times \text{min}^{-1}$. Phytoene was detected with a photodiode array detector using UV absorption at 285 nm and identified by comparing peak retention times with norflurazon-treated and untreated potato plants (Fraser et al., 2000; Holzberg et al., 2002).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY484445.

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LITERATURE CITED

- Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, Smith TH, Vance VB (1998) A viral suppressor of gene silencing in plants. *Proc Natl Acad Sci USA* 95: 13079–13084
- Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant. *Arabidopsis thaliana*. *Nature* 408: 796–815
- Baulcombe DC (1999) Fast forward genetics based on virus-induced gene silencing. *Curr Opin Plant Biol* 2: 109–113
- Birch PRJ, Avrova AO, Lyon GD, Duncan JM, Toth RL (1999) Isolation of potato genes that are induced during an early stage of the hypersensitive response to *Phytophthora infestans*. *Mol Plant Microbe Interact* 12: 356–361
- Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe D (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J* 17: 6739–6746
- Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ, Watson L, Zurcher EJ (1996) Plant viruses online: descriptions and lists from the VIDE database. In AA Brunt, K Crabtree, MJ Dallwitz, AJ Gibbs, L Watson, EJ Zurcher, eds, URL <http://biology.anu.edu.au/Groups/MES/vide/>
- Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 97: 4985–4990
- Cramer A, Whitehorn EA, Tate E, Stemmer WPC (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat Biotechnol* 14: 315–319
- Fraser PD, Pinto ME, Holloway DE, Bramley PM (2000) Technical advance: application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *Plant J* 24: 551–558
- Gidoni D, Fuss E, Burbidge A, Speckmann GJ, James S, Nijkamp D, Mett A, Feiler J, Smoker M, de Vroomen MJ, et al (2003) Multi-functional T-DNA/Ds tomato lines designed for gene cloning and molecular and physical dissection of the tomato genome. *Plant Mol Biol* 51: 83–98
- Goldbach R, Bucher E, Prins M (2003) Resistance mechanisms to plant viruses: an overview. *Virus Res* 92: 207–212
- Harmon FG, Kay SA (2003) The F box protein AFR is a positive regulator of phytochrome A-mediated light signalling. *Curr Biol* 13: 2091–2096
- Hellens RP, Edwards AE, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol Biol* 42: 819–832
- Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J* 30: 315–327
- Jeong DH, An S, Kang HG, Moon S, Han JJ, Park S, Lee HS, An K, An G (2002) T-DNA insertion mutagenesis for activation tagging in rice. *Plant Physiol* 130: 1636–1644
- Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC (1999) RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* 11: 2291–2301
- Kjemtrup S, Sampson KS, Peele CG, Nguyen LV, Conkling MA, Thompson WF, Robertson D (1998) Gene silencing from plant DNA carried by a geminivirus. *Plant J* 14: 91–100
- Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell* 11: 2283–2290
- Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc Natl Acad Sci USA* 92: 1679–1683
- Kumar S, Fladung M (2003) Somatic mobility of the maize element *Ac* and its utility for gene tagging in aspen. *Plant Mol Biol* 51: 643–650
- Lacomme C, Santa Cruz S (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proc Natl Acad Sci USA* 96: 7956–7961
- Lacomme C, Hrubikova K, Hein I (2003) Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *Plant J* 34: 543–553
- Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. *Plant J* 31: 777–786
- Lu R, Martin-Hernandez AM, Peart JR, Malcuit I, Baulcombe DC (2003a) Virus-induced gene silencing in plants. *Methods* 30: 296–303
- Lu R, Malcuit I, Moffett P, Ruiz MT, Peart J, Wu AJ, Rathjen JP, Bendahmane A, Day L, Baulcombe DC (2003b) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J* 22: 5690–5699

- Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiol* **15**: 473–497
- Pan X, Liu H, Clarke J, Jones J, Bevan M, Stein L** (2003) ATIDB: *Arabidopsis thaliana* insertion database. *Nucleic Acids Res* **31**: 1245–1251
- Ratcliff FG, MacFarlane SA, Baulcombe DC** (1999) Gene silencing without DNA: RNA-mediated cross-protection between viruses. *Plant Cell* **11**: 1207–1215
- Ratcliff F, Martin-Hernandez AM, Baulcombe DC** (2001) Tobacco rattle virus as a vector for analysis gene function by silencing. *Plant J* **25**: 237–245
- Santa Cruz S, Chapman S, Roberts AG, Roberts IM, Prior DAM, Oparka KJ** (1996) Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. *Proc Natl Acad Sci USA* **93**: 6286–6290
- Sessions A, Burke E, Presting G, Aux G, McElver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, et al** (2002) A high-throughput *Arabidopsis* reverse genetics system. *Plant Cell* **14**: 2985–2994
- Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM** (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* **407**: 319–320
- Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, et al** (2003) Gene *RB* cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. *Proc Natl Acad Sci USA* **100**: 9128–9133
- Springer PS** (2000) Gene traps: tools for plant development and genomics. *Plant Cell* **12**: 1007–1020
- Takken FLW, Luderer R, Gabriels SHEJ, Westerink N, Lu R, de Wit PJGM, Joosten MHAJ** (2000) A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. *Plant J* **24**: 275–283
- Tani H, Chen X, Nurmberg P, Grant JJ, SantaMaria M, Chini A, Gilroy E, Birch P, Loake GJ** (2004) Activation tagging in plants: a tool for gene discovery. *Integ Funct Genomics* (in press)
- Turnage MA, Muangsan N, Peele CG, Robertson D** (2002) Geminivirus-based vectors for silencing in *Arabidopsis*. *Plant J* **30**: 107–114
- Voinnet O** (2001) RNA silencing as a plant immune system against viruses. *Trends Genet* **17**: 449–458
- Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Signh SP, Abott D, Stoutjesdijk PA, et al** (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* **27**: 581–590
- Xu X, van Lammeren AAM, Vermeer E, Vreugdenhil D** (1998) The role of gibberellin, abscissic acid, and sucrose in the regulation of potato tuber formation *in vitro*. *Plant Physiol* **117**: 575–584
- Yamada K, Lim J, Dale JM, Chen H, Shinn P, Palm CJ, Southwick AM, Wu HC, Kim C, Nguyen M, et al** (2003) Empirical analysis of transcriptional activity in the *Arabidopsis* genome. *Science* **302**: 842–846
- Zhao D, Ni W, Feng B, Han T, Petrusek MG, Ma H** (2003) Members of the *Arabidopsis*-*SKP1*-like gene family exhibit a variety of expression patterns and may play diverse roles in *Arabidopsis*. *Plant Physiol* **133**: 203–217