# The involvement of *cathepsin B-like* genes in disease resistance in *Arabidopsis thaliana*

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# Contents


*Declaration* *vi*
*Acknowledgements* *vii*
*Publications arising from this work* *viii*
*Abstract* *ix*
*List of Figures* *x*
*List of Tables* *xi*
*Abbreviations* *xii*

**1 Introduction**
1.1 Context 1
1.2 The plant immune system 2
1.3 Non-host resistance (PTI) 4
1.4 Suppression of basal resistance (ETS) 9
1.5 R-gene resistance (ETI) 12
1.6 Signalling pathways 15
1.7 The HR: plant PCD 20
1.7.1 The oxidative burst, ROS and NO 22
1.7.2 Developmental PCD: senescence 25
1.7.3 PCD and conserved regulators 26
1.7.4 Proteases and PCD 29
1.8 Previous work 32
1.9 Aims 33

**2 Materials and Methods**
2.1 Plant growth 34
2.1.1 *Arabidopsis thaliana* growth 34
2.1.2 Wheat growth 34
2.1.3 *Nicotiana benthamiana* growth 35
2.2 Pathogen growth and disease resistance assays 35
2.2.1 *Pst* growth and inoculation 35
2.2.2 *Pst* resistance assay 35
2.2.3 *Blumeria graminis* inoculation and disease assay 35
2.2.4 *Erwinia* inoculation and disease assay 36
2.3 Histochemical staining 36
2.3.1 Trypan blue staining 36
2.3.2 DAB staining 37
2.4 RNA extraction 37
2.5 Northern blot 37

2.6 PCR based methods 38
2.6.1 Genotyping of NASC T-DNA knockout mutants 38
2.6.2 RT-PCR 39
2.6.3 Quantitative RT-PCR analysis 40
2.7 Cloning and protein expression 41
2.7.1 Cloning of cathepsin B 41
2.7.2 Protein expression and fraction purification 42
2.7.3 HisBind/ FLAG resin protein purification 42
2.8 SDS PAGE 42
2.9 Western blot analysis 43
2.10 Transgenic line construction and selection 43
2.10.1 Double and triple mutant generation 43
2.10.2 Overexpressor line construction 44
2.10.3 Real time *in planta* imaging of luciferase activity 44
2.10.4 Localisation cloning 44
2.10.5 Transient PVX EPIC expression 45
2.11 Confocal microscopy 45
2.12 Plant protein extraction and activity assays 46
2.12.1 Total protein extraction 46
2.12.2 Apoplastic extraction 46
2.12.3 Cathepsin B substrate assay 46
2.12.4 DCGO4 assay 47
2.12.5 Mass Spectroscopy 47
2.13 Senescence assays 47

**3 Isolation and Construction of Transgenic *Arabidopsis* Lines**
3.1 Arabidopsis *thaliana*: the model plant 48
3.2 Research tools 48
3.3 Isolation of T-DNA insert *cathepsin B* knockout lines 49
3.3.1 Homologue identification 49
3.3.2 Genotyping 50
3.3.3 Knockout confirmation 51
3.4 Double mutant generation and selection 52
3.5 Triple mutant generation and selection 53
3.5.1 Gene silencing using RNA interference 53
3.5.2 RNAi line specificity 54
3.5.3 Triple mutant screening 55
3.6 Overexpressor line construction and selection 56
3.6.1 Splicing of *CathB1* 56
3.6.2 *CathB2* and *3* overexpression 57
3.7 Discussion 59

**4 Cathepsin B in Basal and *R* Gene-Mediated Resistance**

4.1 *Arabidopsis: Pseudomonas* model pathosystem 62
4.2 Forward and reverse genetics 63
4.3 Cathepsin B in basal defence 64
4.3.1 *Cathepsin B* transcription is affected by virulent *Pst* 64
4.3.2 *Cathepsin B* single and double mutants are not susceptible to *Pst* 65
4.3.3 *Cathepsin B* triple knockouts are more susceptible to *Pst* 66
4.3.4 Cell death to *Pst* 67
4.4 Cathepsin B in *R* gene-mediated defence 68
4.4.1 *Cathepsin B* expression is affected by *Pst AvrB* 68
4.4.2 *Cathepsin B* mutants do not enhance susceptibility to *Pst AvrB* 69
4.5 Cathepsin B attenuates the HR to *AvrB* 70
4.6 Discussion 71

**5 Cathepsin B Involvement in Non-Host Resistance**

5.1 *Blumeria graminis* powdery mildews 74
5.2 Non-host resistance to powdery mildews 75
5.3 Previous work and aims 76
5.4 *Cathepsin B* knockout lines are more susceptible to *Bgt* 76
5.4.1 *Bgt* development is more advanced on *cathepsin B* double mutants 76
5.4.2 Quantification of fungal development 78
5.5 Epidermal cell death 78
5.6 Defence gene expression 79
5.7 *Cathepsin B* expression in response to *Bgt* 80
5.8 Discussion 81

**6 Cathepsin B Involvement in Senescence**

6.1 Senescence: developmental PCD 85
6.2 Diverse factors are implicated in senescence 85
6.3 Proteases involved in senescence 86
6.4 *Cathepsin B* mutants show a slight delay in senescence 87
6.5 *Cathepsin B* expression in dark-induced senescence 89
6.6 *SAG12* is down-regulated in *cathepsin B* mutants 90
6.7 Off target silencing in not possible between *CathB2* and *SAG12* 91
6.8 Discussion 92

**7 Cathepsin B Localisation**

7.1 Localisation 95
7.1.1 Where and why? 95
7.1.2 Tools and technology 95
7.2 Mammalian cathepsin localisation 96
7.3 Plant protease localisation 97
7.4 Localisation aims 99
7.5 NbCathB is a secreted protein 100
7.5.1 NbCathB::GFP 100
7.5.2 mRFP Localisation 101
7.5.3 Co-infiltration with a membrane marker 103
7.6. mRFP is Cleaved on Secretion 104
7.7 Secreted cathepsin B from the OX mRFP construct is active 106
7.8 Discussion 107

**8 Cathepsin B Activity and Inhibitors**

8.1 Use of inhibitors to infer protease involvement in plant defence 111
8.2 Pathogen protease inhibitors as effectors 112
8.3 Cathepsin B activity aims 112
8.4 Recombinant *cathepsin B* expression in *E. coli* 113
8.5 Commercial cathepsin B inhibitors 115
8.6 Pathogen-derived cysteine protease inhibitors 117
8.6.1 Avr2 from *C. fulvum* 117
8.6.2 EPIC inhibitors from *P. infestans* 118
8.7 Discussion 120

**9 General Discussion and Future Work**

9.1 Cathepsin B positively regulates PCD in plants 123
9.2 Uncoupling of PCD and defence in *cathepsin B* mutants 127
9.3 Cathepsin B is an extracellular enzyme 128
9.4 Conclusions 130

**10 References** 132

Appendix I Additional primer sequences 160
Appendix II Schematic diagrams of localisation and protein expression constructs 163
Appendix II Gilroy et al., 2007 164

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## Publications arising from this work

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**McLellan, H., Gilroy, E.M., Yun, B-W., Birch, P.R.J., and Loake, G.J.** (2008). Functional redundancy in the cathepsin B gene family contributes to basal and non-host disease resistance and senescence in *Arabidopsis thaliana*. *In preparation*.

# Abstract

Proteases have long been associated with apoptosis, a form of Programmed Cell Death (PCD) in mammals and there is considerable interest in investigating conserved roles for proteases in the Hypersensitive Response (HR), a plant defence response involving PCD, which shares some morphological characteristics with apoptosis. A gene encoding a cysteine protease, with homology to mammalian cathepsin B proteases, was isolated in a screen for genes up-regulated in the HR. Previous work, investigating possible roles of a *Nicotiana benthamiana cathepsin B* (*NbCathB*) homologue in plant defence, found that either *NbCathB* silencing, or peptide inhibition of cathepsin B activity, caused a reduction in the HR and corresponding increase in susceptibility in certain plant-pathogen interactions. The focus of this current research is to examine the roles of cathepsin B genes in the model plant *Arabidopsis*: a more genetically tractable system.

There are three cathepsin B homologues in *Arabidopsis* for which knock-out T-DNA insert lines were isolated. Double mutants were generated using genetic crossing and triple mutants were generated by RNAi. These genes were found to act redundantly with triple mutants showing increased susceptibility to virulent but not avirulent strains of *Pseudomonas syringae* DC3000. Moreover, these genes are also involved in non-host resistance to fungal pathogen *Blumeria graminis* f.sp. *tritici,* where they positively regulate the HR but negatively regulate *Pathogenesis-Related 1* (*PR1*) expression downstream of *Enhanced Disease Susceptible 1* (*EDS1*).

In addition, this work also implicates *cathepsin B* genes in senescence, a developmental form of PCD, via regulation of the senescence marker gene *Senescence Associated Gene 12* (*SAG12*). Furthermore, it was shown that NbCathB is localised to the plant apoplast where it is activated upon secretion. Partially purified cathepsin B protein was inhibited by a variety of peptide inhibitors but evidence of inhibition by several pathogen-derived inhibitors that are secreted during infection was inconclusive.

# List of Figures


| Figure | Title | Page |
|---|---|---|
| 1.1 | A zigzag model illustrates the quantitative output of the plant immune system | 3 |
| 1.2 | A current model for flagellin perception and signalling in plants | 7 |
| 1.3 | Representation of the location and structure of plant disease resistance proteins | 14 |
| 1.4 | Defence signalling pathways and signalling crosstalk | 20 |
| 1.5 | Apoptosis regulation in different Eukaryotes | 28 |
| 3.1 | Position of T-DNA inserts | 50 |
| 3.2 | Examples of T-DNA genotyping using PCR | 51 |
| 3.3 | RT-PCR gene knockout confirmation | 52 |
| 3.4 | RT-PCR showing double knockout mutants | 53 |
| 3.5 | Schematic diagrams of RNAi Constructs | 54 |
| 3.6 | RNAi construct specificity | 55 |
| 3.7 | Quantification of *CathB2* expression levels in homozygous #62 and #57 plants | 56 |
| 3.8 | Alternative Splicing of *At*1g02300 | 57 |
| 3.9 | OX line construction and selection | 58 |
| 3.10 | Northern showing *CathB* expression in putative overexpressor lines | 59 |
| 4.1 | *Cathepsin B* expression in Col-0 in response to challenge with virulent *Pst* DC3000 | 65 |
| 4.2 | *Pst* DC3000 colony counts with *cathb* transgenic lines | 66 |
| 4.3 | *Pst* DC3000 colony counts with triple knockouts | 67 |
| 4.4 | Cell death post-inoculation with *Pst* | 68 |
| 4.5 | *Cathepsin B* expression in Col-0 in response to challenge with *Pst* DC3000 *AvrB* | 69 |
| 4.6 | Colony counts with *Pst AvrB* | 70 |
| 4.7 | Cell death post-inoculation with *Pst AvrB* | 71 |
| 5.1 | Schematic diagram of *Bgt* infection | 75 |
| 5.2 | *Bgt* fungal development on *Arabidopsis* | 77 |
| 5.3 | Quantification of *Bgt* development | 78 |
| 5.4 | Percentage epidermal cell death to *Bgt* | 79 |
| 5.5 | Defence gene expression to *Bgt* | 80 |
| 5.6 | *Cathepsin B* expression in Col-0 and *eds1* in response to *Bgt* challenge | 81 |
| 5.7 | Model integrating cathepsin B into known components of NHR to *Bgt* in *Arabidopsis* | 84 |
| 6.1 | Dark-induced senescence phenotype in *Arabidopsis* lines | 88 |
| 6.2 | Progression of dark-induced senescence quantified using percentage chlorophyll | 88 |
| 6.3 | Relative expression of *cathepsin B* genes in dark-induced senescence in wildtype and *ein2-1 Arabidopsis* | 90 |
| 6.4 | *SAG12* expression in WT and *cathb* triple mutants during dark-induced senescence | 91 |
| 7.1 | Classification of *Arabidopsis* proteases | 99 |
| 7.2 | The effect of BFA on NbCathB::GFP fluorescence | 101 |
| 7.3 | The effect of BFA on mRFP fluorescence | 102 |
| 7.4 | Cathepsin B is localised to the apoplast | 103 |
| 7.5 | mRFP is cleaved from cathepsin B on secretion | 105 |
| 7.6 | Identification of cathepsin B peptides by Mass Spectroscopy | 105 |
| 7.7 | Protease profiling and cathepsin B activity | 107 |

# List of Figures continued


| Figure | Title | Page |
|---|---|---|
| 8.1 | SDS PAGE gel of crude cell culture extract | 113 |
| 8.2 | FLAG-tagged recombinant cathepsin B protein production and purification | 114 |
| 8.3 | FLAG-tagged recombinant cathepsin B protein activity assays | 115 |
| 8.4 | Commercial inhibitors target cathepsin B activity | 116 |
| 8.5 | Avr2 does not target cathepsin B activity | 118 |
| 8.6 | Reduction in HR to *E. amylovora* in PVX-EPIC expressing plants | 119 |
| 9.1 | A model proposing how PCD signalling may be regulated via CathB–EDS1-CPR5 interactions | 126 |


# List of Tables


| Table No. | Title | Page No. |
|---|---|---|
| 1.1 | Pathogen Effectors: functions and targets | 11 |
| 2.1 | *Arabidopsis* control wildtype and transgenic lines | 34 |
| 2.2 | Primers used to generate probes | 38 |
| 2.3 | Gene specific primers designed for T-DNA mutant genotyping | 39 |
| 2.4 | Primers for RT-PCR | 40 |
| 2.5 | qRT-PCR Primers | 41 |
| 2.6 | Transgenic *cathepsin B* lines generated | 44 |

# Abbreviations

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| AGI | *Arabidopsis* genome initiative |
| *At* | *Arabidopsis thaliana* |
| *Avr* | Avirulence gene |
| *Bgt* | *Blumeria graminis* f. sp. *tritici* |
| BLAST | Basic local alignment search tool |
| bp | Base pair |
| BZIP | Basic leucine zipper |
| CathB | Cathepsin B |
| CC | Coiled-coil |
| CD | Cell death |
| Cfu | Colony forming units |
| Col-0 | *Arabidopsis* ecotype Columbia-0 |
| DNA | Deoxyribonucleic acid |
| *E.coli* | *Escherichia coli* |
| EDS1 | Enhanced disease susceptibility 1 |
| EPI | Extracellular protease inhibitor |
| EPIC | Extracellular protease inhibitor cystatin-like |
| ET | Ethylene |
| ETI | Effector-triggered immunity |
| ETS | Effector-triggered susceptibility |
| FLS2 | Flagellin-sensing 2 |
| g | Gram |
| GFP | Green fluorescent protein |
| GSNOR | S-nitrosoglutathione reductase |
| GST | Glutathione S-transferase |
| HR | Hypersensitive response |
| IPTG | Isopropyl-l-thio-3-D-galactoside |

| | |
|---|---|
| JA | Jasmonic acid |
| KB | King's broth media |
| Kda | Kilodalton |
| KO | Knockout |
| LMM | Lesion mimic mutant |
| LRR | Leucine-rich repeat |
| MAPK | Mitogen activated protein kinase |
| MS | Murashige and Skoog media |
| MW | Molecular weight standard |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| *NahG* | Salicylate hydroxylase gene |
| NASC | Nottingham Arabidopsis stock centre |
| *Nb* | *Nicotiana benthamiana* |
| NBS | Nucleotide-binding site |
| NO | Nitric oxide |
| $ONOO^-$ | Peroxynitrite |
| OX | Overexpressor |
| qRT-PCR | Quantitative real-time PCR |
| PAMP | Pathogen associated molecular pattern |
| PCD | Programmed cell death |
| PCR | Polymerase chain reaction |
| PDF | Plant defensin |
| PR | Pathogen related protein |
| *Pst* DC 3000 (*AvrB*) | *Pseudomonas syringae* pv *tomato* DC3000 carrying *AvrB* |
| *Pst* DC 3000 | *Pseudomonas syringae* pv *tomato* DC3000 |
| PTI | PAMP-triggered immunity |
| PVX | Potato virus X |
| *R* | *Resistance* gene |
| RCD | Runaway cell death |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |

| | |
|---|---|
| SA | Salicylic acid |
| SAR | Systemic acquired resistance |
| SDW | Sterile distilled water |
| *St* | *Solanum tuberosum* |
| TAIR | The *Arabidopsis* information resource |
| T-DNA | Transfer DNA |
| TIR | Toll/Interleukin-1 receptor |
| TMV | Tobbacco mosaic virus |

# 1. Introduction

## 1.1. Context

As plants are sessile organisms they must endure or adapt to pressures in the surrounding environment. One such stress is assault by pathogens. Plants are subject to attack by a wide range of different pathogens, including bacteria, fungi, oomycetes, viruses and nematodes. These organisms employ an extensive assortment of infection strategies which must be recognised by the plant before appropriate action can be taken. Remarkably, disease only occurs in the minority of interactions, with resistance being the *status quo*.

Nonetheless, disease in crop plants is still of great economic importance with global pre- and post-harvest crop losses of >12% due to various pathogens, even after the application of diverse chemical pesticides and fungicides (Agrios, 1997; Shah, 1997; Oerke, 2006). Moreover, many farmers in developing countries are unable to afford costly systems of chemical crop protection and so sustain higher losses. There are also growing concerns in more developed countries about the deleterious effects of excessive use of chemicals. With the world's population predicted to increase to 8 billion by 2025 (Huang et al., 2002) there is growing pressure to boost global food production. Traditionally, crop breeding has been used to introduce genes conferring disease resistance. However, breeding programmes can take substantial amounts of time and effort and the resulting resistance is not durable (Pink and Puddephat, 1999).

More recently, biotechnological approaches have been used in order to introduce resistance genes into plants more rapidly, producing genetically modified (GM) crops, despite the growing blacklash against GM technology due to fears for food safety and the 'escape' of transgenes into wild relatives (Malarkey, 2003; Thompson et al., 2003):

21 countries were growing 90 million hectares of approved GM crops by the tenth anniversary of the commercialisation of the first varieties (James, 2006). While there has been considerable success with the generation of GM crops resistant to insect pests and viruses (Gonsalves, 1998; Bates et al., 2005), various attempts to engineer durable, broad-spectrum resistance to bacterial, fungal and oomycete pathogens have failed (Hammond-Kosack and Parker, 2003; Gurr and Rushton, 2005). Plant pathogen interactions are undoubtedly complex. More research into plant disease resistance is required to facilitate the discovery of new genes and development of novel strategies which can be utilised in tandem with sustainable agriculture to ensure crop production meets global demand, as well as adding to our existing knowledge of plant pathology.

## 1.2. The plant immune system

Currently, two types of plant disease resistance are recognised: host and non-host resistance (NHR), although there is a move in the current literature to refer to these as effector-triggered immunity (ETI) and pathogen associated molecular pattern (PAMP)-triggered immunity (PTI), respectively (Jones and Dangl, 2006). Host resistance has been well studied and is dependent on specific genetic interactions, also called *Resistance* (*R*) gene-mediated resistance or ETI. This works on a gene-for-gene basis and permits a single plant ecotype resistance against a single race of pathogen (Flor, 1971). In contrast, relatively little is known about NHR or PTI which is said to occur when an entire plant species is resistant to all the given races of a pathogen. NHR is thought to be broad-spectrum, enduring and involving multiple genes (Heath, 2000; Zimmerli et al., 2004).

A model has been put forward by Jones and Dangl (2006) to explain how these different plant resistance responses integrate to form the plant immune system. The zigzag model (Fig. 1.1) proposes that perception of conserved PAMPs enables plants to mount an

effective NHR/ PTI response which restricts the growth of the majority of potential pathogens. Determined pathogens have evolved to produce effector molecules, also called Avirulence (Avr) proteins, which can suppress this basal resistance causing effector triggered susceptibility (ETS). However, plants have concomitantly evolved mechanisms to recognise these effectors: *R* gene-mediated resistance or ETI and increase the amplitude of the defence response. This response can subsequently be suppressed by the development of new pathogen effectors and then re-activated by the acquisition of new plant recognition specificity: and so the evolutionary arms race continues (Jones and Dangl, 2006).

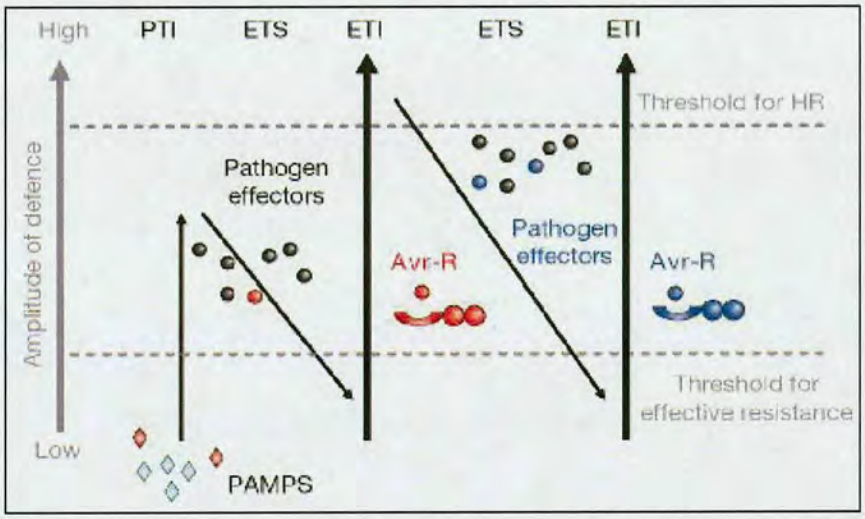


**Figure 1.1. A zigzag model illustrates the quantitative output of the plant immune system.** Plants detect pathogen-associated molecular patterns (PAMPs, red diamonds) to trigger PAMP-triggered immunity (PTI). Successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). One effector (indicated in red) is recognized by *R* gene-mediated resistance, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR): an effective defence response. Pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favours new plant R gene alleles that can recognize one of the newly acquired effectors, resulting again in ETI. Source: (Jones and Dangl, 2006)

## 1.3. Non-host resistance (PTI)

The facts that disease only results in a small minority of plant–pathogen interactions and that most pathogens are unable to widen their range of host species show the effectiveness of NHR (Nurnberger and Lipka, 2005). Currently, there are two categories of NHR: Type I which shows no apparent symptoms and Type II which is mediated via non-host hypersensitive cell death; the type of response which occurs tends to depend on both the pathogen and plant involved (Mysore and Ryu, 2004). The first line of defence in non-host interactions comprises preformed defences. These can include physical barriers such as the waxy cuticle, cell wall and the cytoskeleton or the constitutive accumulation of secondary metabolites such as phytoanticipins which have antimicrobial activity (Mysore and Ryu, 2004; Nurnberger and Lipka, 2005; Ingle et al., 2006). The second line of defence consists of a plethora of pathogen-inducible mechanisms which are thought to include, but are not limited to, production of reactive oxygen species (ROS), cell wall cross-linking and reinforcement, the accumulation of phytoalexins such as camalexin and the hypersensitive response (HR): a form of programmed cell death (PCD), which will be discussed in detail subsequently (Mysore and Ryu, 2004; Nurnberger and Lipka, 2005; Ingle et al., 2006).

In order for such inducible defences to be triggered the plant must first perceive the presence of potential pathogens. Emerging evidence suggests that this perception is mediated by detection of PAMPs, suggesting similarities with mammalian innate immunity (Zipfel, 2008). PAMPs are microbial elicitors which tend to be conserved between related microbial groups and perform an essential function so that they cannot easily be jettisoned in order to evade recognition. Examples of known PAMPs include flagellin and lipopolysaccharide (LPS) from bacteria, chitin and ergosterol from fungi, and glucans from oomycetes (Nurnberger et al., 2004). Resistance prompted by PAMP detection is termed PTI and occurs via pattern recognition receptors (PRRs). The best studied example of a PRR is flagellin-sensing 2 (FLS2) from *Arabidopsis* which

recognises the epitope flg22: a conserved peptide of the protein flagellin. *FLS2* encodes a receptor-like kinase (RLK) comprising an extracellular leucine rich repeat (LRR) domain, a single transmembrane spanning region and an intracellular kinase domain (Gomez-Gomez and Boller, 2000, 2002). *Arabidopsis* and *Nicotiana benthamiana fls2* mutants show increased susceptibility to a range of non-host bacterial pathogens (de Torres et al., 2006; Hann and Rathjen, 2007). There are >600 *RLK* genes present in the *Arabidopsis* genome (Zipfel, 2008) and another of these has been shown to be the receptor for the recognition of a second bacterial PAMP, elongation factor tu (EF-Tu). The EF-Tu receptor (EFR) belongs to the same sub-family as FLS2 and recognises the elf18 epitope of EF-Tu (Zipfel, 2006). Interestingly, these two PRRs activate similar downstream responses and show a requirement for BRI1-associated receptor kinase 1 (BAK1). *Arabidopsis bak1* mutants show perturbed responses to flg22 and elf18 perception (Chinchilla et al., 2007; Heese et al., 2007). BAK1 is a RLK required for signal transduction by brassinosteroid insensitive 1 (BRI1), another RLK which is a hormone receptor involved in the regulation of plant growth and development (Li, 2002; Li and Jin, 2007). To date, no other PRRs have been identified although many different PAMPs are known to trigger PTI responses in plants (Zipfel, 2008).

Downstream signal transduction in response to non-host pathogen perception is thought to be mediated by calcium, ROS generation, mitogen-activated protein kinase (MAPK) cascades and WRKY transcription factor expression (Ingle et al., 2006; Chinchilla et al., 2007). Calcium influxes are one of the most rapid responses to pathogen or elicitor treatment and are known to be required for the subsequent generation of ROS and phytoalexins, as treatment with calcium channel blockers can eliminate these responses (Blume et al., 2000). The oxidative burst which generates ROS, such as superoxide and hydrogen peroxide, is also a rapid response to pathogen challenge. These ROS are predicted to play various roles in signalling, cell wall reinforcement and microbial toxicity, as reviewed in Grant and Loake (2000) and Torres *et al.* (2006). The accumulation of phytoalexins such as camalexin is thought to be important in NHR to

some pathogens, as *phytoalexin deficient 3* (*pad3*) *Arabidopsis* mutants are more susceptible to the necrotrophic fungal pathogen *Alternaria brassicicola* (Thomma et al., 1999). MAPK cascades are ubiquitous pathogen response signal transduction mechanisms which work by transferring a phosphate group sequentially from a MAPK kinase kinase (MAPKKK) to a MAPK kinase (MAPKK) to a MAPK and on to downstream targets thus regulating their activity (Ingle et al., 2006). A complete MAPK cascade in *Arabidopsis* which is triggered by FLS2-mediated PAMP perception has been identified (Asai et al., 2002; Meszaros et al., 2006). This cascade has been shown to increase the expression of WRKY transcription factors which are known to be important for the induction of various defence genes (Eulgem et al., 2000; Eulgem, 2006). Figure 1.2 taken from Ingle et al. (2006) summarises some of the information known about PAMP perception and signal transduction via FLS2.

Another intriguing gene which has emerged in recent studies searching for mediators of NHR is *non-host 1 (NHO1)*. Pathogen and flg22-induced *NHO1* encodes a glycerol kinase which is required to arrest the growth of non-host strains of *Pseudomonas syringae* bacteria *P. syringae* pv *phaseolicola, P. syringae* pv *tabaci* and *P. syringae* pv *fluorescens* in *Arabidopsis* (Lu et al., 2001; Kang et al., 2003).

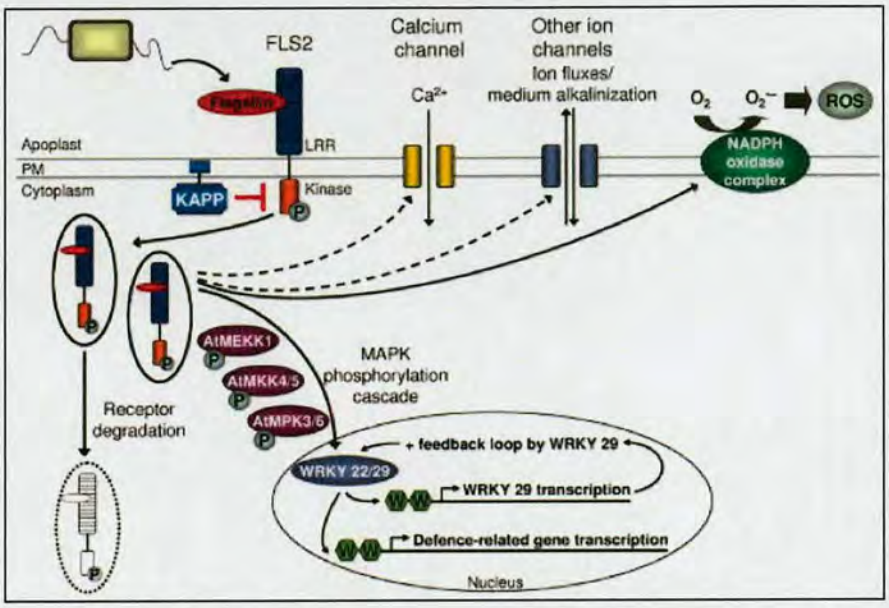


**Figure 1.2. A current model for flagellin perception and signalling in plants.** Flagellin interacts with the extracellular LRR domain of FLS2. Autophosphorylation of the FLS2 kinase domain is crucial for ligand binding and signalling. KAPP acts as a negative regulator of signal transduction. After ligand binding, FLS2 accumulates in mobile intracellular vesicles and is then degraded. Calcium fluxes occur in response to flagellin. Plasma membrane calcium channels are illustrated but the source of the calcium increase has not yet been determined. Other cellular responses include medium alkalinization and production of ROS by NADPH oxidase. Dashed arrows indicate flagellin-induced responses for which the requirement of flagellin binding has not been directly shown. FLS2 kinase activity is required for the activation of a MAPK cascade. The phosphorylated AtMEKK1 phosphorylates AtMKK4 and AtMKK5 that phosphorylate and activate AtMPK3 and AtMPK6 leading to expression of the transcription factors WRKY22 and WRKY29 which regulate the expression of defence genes. Source: (Ingle et al., 2006)

Three *penetration* (*PEN*) genes in *Arabidopsis* were found to impose NHR to epidermal cell penetration by the fungal pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*) (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). *PEN1* encodes a syntaxin which was found to be localised to the plasma membrane: syntaxins are part of the SNARE complex which is responsible for membrane fusion events. *Pen1* mutants are delayed in the formation of cell wall appositions which prevent *Bgh* penetration. Therefore, PEN1 is proposed to mediate polarised secretion, although it appears to have additional roles as a negative regulator of the HR and defence signalling (Collins et al., 2003; Assaad et al., 2004; Zhang et al., 2007).

*PEN2* encodes a glycosyl hydrolase which localises to peroxisomes which have been observed to accumulate at potential *Bgh* entry sites (Lipka et al., 2005). As a catalytically inactive form of PEN2, $PEN2_{E183D}$ is unable to complement *pen2* mutants it has been suggested that PEN2 activity is responsible for production of a toxic metabolite which is then released from peroxisomes in response to fungal attack (Lipka et al., 2005).

*PEN3* encodes an ATP binding cassette transporter which is also known as PDR8 and *pen3* mutants display increased penetration and fungal development in response to *Bgh* infection (Stein et al., 2006). PEN3 is normally dispersed throughout the plasma membrane but re-localises in infected cells to concentrate around attempted penetration sites. Stein *et al.* (2006) propose that PEN3 exports a toxic compound in a targeted manner which poisons the fungal penetration peg halting its growth. Moreover, the increased fungal development observed in *pen3* mutants but not in *pen1* and *pen2* mutants can be explained by the fact that PEN3 was also observed to localise around the haustorium where it is expected to export toxins into the extrahaustorial matrix, thus halting post-invasive fungal growth (Stein et al., 2006).

In spite of the discovery of these genes through screens specifically for factors which are involved in NHR, some have also been demonstrated to show responses to host-adapted pathogens (Lu et al., 2001; Kang et al., 2003; Stein et al., 2006). Furthermore, genes which are already known components of other signalling pathways, which will be discussed later, are also predicted to play various roles in NHR. This suggests that there may be many conserved regulators of plant disease resistance, and host resistance and NHR may be differentiated partially by the means of pathogen perception.

## 1.4. Suppression of basal resistance (ETS)

It is now known that many of the components of basal resistance are also utilised in NHR/ PTI leading to the theory that: (1) basal defence is essentially NHR which is ineffective due to pathogen suppression and (2) that non-host pathogens are able to become host pathogens by manipulating plant basal defences using effectors: this is said to be effector triggered susceptibility (ETS) (Ingle et al., 2006; Jones and Dangl, 2006). A recent study by Ham et al. (2007) demonstrates that NHR to *P. syringae* pv *phaseolicola* in *Arabidopsis* is comprised of multiple layers of basal defences. Furthermore, they show that mutation in several of these layers or pathways in combination with the expression of effectors from virulent *P. syringae* strains is necessary and sufficient to disrupt NHR and allow *P. syringae* pv *phaseolicola* to grow on *Arabidopsis* as a host pathogen (Ham et al., 2007).

It is thought that bacterial pathogens such as *P. syringae* and *Xanthomonas* spp. are able to translocate their effector proteins inside plant cells using a type three secretion system (T3SS), as it has long been known that T3SS mutants trigger basal defence and T3SS wildtype bacteria can suppress this induction (Jakobek et al., 1993; Brown et al., 1995; Keshavarzi et al., 2004; Nomura et al., 2005). The T3SS is encoded by a set of *hypersensitive response* and *pathogenicity* (*HRP*) genes, which were identified for their roles in virulence. The T3SS acts as a molecular syringe, injecting effectors into the cytoplasm. Similar T3SS are used by animal bacterial pathogens to introduce virulence factors inside their host's cells (Galan and Collmer, 1999).

However, fungal and oomycete pathogens do not possess a T3SS to translocate their effectors, so have developed other mechanisms. Some fungal effectors are known to be secreted into the plant apoplast where they are able to interact with and/ or inhibit the action of extracellular targets. An example from the fungal pathogen of tomato, *Cladosporium fulvum*, is Avr2, which interacts with and inhibits the apoplastic cysteine

protease RCR3 (Rooney et al., 2005). A second secreted *C. fulvum* effector, Avr4, is able to bind chitin, a fungal PAMP, in the *C. fulvum* cell wall, thus preventing its hydrolysis by plant defensive chitinases. Furthermore, expression of Avr4 in tomato and *Arabidopsis* renders them more susceptible to other fungal pathogens (van Esse et al., 2007). Nevertheless, some fungal effectors such as AvrPita from *Magnaporthe grisea* are detected in the cytoplasm (Jia et al., 2000) suggesting that fungi have developed some unknown mechanism for translocating effectors.

Oomycetes also secrete some effectors into the apoplast. *Phytophthora infestans*, an oomycete pathogen of potato and tomato, secretes both serine and cysteine protease inhibitors into the apoplast upon infection; these effectors interact with and inhibit plant defensive proteases such as P69 and PIP1 (Tian et al., 2004; Tian et al., 2005; Tian et al., 2007). Moreover, *P. infestans* and other oomycete pathogens have developed a mechanism of translocating their effectors inside plant cells where they have access to more potential targets. This translocation mechanism is based on an RXLR-EER protein motif which is similar to that employed by the malaria parasite *Plasmodium* spp. which allows translocation of its effectors into host red blood cells (Whisson et al., 2007).

There is currently significant interest in the means by which secreted pathogen effectors are able to target and manipulate plant basal defences to their own ends. In order to investigate the spectrum of potential effector targets several transcriptome studies have been carried out using the *Arabidopsis-P. syringae* model system. Truman et al. (2006) indentified >800 plant genes that appear to be regulated by bacterial effectors with 20% of these being transcripts previously up-regulated in basal defence. Interestingly, one known target of suppression by bacterial effectors appears to be *NHO1* which is induced in NHR/ PTI and is suppressed by virulent *P. syringae* pv. *tomato* DC3000 (*Pst*) effectors (Kang et al., 2003; Navarro et al., 2004; Truman et al., 2006). Several screens carried out in *Pst* have identified >30 effectors predicted to be secreted via the T3SS (Guttman et al., 2002; Chang et al., 2005; Schechter et al., 2006). The roles of many of

these effectors are unknown, although several have homology to eukaryotic enzymes and possible functions can be predicted accordingly (Table 1.1). Host targets have even been validated experimentally for a few effectors.

**Table 1.1. Pathogen Effectors: functions and targets.**

| Effector | Pathogen | Function | Target | Reference |
|---|---|---|---|---|
| HopU1 | *P. syringae* | mono-ADP-ribosyltransferase | GRP7 | (Fu et al., 2007) |
| HopAO1 | *P. syringae* | tyrosine phosphatase | | (Espinosa et al., 2003) |
| HopAB2 | *P. syringae* | ubiquitin E3 ligase | | (Janjusevic et al., 2006) |
| HopZ2 | *P. syringae* | SUMO protease | | (Lewis et al., 2008) |
| AvrRpt2 | *P. syringae* | staphopain cysteine protease | RIN4 | (Mackey et al., 2003) |
| AvrRpm1 | *P. syringae* | | RIN4 | (Mackey et al., 2002) |
| AvrB | *P. syringae* | | RIN4 | (Mackey et al., 2002) |
| AvrPphB | *P. syringae* | cysteine protease | PBS1 | (Shao et al., 2003) |
| AvrBs3 | *Xanthomonas spp.* | transcription factor | | (Szurek et al., 2001) |
| AvrBs2 | *X. spp.* | glycerophosphoryldiesterase | | (Swords et al., 1996) |
| XopD | *X. spp.* | ubiquitin-like SUMO protease | | (Hotson et al., 2003) |
| EPI | *P. infestans* | serine protease inhibitor | P69 | (Tian et al., 2004; Tian et al., 2005) |
| EPIC | *P. infestans* | cysteine protease inhibitor | PIP1 | (Tian et al., 2007) |
| GIP1/2 | *Phytophthora sojae* | glucanase inhibitor | EGaseA | (Rose et al., 2002) |
| Avr2 | *C. fulvum* | | RCR3 | (Rooney et al., 2005) |
| Avr4 | *C. fulvum* | chitin binding protein | | (van Esse et al., 2007) |

One recent example of a bacterial T3SS effector for which a putative mechanism of manipulation of plant defence has been demonstrated is HopU1 which is a mono-ADP-ribosyltransferase. Fu et al. (2007) have demonstrated that this activity is necessary for full virulence of *Pst* on wild-type *Arabidopsis* and that it is able to ADP-ribosylate a glycine rich RNA binding protein AtGRP7. It is proposed that this modification alters the RNA binding capacity of GRP7 which in turn modifies the stability of pathogen induced transcripts allowing *Pst* to manipulate basal defence. Moreover, *grp7* *Arabidopsis* mutants were found to be impaired in basal defence responses, such as

callose deposition, and were more susceptible to both wild-type *Pst* and T3SS mutants (Fu et al., 2007).

Despite their partial suppression by pathogen effectors, some basal defence responses are still induced in host plants in response to virulent pathogen attack. These defences are often insufficient to provide successful resistance to these pathogens, but can restrict their growth to a certain extent. This is exemplified by *enhanced disease susceptibility* (*eds*) mutants or *fls2* which have been demonstrated to permit the growth of virulent pathogens to higher levels than in wildtype plants (Glazebrook et al., 1996; Zipfel, 2004); indicating that these genes still perform some basal defensive function. In addition, plants have developed mechanisms to recognise some of these effectors or their modified targets *in planta* and are able to over-ride their manipulation by employing rapid induction of heightened defence responses typically heralded by the HR. This second tier of defence response is termed effector triggered immunity (ETI) or *Resistance* (*R*) gene-mediated resistance after the class of genes which execute this response (Jones and Dangl, 2006).

### 1.5. *R* gene-mediated resistance (ETI)

The specific genetic interactions involved in *R* gene resistance were first described by Flor (1971) and given the name gene-for-gene resistance. For the resistance response, an incompatible reaction, to occur a plant *R* gene product must recognise a corresponding pathogen effector or *Avirulence* (*Avr*) gene product; the plant is termed resistant and the pathogen avirulent. If either gene is absent then the outcome is disease: a compatible reaction, where the plant is termed susceptible and the pathogen virulent.

There are five classes of *R* genes currently recognised (Fig. 1.3). The largest class of *R* genes encodes cytoplasmic proteins comprising leucine rich repeat (LRR) and

nucleotide binding site (NB) domains as well as one or the other of a coiled coil (CC) or toll/ interleukin receptor-like (TIR) domain. LRRs are thought to mediate protein-protein interactions and NB domains typically bind ATP or GTP (Dangl and Jones, 2001). Examples of these NB-LRR *R* gene encoded proteins include RPM1 and RPP13 which recognise effectors from un-related pathogens *P. syringae* and *Hyaloperonospora parasitica* respectively (Grant et al., 1995; Allen et al., 2004). A second class is represented by the tomato resistance to *C. fulvum R* genes (*Cf-2/ Cf-4*) which encode transmembrane proteins with an extracellular LRR domain; these recognise the extracellular *C. fulvum* effectors Avr2 and Avr4 respectively (Dixon et al., 1996; Thomas et al., 1997). A third class encodes transmembrane proteins with an extracellular LRR domain and an intracellular kinase domain. An example of this type of *R* gene is *Xa21* which confers resistance to an unknown effector from *Xanthomonas oryzae* pv. *oryzae* in rice (Song et al., 1995). These genes are also similar in structure to the PAMP receptors FLS2 and EFR. The fourth class is represented by the membrane bound RPW8 gene product which possesses an intracellular CC domain. RPW8 unexpectedly controls resistance to a broad range of powdery mildew pathogens in *Arabidopsis* (Xiao et al., 2001). The final class of *R* gene encodes cytoplasmic serine/threonine kinase domain proteins such as Pto which directly interacts with the bacterial effector AvrPto thus conferring resistance, although additional proteins are needed for signal transduction (Tang et al., 1996; Rathjen et al., 1999).

The most straightforward way to account for the gene-for-gene phenomenon is to propose a direct interaction between the two gene products which can trigger the transduction of resistance signalling pathways. However, evidence for a physical interaction has only been found in a few instances (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006; Ellis et al., 2007). A new hypothesis has since been suggested and is becoming more widely accepted. The guard hypothesis proposes that the plant *R* gene products 'guard' the cellular targets of pathogen avirulence factors i.e. they can recognise interactions between these cellular targets and *Avr* gene products and initiate

defence signalling (Van der Biezen and Jones, 1998). Or, expressed in another manner, *R* gene products are able to perceive the manipulation of basal defences by pathogen effectors and subsequently re-route an amplified defence response (Jones and Dangl, 2006).

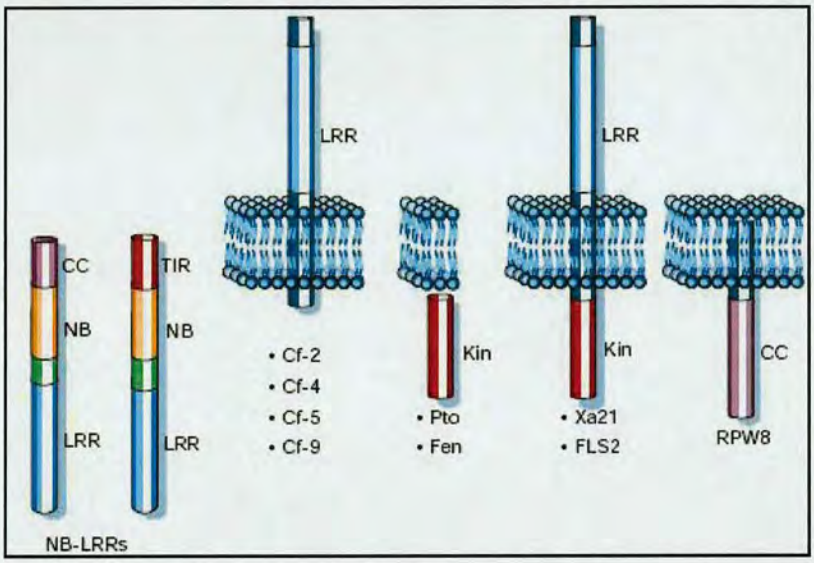


**Figure 1.3. Representation of the location and structure of plant disease resistance proteins.** Xa21 and Cf-X proteins carry transmembrane domains and extracellular LRRs. The RPW8 gene product carries a putative signal anchor at the N terminus. The *Pto* gene encodes a cytoplasmic Ser/Thr kinase, but may be membrane associated through its N-terminal myristoylation site. The largest class of R proteins, the NB-LRR class, are presumably cytoplasmic (although they could be membrane associated) and carry distinct N-terminal domains. Source: (Dangl and Jones, 2001).

There are a number of factors which support the guard hypothesis. Firstly, *Avr* gene products are in fact virulence factors or effectors; they target aspects of the host's cellular machinery. These effectors in the absence of *R* gene recognition are required for manipulation of NHR/ basal defences for successful infection (Jones and Dangl, 2006). Secondly, there are just five classes of *R* genes, which are thought to comprise only a few hundred plant *R* gene products (Dangl and Jones, 2001). Thus, it seems unlikely that these are sufficient to interact directly with thousands of *Avr* gene products from diverse types of pathogens. It seems more plausible that the *R* genes would be able to recognise the more limited number of cellular targets of *Avr* genes (Innes, 2004).

A clear illustration of the arms race which exists between plant and pathogen involving the development of effectors which manipulate plant defence and the acquisition of *R* gene specificities which recognise and circumvent this is highlighted in the following example. RIN4 is a negative regulator of basal defence targeted by two bacterial effectors AvrB and AvrRpm1 which induce its phosphorylation, presumably enhancing its function as a repressor. This pathogen-induced modification is recognised by *R* gene product RPM1 leading to a rapid induction of the HR and resistance to the pathogen. The pathogen has, however, acquired a third effector, AvrRpt2, which encodes a cysteine protease. This cleaves RIN4 blocking recognition by RPM1, leading to the development of disease. This manipulation of RIN4 can be recognised by the plant after gaining a second NB-LRR gene specificity, RPS2, which recognises the cleavage of RIN4 and again leads to the HR and resistance to the pathogen (Axtell and Staskawicz, 2003; Mackey et al., 2003; Kim et al., 2005). This example also supports the guard hypothesis as it is not the pathogen effector which the plant is able to recognise but it is the detected manipulation of host targets which triggers the subsequent defence response.

### 1.6. Signalling pathways

There are two major defence signalling pathways in plants: salicylic acid (SA) tends to mediate responses to biotrophic pathogens, while jasmonate and ethylene (JA and ET) tend to mediate responses to necrotrophic pathogens. This leads to the induction of two distinct sets of defence responses which are tailored to eliminate the threat of a given pathogen (Glazebrook, 2005).

SA is involved in transducing signals from *R* gene-mediated resistance. This leads to a HR and the induction of *pathogenesis related* (*PR*) gene expression. Some *PR* genes encode known products: *PR2* and *PR3* encode the antimicrobial proteins β-1,3-glucanase

and chitinase respectively, whereas many, like *PR1*, encode proteins with unknown function. Nevertheless, they are required for and are used as markers of resistance (Hammond-Kosack and Jones, 1996). Genes encoding *Non-race specific disease resistance1* (*NDR1*) and *enhanced disease susceptibility1* (*EDS1*) are signalling elements upstream of SA accumulation and were isolated in screens for mutants defective in *R* gene-mediated defence. *NDR1* encodes a protein with transmembrane domains required for signalling mediated by CC-NB-LRR *R* genes. EDS1 has similarity to eukaryotic lipases and is required for signalling via TIR-NB-LRR *R* genes (Century et al., 1995; Parker et al., 1996; Aarts et al., 1998; Falk et al., 1999). EDS1 interacts with two other proteins with lipase homology: phytoalexin deficient4 (PAD4) and senescence associated gene101 (SAG101). Mutations in these genes compromise resistance to both host and non-host pathogens, demonstrating a role for SA signalling in both forms of resistance (Wiermer et al., 2005). Moreover, EDS1 and PAD4 are also known to act upstream of the oxidative burst and the HR (Rusterucci et al., 2001). Recent studies also show that SA signalling is also required for PAMP signalling in PTI (Tsuda et al., 2008). The ankyrin repeat containing protein encoded by *non-expresser of PR1* (*NPR1*) is a common signalling node downstream of SA (Cao et al., 1994; Cao et al., 1997). *Npr1* mutants are unable to express *PR* genes and are susceptible to many pathogens. Ankyrin repeats mediate protein-protein interactions and NPR1 has been shown to localise to the nucleus where it interacts with basic leucine zipper transcription factors mediating *PR1* gene expression (Zhang et al., 1999; Kinkema et al., 2000).

Many additional *Arabidopsis* mutants have been isolated and characterised which have elevated SA levels leading to enhanced disease resistance; among these are: *constitutive expresser of PR genes* (*cpr*) (Bowling et al., 1994); *constitutive expression of PR1* (*cep*) (Silva et al., 1999); *activated disease resistance 1* (*adr1*) (Grant et al., 2003) and mutants displaying spontaneous cell death such as *lesions simulating disease* (*lsd*) (Dietrich et al., 1994). Conversely, plants unable to accumulate SA, such as the *SA induction deficient* (*sid*) mutants have also been isolated. *SID2* encodes an

isochorismate synthase important for SA biosynthesis. *sid2* mutants exhibit enhanced susceptibly to pathogens concomitant with reduced *PR* gene expression; both phenotypes can be reversed by exogenous SA application (Nawrath and Metraux, 1999; Wildermuth et al., 2001). The discovery and characterisation of such mutants allows the dissection of the SA signalling pathways and the identification of the components involved (Dong, 2001).

Following successful resistance to a pathogen incursion, the plant enters a heightened resistance state, where it is able to fend off attacks from formerly virulent pathogens. This state is termed systemic acquired resistance (SAR) and is also mediated by SA. SAR is defined by the systemic expression of PR proteins and by the range of pathogens which resistance is maintained against (Ryals et al., 1996). SA is known to be essential for SAR through the study of a transgenic plant line, (*nahG*), containing the bacterial salicylate hydroxylase gene which prevents the accumulation of SA. These plants are unable to exhibit SAR and are very susceptible to pathogen attack (Friedrich et al., 1995). Spraying plants with SA and chemical analogues such as INA (2,6-dichloroisonicotinic acid) and BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester) is able to induce SAR (Gorlach et al., 1996) and has been used as a form of crop protection. Although SA is required in both local and systemic tissues for SAR to occur, grafting experiments have shown that it is not the systemic signal responsible for transmission from the initial site of pathogen infection to the rest of the plant (Vernooij et al., 1994). A mutant defective in systemic but not local SAR signalling *Defective in induced resistance1* (*DIR1*) was isolated and encodes an apoplastic lipid transfer protein proposed to produce or transmit the elusive SAR signal (Maldonado et al., 2002) leading to systemic resistance. More recently a role in SAR has been uncovered for JA which accumulates in the phloem exudates of leaves challenged with SAR-inducing stimuli (Truman et al., 2007).

An additional defence pathway termed induced systemic resistance (ISR) has also been discovered. ISR confers enhanced growth and resistance to certain pathogens and can be triggered in response to root colonisation by non-pathogenic rhizobacteria as well as other stimuli including volatiles (Ryu et al., 2004). This pathway is mediated by JA/ ET but not by SA although one of the components of this pathway is known to be *NPR1* which is also a component of SA-mediated SAR (Pieterse et al., 1998; Pieterse and van Loon, 1999), demonstrating that the interactions between the SA pathway and other JA/ET dependent pathways are undoubtedly complex and as yet poorly understood (Dong, 1998).

JA and ET are hormonal regulators of plant growth and development which are also strongly induced by wounding and necrotrophic pathogen challenge. A distinct subset of pathogen-inducible genes are activated by JA/ET including proteinase inhibitors (Doares et al., 1995), *thionin2.1* (*THI2.1*) and *plant defensin1.2* (*PDF1.2*) (Epple et al., 1995; Penninckx et al., 1996). JA and ET mutants are impaired in defence against a certain spectrum of pathogens including necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* and are unable to induce expression of *PDF1.2*, which has become a marker gene for JA/ET-mediated resistance, much as *PR1* is a marker for SAR (Thomma et al., 1998; Thomma et al., 1999; Berrocal-Lobo and Molina, 2004). The *Arabidopsis* mutant *coronatine insensitive* (*coi1*) is insensitive to JA and encodes a component of an SCF ubiquitin-ligase complex, suggesting that COI1 is required to target JA signalling repressors for ubiquitin-mediated proteasome degradation (Feys et al., 1994; Devoto et al., 2002). *Jasmonate insensitive 3* (*JIN3*) and related *jasmonate ZIM domain* (*JAZ*)-gene encoded proteins have finally been uncovered as the direct targets of COI1-mediated destruction; JIN3 is a repressor of MYC2; a transcriptional activator of JA-responsive genes (Chini et al., 2007).

A selection of ET response mutants defective in synthesis or perception of ET have also been isolated allowing the assembly of a putative signal transduction pathway. ET is

perceived by a family of five receptors: ethylene receptor 1 (ETR1), ETR2, ethylene insensitive 4 (EIN4), ethylene response sensor 1 (ERS1), and ERS2 (Hua and Meyerowitz, 1998; Stepanova and Ecker, 2000). These are histidine kinases which, in the absence of their ligand, constitutively inhibit ET signalling via activation of an additional negative regulator, constitutive triple response 1 (CTR1) (Kieber et al., 1993). Conversely positive regulator *ethylene-insensitive 2* (*ein2*) encodes a membrane associated protein with similarity to NRAMP metal ion transporters and is required for ET signal transduction (Alonso et al., 1999). Downstream of EIN2, ethylene response factor 1 (ERF1) is a transcription factor which is required for the expression of genes mediated by both JA and ET, integrating signals from these two hormones (Lorenzo et al., 2003).

Considerable cross-talk is thought to be required in order to integrate SA and JA/ET signalling pathways to prioritise the allocation of resources during the induction of the distinct defence responses required for effective protection against different pathogens (Kunkel and Brooks, 2002). Many studies provide evidence that these hormones act antagonistically: JA has been shown to suppress SA signalling and *vice versa* (Doares et al., 1995; Niki et al., 1998; Gupta et al., 2000). However, recent microarray analysis has shown that SA and JA can also act in concert to induce the expression of an overlapping set of genes (Schenk et al., 2000). In addition, JA is now thought to be necessary in the induction and establishment of SAR mediated by SA (Truman et al., 2007). Figure 1.4 is a simplified model of SA and JA/ET signalling and shows several recently identified nodes which influence the flow of defence signalling. The WRKY70 transcription factor is up-regulated by SA and down-regulated by JA, possibly via COI1. Moreover, WRKY70 acts downstream of NPR1 to repress the induction of JA signalling (Li et al., 2004). The ESD1-PAD4 complex is upstream of, and positively regulates, SA signalling, and concomitantly exerts an inhibitory effect on JA signalling (Wiermer et al., 2005). Both of these EDS1-PAD4 functions are thought to be mediated by mitogen-activated protein kinase 4 (MAPK4) which has been demonstrated to inhibit SA

signalling and positively regulate the JA/ET pathway (Brodersen et al., 2006). Moreover, plant pathogenic bacteria have developed mechanisms to alter the balance between these two pathways to their own advantage. *P. syringae* produces the phytotoxin coronatine which mimics the action of JA thus inhibiting SA signalling which reduces the effectiveness of the defence response to this pathogen (Brooks et al., 2005). Coronatine also functions by over-riding stomatal closure during plant defence (Melotto et al., 2006).

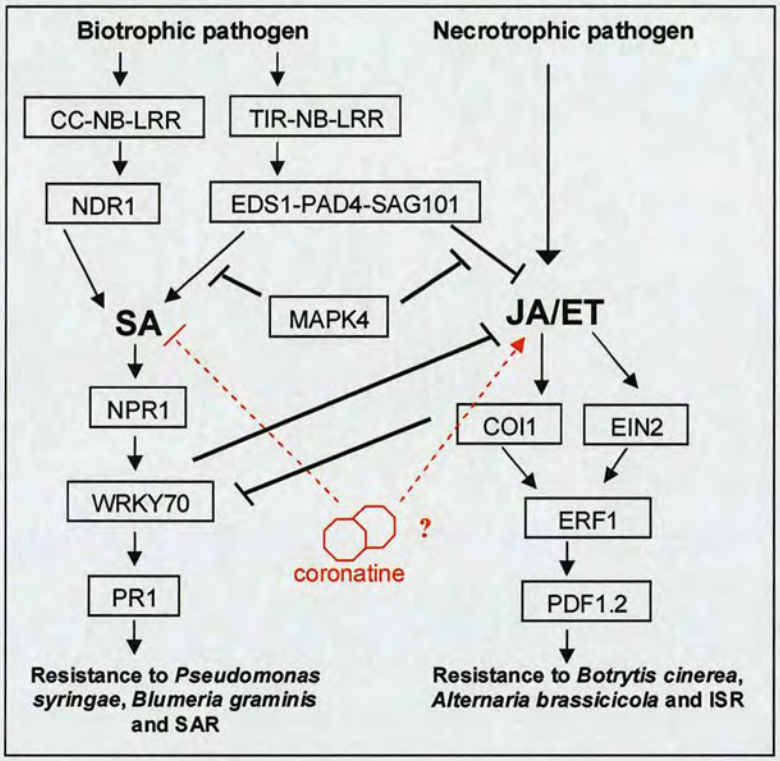


**Figure 1.4. Defence signalling pathways and signalling crosstalk.** A simple model showing known defence signalling nodes in response to different pathogens and how these may be interlinked to allow cross-talk and appropriate allocation of defence resources.

## 1.7. The HR: plant PCD

The hypersensitive response (HR) is a plant defence response which involves localised cell death surrounding the site of attempted pathogen incursion within a few hours.

However, the HR is more than cell death and comprises additional functions such as the coordination of local and systemic defence signals and the induction of oxidative and nitrosative bursts (Levine, 2004). The HR is triggered in both *R* gene-mediated resistance and in type II NHR where it is effective at establishing resistance to biotrophic pathogens which require living plant cells to obtain nutrition. Unsurprisingly, the HR is not effective at providing resistance to necrotrophic pathogens which typically kill cells to obtain nutrients (Glazebrook, 2005). Indeed there is considerable evidence that necrotrophic pathogens such as *Alternaria alternata* and *Cochliobolus victoriae* produce the toxins AAL and victorin specifically to induce the HR and enhance their virulence (Wang et al., 1996; Navarre and Wolpert, 1999). The HR can range from the death of a single cell in contact with the pathogen to larger localised lesions. It is sensitive to environmental influences, particularly humidity and has been shown to be distinct from necrosis: which is an uncontrolled passive form of cell death, not requiring energy, caused by trauma or toxin accumulation (Dangl et al., 1996; Evans, 2004).

Instead, the HR is thought to be a form of programmed cell death (PCD), under strict genetic control much like apoptosis in animals. PCD is an active process and requires metabolically active cells which have the ability to undergo transcription and translation; as demonstrated using inhibitors of protein synthesis and transcription to block the HR (He et al., 1993). Other evidence which indicates that the HR is under genetic control includes the discovery of mutant plants which exhibit spontaneous cell death; these so-called paranoid plants undergo the HR in the absence of pathogen challenge. These lesion mimic mutants (LMMs), of which there are 37 in *Arabidopsis*, belong to two distinct classes: 1) initiation class mutants form localised lesions of a determinate size and are thought to be involved in the regulation of signals required for the perception or induction of the HR; 2) propagation class mutants are unable to stop the proliferation of cell death after initiation and are thought to be involved in feedback regulation to suppress the HR spread (Dietrich et al., 1994; Lorrain et al., 2003). Only 6 propagation class mutants are known in *Arabidopsis*, these include *accelerated cell death 2* (*acd2*)

and *lesions simulating disease resistance response 1* (*lsd1*). These mutants exhibit runaway cell death in the absence of pathogen and are thought to negatively regulate PCD (Dietrich et al., 1994; Greenberg et al., 1994). ACD2 encodes a protein involved in red chlorophyll metabolism (Mach et al., 2001), whereas LSD1 is a zinc finger protein which negatively regulates cell death via control of the sub-cellular localisation of the positive regulator bZIP10, a transcription factor (Kaminaka et al., 2006).

Initiation class mutants are too numerous to discuss here fully but include the *constitutive expressor of PR* mutants (*cpr*) (Bowling et al., 1997). *Cpr* mutants were identified in screens for enhanced *PR* gene expression but also form spontaneous HR-like lesions. *CPR5* encodes a Type IIIa transmembrane protein with a putative nuclear localisation signal (NLS) and *cpr5* mutants show pleiotropic phenotypes, including the aforementioned constitutive defence and spontaneous PCD as well as hyper-senescence, aberrant trichome formation and reduced cell proliferation (Kirik et al., 2001; Yoshida et al., 2002). Many mutants that constitutively express defence genes also exhibit spontaneous development of HR-like lesions and SAR, implying a role for the HR in SA-mediated defence signalling (Lorrain et al., 2003). However, mutants where defence signalling is uncoupled from cell death, including *defence, no death 1* (*dnd1*), have been identified. *DND1* encodes a cyclic nucleotide gated ion channel (AtCNGC2) with a calmodulin binding domain, although how CNGCs negatively influence defence but positively regulate PCD is unknown (Yu et al., 1998; Clough et al., 2000). The study of LMMs should help to determine the components and signalling pathways which control the HR, for example genetic crossing with *Arabidopsis R* gene or hormone signalling mutants allows signal transduction networks to be drawn up and the relative placement of these genes proposed.

### 1.7.1. The oxidative burst, ROS and NO

The generation of reactive oxygen species (ROS) such as superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) via the oxidative burst is an early defence signalling event associated

with the HR. ROS are produced in a biphasic burst: a rapid but small burst is non-specifically triggered by pathogen challenge or wounding and a second larger and sustained burst occurs only after challenge with avirulent pathogens. This second phase of ROS generation is required for the HR and resistance (Chandra et al., 1996; Grant and Loake, 2000; Levine, 2004). $O_2^-$ is produced in animal macrophages by an NADPH oxidase, known as the respiratory burst complex (RBO) and mediates the destruction of microbial invaders (Segal and Abo, 1993). *Arabidopsis* has 10 homologues of $gp91^{phox}$, the plasma membrane bound enzymatic subunit of this complex, and several RBO homologues (*At*RBOH) are required for ROS accumulation in plants (Torres et al., 2002; Torres and Dangl, 2005). Plant RBOH possess an additional intracellular EF-hand calcium binding domain and it has been predicted that these proteins are activated by the intracellular $Ca^{2+}$ influx which occurs on pathogen perception (Grant et al., 2000a; Torres and Dangl, 2005). However, $O_2^-$ is unstable and is rapidly dismutated by superoxide dismutase (SOD) to produce $H_2O_2$ which is more stable and able to cross cell membranes (Grant and Loake, 2000). An alternative mechanism for $H_2O_2$ production is via apoplastic peroxidases which appear to be activated in alkaline conditions such as those experienced in the apoplast upon pathogen challenge (Bolwell et al., 2002). The mechanism employed is likely to depend on the particular plant pathogen interaction. For example, transgenic wheat over-expressing a plant peroxidase using a pathogen inducible promoter show increased $H_2O_2$ production, HR and enhanced resistance to fungal pathogen *Blumeria graminis* f. sp. *tritici* (Schweizer, 2008). In contrast, *AtrbohD* and *F* knockouts show reduced HR to avirulent bacteria but not to the oomycete *Hyaloperonospora parasitica,* whereas *Nbrboh* silencing reduces the HR and increases susceptibility to the oomycete *P. infestans* (Torres et al., 2002; Yoshioka et al., 2003; Torres et al., 2006).

$H_2O_2$ is proposed to have additional roles in the oxidative cross-linking of both cell wall proteins (Bradley et al., 1992) and polysaccharides (Fry et al., 2000) which presumably act to strengthen the cell wall in response to pathogen challenge. High concentrations of

$H_2O_2$ are expected to induce HR, whereas low concentrations in neighbouring cells can induce the expression of cellular defence genes and may limit HR spread (Levine, 2004) Furthermore, a role has also been proposed for the $H_2O_2$-triggered HR in the instigation of SAR as both avirulent pathogen challenge and a $H_2O_2$-generating system in local leaves lead to micro-oxidative bursts and micro-HRs prior to the establishment of SAR. Infiltration with the RBOH inhibitor diphenylene iodinium (DPI) blocked this pathway (Alvarez et al., 1998). However, although ROS are necessary for the HR they are not always sufficient to induce PCD and act in concert with nitric oxide (NO) to trigger the HR (Delledonne et al., 1998).

NO is a gaseous free radical, able to diffuse through cell membranes making it an ideal cell-to-cell signal. In animals, NO is synthesised by nitric oxide synthase (NOS) enzymes from the precursors L-arginine and L-citruline (Romero-Puertas et al., 2004). In plants the mechanism of NO synthesis is unclear but possible sources include nitrate reductase (NR) which is involved in nitrogen assimilation in roots (Rockel et al., 2002) or a plant NOS-like enzyme. *AtNOS1* was identified by Guo et al. (2003) and suggested to encode a plant NOS and, although *Atnos1* mutants are deficient in NO synthesis and accumulation, subsequent experiments have failed to detect NOS activity (Crawford et al., 2006; Zemojtel et al., 2006). However NO is synthesised, it accumulates following pathogen challenge particularly with avirulent pathogens which subsequently show PCD (Delledonne et al., 1998; Zhang et al., 2003). The balance between NO and $H_2O_2$ is critical for the induction of the HR (Delledonne et al., 2001). This is perhaps unexpected as in animal systems it is the accumulation of peroxynitrite $ONOO^-$ a toxic reaction product of $O_2^-$ and NO which mediates microbial cell death by macrophages. However $ONOO^-$ does not seem to be particularly toxic to plants (Delledonne et al., 2001; Romero-Puertas et al., 2004). Not only is NO required for the induction of the HR but it reacts with cysteine residues in proteins and thus could play an important role in the regulation of protein activity through modifications such as S-nitrosylation (Durner and Klessig, 1999; Feechan et al., 2005). *Arabidopsis* mutants which are defective in

the enzyme S-nitrosoglutathione reductase (GSNOR) which regulates cellular S-nitrosothiol (SNO) levels show increased pathogen susceptibility and a perturbed HR (Feechan et al., 2005; Wang et al., 2006).

### 1.7.2. Developmental PCD: senescence

In addition to the involvement of PCD in the HR and pathogen defence plants, like animals, also utilise PCD to regulate many developmental processes. Examples of these include the formation of xylem vessels, where tracheary element cells undergo secondary cell wall reinforcement then lose their cell contents to form hollow tubes (Obara and Fukuda, 2004). PCD also occurs during plant reproductive development (Riggs, 2004), seed development and germination (Gallie, 2004), as well as during leaf and root development (Huelskamp and Schnittger, 2004) and senescence (Orzaez and Granell, 2004). Senescence is a controlled process involving PCD which is used to mobilise and recycle nutrients from organs at the end of their development. This process proceeds to an ordered timetable and is an active process requiring energy and gene expression (Buchanan-Wollaston, 1997). Many plant hormones have emerged as playing a regulatory role in senescence including SA, ET, JA and abscisic acid (ABA) which are positive regulators whereas cytokinin is a negative regulator (Weaver et al., 1998; He et al., 2001; Buchanan-Wollaston et al., 2005). Transcriptomic analysis carried out in Arabidopsis has identified different subsets of genes involved in the following processes presumed to be required for senescence (Guo et al., 2004; Buchanan-Wollaston et al., 2005). Many up-regulated genes are predicted to be involved in macromolecule degradation; this includes proteases, nucleases, and lipid and chlorophyll degradation factors. Another group of genes is predicted to be involved in regulation, including those encoding for transcription factors, protein kinases/ phosphorylases, ubiquitin pathway components and calcium binding factors. Carbohydrate metabolism and transport protein encoding genes represent another important subset up-regulated. Many stress-related genes, including those with antioxidant and pathogen defence functions, are also induced, presumably to ensure cells

remain viable long enough to remobilise all useful components (Buchanan-Wollaston et al., 2003; Orzaez and Granell, 2004). One of the most highly up-regulated groups of senescence associated genes (SAGs) encode cysteine proteases such as SAG12 (Lohman et al., 1994; Guo et al., 2004). Cysteine proteases are also heavily involved in animal PCD, particularly the regulation and execution of apoptosis. Therefore, it is possible that these proteases may represent a conserved link between animal and plant forms of PCD.

### 1.7.3. PCD and conserved regulators

Although the HR is known to be a genetically controlled process, its morphological and biochemical characteristics are still unclear, as is whether any mechanisms are shared in common with animal PCD (Heath, 1998). In animals the only form of PCD originally recognised was apoptosis which has very specific morphological characters including condensation of the nucleus and cytoplasm, DNA laddering, formation of apoptotic bodies and phagocytosis by neighbouring cells. Cell death with other characters was regarded as passive necrosis (Kerr, 2002). However, three types of cell death are now recognised in animals: type 1) apoptosis, type 2) autophagic cell death and type 3) non-lysosomal disintegration (Kitanaka and Kuchino, 1999). Some of the morphological features of apoptosis have been observed in plant cells during the HR and other forms of developmental PCD. However, this varies to a great extent with the plant: pathogen system under investigation (Heath, 2000b; Christopher-Kozjan and Heath, 2003; Krzymowska et al., 2007), but traditional apoptotic features such as DNA laddering have been observed (Khanna et al., 2007). The presence of a cell wall in plants would necessitate changes in the latter stages of PCD as dying cells cannot be phagocytosed by macrophages (Evans, 2004).

The simplest regulatory pathway leading to apoptosis was uncovered in studies of *cell death defective* (*ced*) mutants of the nematode *Caenorhabditis elegans* (Fig. 1.5). PCD is triggered by activation of the caspase CED3 by CED4 an ATPase which cleaves the

prodomain from CED3. The activity of CED4 is inhibited by the binding of CED9 which in turn can be negatively regulated by EGL-1 (Conradt and Horvitz, 1998; Williams and Dickman, 2008). The situation in mammals is more complex but essentially follows the same pattern. Homologues of all *C. elegans* proteins have been identified and there are 14 CED3 caspase homologues. Caspases are cysteine proteases which cleave substrates after an aspartic acid residue. They are synthesised as inactive proproteins which are post-translationally activated by cleavage of the prodomain. These proteases are further subdivided into initiator and effector caspases depending on their roles in initiation of caspase signalling cascades or in cleavage of intracellular targets (Earnshaw et al., 1999; Boatright and Salvesen, 2003). Apoptotic protease activating factor 1 (Apaf-1) is a homologue of CED4 and can activate procaspase-9. B-cell lymphoma 2 (Bcl-2) family members can have either pro- or anti-apoptotic activities depending on their structural features. Bcl-2 and Bcl-$X_L$ have anti-apoptotic activity, like CED9, and inhibit Apaf-1-mediated caspase activation, whereas Bik and Bax have pro-apoptotic activity and are homologous to EGL-1 (Fig. 1.5) (Earnshaw et al., 1999; Dickman and Reed, 2004). Bik and Bax have homology with the bacterial toxins colicins which kill competing bacteria by introducing pores in their cell membranes, causing depolarisation. It is proposed that Bax may target mitochondrial membranes to the same effect (Dickman and Reed, 2004; Williams and Dickman, 2008). Apoptosis occurs via intrinsic or extrinsic pathways, both pathways subsequently converge and cell death is mediated by the action of caspases. The extrinsic pathway is activated by cell death signal recognition by a family of tumour necrosis factor (TNF) receptors (TNFR). TNFR then form complexes which results in the activation of procaspase-8. The intrinsic pathway is mediated by the perception of various cell stresses by the mitochondria which lose membrane potential and become permeable, releasing pro-death factors such as apoptosis inducing factor (AIF) and cytochrome c, which ultimately result in caspase-3 activation and cell death (Williams and Dickman, 2008).

Very few homologues of genes involved in apoptosis have been found in plants. One such gene *defender against death 1* (*DAD1*), is thought to block apoptosis in mammalian cells (Nakashima et al., 1993; Sugimoto et al., 1995). Not much is known about the roles of plant DAD1 except that it is down-regulated during senescence (Orzáez and Granell, 1997). A plant homologue of Bax-inhibitor 1 (BI-1) has also been identified which is able to suppress Bax-induced PCD in yeast (Kawai et al., 1999). BI-1 appears to be involved in regulation of plant stress-induced PCD (Watanabe and Lam, 2006, 2008). No other homologues have yet been discovered, although studies of transgenic plants expressing anti-apoptotic genes CED9 and Bcl-$X_L$ show reduced PCD to both biotic and abiotic inducers (Mitsuhara et al., 1999; Dickman et al., 2001) and in cell cultures expressing CED9 and Bcl-$X_L$ cell death is reduced and *Agrobacterium*-mediated transformation efficiency is enhanced (Khanna et al., 2007). Moreover pro-apoptotic genes such as Bax trigger PCD in plant cells (Lacomme and Santa Cruz, 1999; Baek et al., 2004). This suggests that there is functional conservation of PCD regulation between plants and animals (Fig. 1.5) even if there is not always sufficient homology at the amino acid level.

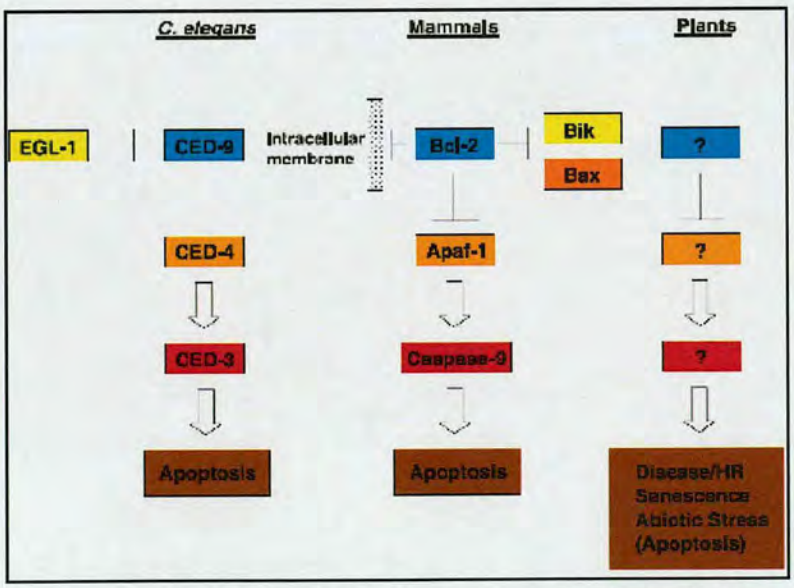


**Figure 1.5. Apoptosis regulation in different Eukaryotes.** Source (Williams and Dickman, 2008).

Type 2 autophagic cell death is proposed to play a role in forms of plant developmental PCD such as senescence due to a resemblance of morphological features (Orzaez and Granell, 2004). Autophagy comprises the engulfment of cytoplasm and organelles in double membrane vesicles, or autophagosomes, which are then acidified and targeted to either lysosomes (animals) or the vacuole (yeast and plants) for degradation (Greenberg, 2005). In contrast to the lack of highly conserved apoptosis regulatory genes in plants, *autophagy* (*ATG*) genes are highly conserved in eukaryotes and 25 homologues of essential yeast *ATG* genes were identified in *Arabidopsis*. Several *atg* mutants: *atg4*, *atg7* and *atg9* show early senescence phenotypes (Doelling et al., 2002; Hanaoka et al., 2002). Furthermore, recent data suggests that autophagy in plants is essential for limiting the spread of pathogen-induced cell death (Liu et al., 2005; Patel and Dinesh-Kumar, 2008). *ATG6* is the homologue of mammalian *Beclin1* and silencing of this gene in *N. benthamiana* leads to increased local and systemic HR in response to TMV infection as does silencing of the additional autophagy components *ATG3* and *ATG7*. Autophagy via autophagosome formation is observed in control pathogen-challenged cells and in cells surrounding the infection site, but does not occur in *Beclin1*-silenced plants. Therefore, it is proposed that the autophagic process in plants is required to mediate the destruction of a pro-death signal in order to limit the spread of PCD out-with the infection site (Liu et al., 2005). There may also be links in plants between autophagy and apoptosis; as in animal systems blocking of autophagy after treatment with autophagic cell death inducers causes cells to die by apoptosis (Boya et al., 2005). Moreover Beclin1 triggered autophagy in yeast and mammalian cells can be inhibited by binding of Bcl-2, an inhibitor of apoptosis (Pattingre et al., 2005). Finally, caspases which regulate apoptosis have also been shown to be involved in the regulation of autophagy in animals (Martin and Baehrecke, 2004).

### 1.7.4. Proteases and PCD

Another facet which has the potential to be conserved between animal and plant PCD is the involvement of cysteine proteases such as caspases which are crucial components of

apoptosis and may even regulate autophagy in some cases. There are no true caspase gene homologues present in plants but caspase activity was first measured during the HR by del Pozo and Lam (1998) who demonstrated that caspase specific peptide inhibitors could block HR cell death triggered by bacteria and moreover that several caspase-like activities could be measured during the HR induced by TMV. Throughout the subsequent ten years eight distinct caspase activities have been measured in plants although often the proteases which exhibit these activities are not known (D'Silva et al., 1998; Chichkova et al., 2004; Coffeen and Wolpert, 2004; Hatsugai et al., 2004; Bonneau et al., 2008). One protein with known caspase activity is a subtilisin-like serine protease or saspase which has been shown to cause PCD induced by the toxin victorin in oat, *Avena sativa*, cells (Coffeen and Wolpert, 2004). On induction of PCD saspases are rapidly secreted into the apoplast where they are hypothesised to play a role in a protease cascade leading to cell death, as they account for only one type of protease activity induced in this system and they are themselves unable to cleave Rubisco (Coffeen and Wolpert, 2004). Vacuolar processing enzymes (VPEs) have also been demonstrated to possess caspase-like activity and have been proposed to control PCD triggered by vacuole collapse (Hatsugai et al., 2006). Silencing of VPE in *N. benthamiana* suppressed the HR in response to infection with TMV (Hatsugai et al., 2004), and null *vpe* mutants of the four VPEs in *Arabidopsis* inhibit PCD induced by the mycotoxin fumonisin b1 (Kuroyanagi et al., 2005). Furthermore, these enzymes have been implicated in various forms of developmental PCD including senescence and lateral root emergence (Kinoshita et al., 1999). VPE proteins have no significant amino acid homology with caspases but do share similar 3D structural properties which may account for their ability to cleave caspase substrates (Hatsugai et al., 2006). Database searches have identified a group of plant metacaspase genes with conservation of caspase-like structural features (Uren et al., 2000). It has since been demonstrated that metacaspases do not possess caspase substrate specificity and prefer to cleave arginine or lysine residues rather than aspartic acid residues like caspases (Vercammen et al., 2004). Evidence for the roles of metacaspases in cell death is variable. The expression

of several metacaspases correlates with cell death in response to necrotrophic pathogens in tomato and *N. benthamiana* but not with chemically-induced or HR cell death (Hoeberichts et al., 2003; Hao et al., 2007). However in other studies, one of the nine *Arabidopsis* metacaspases (AtMC), AtMC8, has been shown to be up-regulated by oxidative stress, and is able to complement a yeast mutant defective in cell death. Furthermore, knockouts show reduced cell death whereas there is evidence of increased cell death in overexpresser derived protoplasts (He et al., 2008). Metacaspases may also be required for developmental PCD as repression of these proteins interferes with PCD of certain cells during embryogenesis in pine (Suarez et al., 2004; Bozhkov et al., 2005). More recently proteases required for proteasome function have been shown to be required for PCD activation, upstream of caspase activity, in tobacco cell cultures induced to die via heat shock treatment (Vacca et al., 2007).

These studies provide evidence that proteolytic activity other than that of caspases is required for PCD in plants. Cathepsins are a family of lysosomal non-caspase cysteine and aspartate proteases which are thought to be involved in mammalian apoptosis, both alongside and independently of caspases (Leist and Jaattela, 2001; Turk and Stoka, 2007; Vasiljeva and Turk, 2007). Cathepsin B has been shown to activate caspases by cleaving the prodomains from procaspases 1 and 11 which belong to the group of initiator caspases and treatment with cathepsin B induces nuclear apoptosis *in vitro* (Vancompernolle et al., 1998). Moreover cathepsin B also induces neuronal apoptosis following its secretion from microglial cells (Kingham and Pocock, 2001). During the extrinsic apoptotic pathway cathepsin B is able to execute caspase-independent apoptosis following TNFR activation, and is required for the translocation of phosphatidylserine outside the cell which acts as the 'eat me' signal to phagocytes (Foghsgaard et al., 2001). Moreover, mice mutants where cathepsin B has been knocked out exhibit reduced apoptosis triggered by TNF (Guicciardi et al., 2001; Reinheckel et al., 2001). Other studies have shown that cathepsins, including cathepsin B, can cleave Bid, a pro-apoptotic factor, thus activating it and triggering cytochrome c release from

mitochondria and subsequent cell death (Cirman et al., 2004; Blomgran et al., 2007). Moreover cathepsins are able to degrade anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-$X_L$ (Turk and Stoka, 2007).

Unlike caspases, genes encoding cathepsin B-like proteases are also present in plants. Several pieces of evidence support the involvement of these papain class cysteine proteases in plant PCD. Cathepsin B and other papain-like cysteine proteases such as SAG12 are strongly expressed during senescence, a form of plant developmental PCD (Guo et al., 2004). SAG12 is also expressed during the HR along with other cell death marker genes (Pontier et al., 1999). Lastly over expression of cystatins, which are endogenous plant papain-like cysteine protease inhibitors, blocks PCD induced by various stimuli including $H_2O_2$, NO and avirulent bacteria which induce the HR (Solomon et al., 1999; Belenghi et al., 2003). Cumulatively this evidence suggests that papain-like cysteine proteases such as cathepsin B may play a role in plant PCD processes such as the HR.

## 1.8. Previous Work

A screen to identify genes up-regulated in an incompatible HR between *Solanum tuberosum* and the major pathogen *Phytophthora infestans* was carried out using suppression subtractive hybridisation (SSH). One of the genes identified in this study was predicted to encode a protein with homology to mammalian cathepsin B cysteine proteases (Avrova et al., 2004). Further investigation of possible roles for cathepsin B in plants has been carried out in our laboratory using virus-induced gene silencing (VIGS). *N. benthamiana* plants where *cathepsin B* is silenced exhibit a reduction in the non-host HR triggered by bacterial pathogens *Erwinia amylovora* and *P. syringae* pv. *tomato* (DC3000) and concomitant increase in susceptibility to these pathogens. *Cathepsin B* silencing also suppressed an *R* gene-triggered HR using the potato–*P. infestans R3a*–

*Avr3a* interaction but did not suppress the HR mediated by *Cf4–Avr4* recognition (Gilroy et al., 2007).

## 1.9. Aims

The number of *cathepsin B* (*CathB*) genes present in potato and *N. benthamiana* is unknown. In this project the model plant *Arabidopsis* was employed to characterise the three *AtCathB* homologues present in this species with respect to their phenotypes during various forms of plant PCD, both pathogen-induced and developmental, as well as other possible roles in plant disease resistance.

The specific aims of this project were as follows:

- Construction and selection of transgenic *AtCathB* plant lines.
- Characterise their response to infection with both virulent and avirulent *P. syringae* DC3000 bacterial pathogens.
- Characterise their roles in response to non-host fungal pathogen *Blumeria graminis* f. sp. *tritici*.
- Assess any possible roles in senescence: a developmental form of PCD.
- Examine the sub-cellular localisation of NbCathB using confocal microscopy.
- Expression of recombinant CathB and assessment of its inhibition using a range of commercial and pathogen-derived cysteine protease inhibitors.

# 2. Materials and Methods

## 2.1. Plant growth

### 2.1.1. *Arabidopsis thaliana* growth

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used; all other transgenic lines and mutant strains used were in a Col-0 background unless indicated (Table 2.1.). Seeds were sown in peat moss, vermiculite and sand (4:1:1), and vernalised for 48 hours at 4°C then transferred to 20°C. Plants were placed 6 to a pot and grown in long days (16 hours light, 8 hours dark), with 130-150 uE m-2 sec-1 light intensity and 40% humidity.

**Table 2.1 *Arabidopsis* control wildtype and transgenic lines**

| Line | Gene | Phenotype/function | Source | Reference |
|---|---|---|---|---|
| Col-0 | | Wildtype | NASC | (AGI, 2000) |
| *nahG* | NCBI_X83926 | Blocked SA accumulation | Novartis USA | (Lawton et al., 1995) |
| *gsnor1-3* | *At*5g43940 | High levels of protein S-nitrosylation | Loake, Edinburgh | (Feechan et al., 2005) |
| *ein2-1* | *At*1g47240 | Ethylene insensitive | NASC | (Guzman and Ecker, 1990) |
| *nos1* | *At*3g47450 | Nitric oxide responsive | NASC | (Guo and Crawford, 2005) |
| *cathb1-1* | *At*1g02300 | *Cathepsin B* TDNA insert | NASC | (Alonso et al., 2003) |
| *cathb1-2* | *At*1g02300 | *Cathepsin B* TDNA insert | NASC | (Alonso et al., 2003) |
| *cathb2-1* | *At*1g02305 | *Cathepsin B* TDNA insert | NASC | (Alonso et al., 2003) |
| *cathb2-2* | *At*1g02305 | *Cathepsin B* TDNA insert | NASC | (Alonso et al., 2003) |
| *cathb3-1* | *At*4g01610 | *Cathepsin B* TDNA insert | NASC | (Alonso et al., 2003) |
| *cathb3-2* | *At*4g01610 | *Cathepsin B* TDNA insert | NASC | (Alonso et al., 2003) |
| *Ler* | | Wildtype | NASC | (AGI, 2000) |
| *eds1-2 (Ler)* | *At*3g48090 | Defective TIR-NBS-LRR signalling | Parker, Max Planck | (Parker et al., 1996) |

### 2.1.2. Wheat growth

Wheat seeds of variety Hereward were germinated two to a pot every 2-3 weeks for *Blumeria graminis* f sp *tritici* maintenance. These plants were grown in a glasshouse at 22°C with a 16 hour photoperiod.

### 2.1.3. *Nicotiana benthamiana* growth

SCRI glasshouse staff provided weekly supplies of *N. benthamiana* seedlings which were grown in individual pots in a glasshouse at 22°C with a 16 hour photoperiod.

## 2.2 Pathogen growth and disease resistance assays

### 2.2.1. Growth and inoculation of *Pseudomonas syringae* pv *tomato* DC3000

*Pseudomonas syringae* pv *tomato* DC3000 virulent and *AvrB* were grown in King's broth (KB) liquid media (King et al., 1954) with 50 $mg.l^{-1}$ rifampicin (supplemented with 50 $mg.l^{-1}$ kanamycin for *AvrB* only) at 30°C. Cells were harvested at $OD_{600}$ equal to 0.2 (the equivalent of $10^6$ $cfu.cm^{-2}$) and pelleted by centrifugation before re-suspension in 10 mM $MgCl_2$. Four week old plants were infiltrated with a *Pst* DC3000 *virulent* suspension ($OD_{600}$= 0.0002 for resistance assay) and *AvrB* ($OD_{600}$=0.002 for resistance assay and 0.02 for leaf staining) on the abaxial side of the leaf using a 1 ml syringe (Cao et al., 1994; Grant et al., 2000b).

### 2.2.2. *Pseudomonas syringae* pv *tomato* DC3000 resistance assay

*Pst* DC3000 was inoculated into plants as described above. After four days, plants were examined for disease symptoms and leaves were harvested for analysis of bacterial growth. One leaf disc (1 $cm^2$) was collected per plant with 3 discs per sample and ground in 3 ml 10 mM $MgCl_2$ using a pestle and mortar. Serial dilutions were made of the resulting bacterial suspension and 100 µl of each dilution plated onto KB plates containing the appropriate antibiotics as above. The plates were incubated for 2 days at 30°C and the number of bacterial colonies for each sample counted and recorded.

### 2.2.3. *Blumeria gramis sp tritici* inoculation and disease assay

*Blumeria graminis* f. sp. *tritici* (*Bgt*) was obtained from Syngenta (Jealott's Hill, UK). *Bgt* races were maintained on wheat cultivar Hereward. The *Bgt* race 1 (Sav GH135)

was routinely employed, *Bgt* race 2 (W11S), was also used occasionally. *Bgt* conidia were dust-inoculated directly on the leaf surface of *Arabidopsis* plants, which were left for 7-10 days before harvesting leaf samples for staining. Samples for Northern analysis were collected at 1 and 2 dpi. The percentage of haustorial formation, secondary hyphae formation and epidermal cell death were normalized to the number of germinated conidia.

### 2.2.4. *Erwinia amylovora* inoculation and disease assay

*Erwinia amylovora* 1430 (apple fire blight) (Eastgate, 2000) was grown overnight at 30°C in L broth (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 litre at pH 8.5) inoculated with single colonies from plates streaked from glycerol stocks. This was centrifuged at 4000g and the pellet resuspended in 5 mM MES (2-morpholinoethanesulfonic acid) buffer at an $OD_{600}$=0.002 and inoculated into the abaxial leaf surface of *N. benthamiana* carrying pGrab::PVX vectors expressing either GFP, EPIC2, EPIC3 or EPIC4. The percentage of inoculation sites showing HR (50% of the inoculated area showing total tissue collapse) was scored 4-7 days later.

## 2.3. Histochemical staining

### 2.3.1. Trypan blue (TB) staining

Dead cells and fungal growth were visualised by TB staining (Yun et al., 2003). Leaves were boiled for 2 minutes in TB solution (2.5 mg/ml trypan blue, 25% (w/v) lactic acid, 23% water saturated phenol, 25% glycerol and water). Leaves were then rinsed in water before destaining with chloral hydrate solution (250 g/l) for 24 hours. Samples were mounted onto microscope slides with 60% glycerol. The slides were examined for cell death using a Leica Wild M3C microscope.

### 2.3.2. Diaminobenzidine (DAB) staining

$H_2O_2$ accumulation was visualised by 3,3-diaminobenzidine (DAB) staining (Yun et al., 2003). DAB is polymerised through the action of peroxidase locally in the presence of $H_2O_2$ producing a visible brown stain. Leaves were placed in 20 ml glass McCartney bottles, covered with 0.1% (w/v) DAB solution and incubated overnight in the dark. The stain was removed and the chlorophyll cleared by boiling in 96% (v/v) ethanol for 10 minutes and then leaves were mounted under coverslips in 60% glycerol and examined under a Leica Wild M3C microscope.

## 2.4. RNA extraction

Total RNA was extracted from 4 week old plant leaves using Tri Reagent (Chomczynski and Sacchi, 1987). 100 mg of tissue was ground in liquid nitrogen using a pestle and mortar and 1 ml Tri Reagent was added. Samples were vortexed and 200 µl of choroform added before vortexing again, samples were then centrifuged at 10,000 g for 15 minutes at 4°C. The aqueous phase (600 µl) was retained and 300 µl each of isopropanol and 0.8 M sodium citrate/1.2 M sodium chloride were added and mixed by inversion. After incubation at room temperature for 10 minutes samples were centrifuged at 10,000 g for 10 minutes. The pellet was washed in 1 ml of 75% ethanol and re-centrifuged as before and supernatant discarded. The pellet was resuspended in ~80 µl DEPC-treated water. The absorbance of each sample was measured at 260 nm and used to calculate the RNA concentration of each sample.

## 2.5. Northern blot

RNA (10 µg) was separated by formaldehyde-agarose (Sambrook et al., 1989) electrophoresis and transferred to a Hybond$^{TM}$-N hybridisation membrane according to

the supplier's instructions (Amersham Life Sciences, UK). Equal RNA loading was confirmed by methylene blue staining (0.3 M sodium acetate pH 5.5, 0.03% w/v methylene blue). Probes (described below, table 2.2) for hybridisation were labelled with $\alpha$-$^{32}$P-dCTP by random priming prepared using a Prime-a-Gene® labelling kit (Promega). The pre-hybridisation/hybridisation buffer contained dextran sulphate (10% w/v) to improve probe binding (Sambrook et al., 1989). After hybridisation overnight at 65°C, membranes were washed at 65°C, twice for 30 minutes in 1X SSC, 1% (w/v) SDS. Blots were exposed to X-Omat-AR ™ imaging film (Kodak, USA) and the film was developed on an X-ray developer.

**Table 2.2 Primers used to generate probes**

| Gene | Loci | Primer name | Primer sequence (5'-3') | Probe |
|---|---|---|---|---|
| *Cathepsin B* | At4g01610 | At4g01610-F | ACTCTGTTTCCATGGCTGTTT | 1Kb |
| | | At4g01610-R | TAACTCGAGGCAACCGGAA | |
| *PR1* | At2g14610 | PR1-F | TGCAGACTCATACACTCTGG | 0.3Kb |
| | | PR1-R | TATGTACGTGTGTATGCATGATC | |
| *GST1* | At1g02930 | GST1-F | GGTTCTTTAAGTGAATCTCAAAC | 1Kb |
| | | GST1-R | CAAGACTCATTATCGAAGATTAC | |

## 2.6. PCR based methods

### 2.6.1. Genotyping of NASC T-DNA knockout mutants

T-DNA insert mutants were ordered from Nottingham *Arabidopsis* Stock Centre (NASC); seeds arrive as progeny of a heterozygous parent. The T-DNA primer express website was used to design primers (Table 2.3). 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) as described in Doyle and Doyle (1990). Gene specific primers were used together with left border specific primer LBB1 in one PCR tube. The 800 bp band represented the wild type

allele whereas a 400 bp band represented the mutant allele. Seed was collected from plants homozygous for the insert. Knockout status was confirmed by RT-PCR.

**Table 2.3: Gene specific primers designed for T-DNA mutant genotyping**

| Gene | Loci | Description | Primer name | Primer sequence (5'-3') |
|---|---|---|---|---|
| Left Border | T-DNA | pROK2 | LBb1 | GCGTGGACCGCTTGCTGCAACT |
| *CathB1* | At1g02300 | SALK_049118 | CathBGenFor | AGAACCGCTTGGTCACATTTGC |
| | | | CathBGenRev | TGTGCAAAGTCCTGAAGAACAAAA |
| *CathB2* | At1g02305 | SALK_138261 | CathBbGenFor | GAGGAACGAACGAATGTGGCA |
| | | | CathBbGenRev | TTTTCAGCTGCAATACCCTGAGA |
| *CathB3* | At4g01610 | SALK_019630 | CathBcGenFor | GGTTTTGGTTTAGGGACATTGTGG |
| | | | CathBcGenRev | GCCTCTGTTCCATTGATTTGCC |
| *CathB1* | At1g02300 | SALK_151526 | BaKOF | CAAGCCAAGATTGACGATTGC |
| | | | BaKOR | TTCAATGCCACATTCGTTCGT |
| *CathB2* | At1g02305 | SALK_089039 | BbKOF | TGCGTGGCGGTACTTTAAGC |
| | | | BbKOR | TGGTAACAACAACACAGTGGGAA |
| *Cath B3* | At4g01610 | SALK_019629 | BcKOF | GGACATTGTGGTTCTTGCTGG |
| | | | BcKOR | CCCAGCCTCTGTTCCATTGAT |

### 2.6.2. RT-PCR

RT-PCR was carried out using Omniscript RT-PCR kits (QIAGEN, CA USA) according to the manufacturer's instructions: a half scale reaction was used. The PCR programs were as follows; Reverse Transcription step for 1 hr at 37°C. 2µl of these reactions were used as templates for semi quantitative 20 µl PCR reactions. Primers used for probes are shown (Table 2.4). Control reactions were carried out using primers for *actin* as a loading control and *PR-1* as a disease inducible control (using 1/10 dilutions of RT step as template for both). PCR reaction was: 30 seconds at 94°C, 3-step cycling x 30; (20 seconds at 94°C, 20 seconds at 50°C, 30 seconds at 72°C) and final extension 7 minutes at 72°C.

**Table 2.4. Primers for RT-PCR**

| Gene | Loci | Primer name | Primer sequence (5'-3') |
|---|---|---|---|
| *CathB1* | At1g02300 | At1g02300F | GGCCATGGCTGATAGTTGTTG |
| | | At1g02300R | GACTCGAGACTGAGGAAACCAGA |
| *CathB2* | At1g02305 | At1g02305F | AAAGATCCATGGCTGATAATGTAT |
| | | At1g02305R | GTGCTCGAGGAAACAAGAAGA |
| *CathB3* | At4g01610 | At4g01610F | ACTCTGTTTCCATGGCTGTTT |
| | | At4g01610R | TAACTCGAGGCAACCGGAA |
| *Actin* | At2g37610 | AtActin1-F | AAAGGATGCTTATGTTGGCG |
| | | AtActin1-R | AGCCACATACATAGCAGGGG |
| *PR1* | At2g14610 | PR1-F | TGCAGACTCATACACTCTGG |
| | | PR1-R | TATGTACGTGTGTATGCATGATC |

### 2.6.3. Quantitative real time RT-PCR (qRT-PCR) analysis

First strand cDNA was synthesised from 3 μg of RNA using Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen, UK) according to manufacturer's instructions. Real time RT-PCR reactions were performed using SYBR Green (QuantiTect SYBR GreenPCR kit, Qiagen, CA USA) and run on a Chromo4 thermal cycler (MJ Research, UK) using Opticon Monitor 3 software. Primer pairs were designed outside the region of cDNA targeted for silencing following the manufacturer's guidelines (Table 2.5). 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 2.5. qRT-PCR primers**

| Gene | Loci | Primer name | Primer sequence (5'-3') |
|---|---|---|---|
| *CathB1* | At1g02300 | CathB1F | CAAGACGATGTGTCCCTACTTC |
| | | CathB1R | TTGAAGGTGATTCTTTCAATGGT |
| *CathB2* | At1g02305 | CathB2F | GTGTTAGCGGAAACCAGCTT |
| | | CathB2R | CAGTGAAGGCAACCTCAACA |
| *CathB3* | At4g01610 | CathB3F | GAAATGCGTTAGCGACAACA |
| | | CathB3R | CTGCCATGATATCTTGTGGATT |
| *Actin1* | At2g37620 | ACTIN1F | CTCTTGTTTGCGACAATGGA |
| | | ACTIN1R | CGAGGACGACCCACAATACT |
| *Sag12* | At5g45890 | SAG12F | TTCTCGTCCACTCGACAATG |
| | | SAG12R | GCGATTGTTTTCCTCCTTCA |

## 2.7. Cloning and protein expression

### 2.7.1. Cloning of cathepsin B

The cDNA clones for At1g02305 and At4g01610 were ordered from RIKEN BioResource Centre (Japan). The At1g02300 cDNA was obtained from Col-0 RNA and *NbCathB* cDNA was obtained from *N. benthamiana* RNA. The cDNAs were sequenced to ensure correct sequence. Each CathB cDNA were amplified with *XhoI* and *NcoI* restriction sites in their primers and ligated into pGEM-T easy (Promega, UK) fragments were cut out and ligated into the pET-32 Expression vector (Novagen, UK) with and without signal peptides using *Xho I* and *Nco I* restriction sites and enzymes (Promega, UK). Cathepsin B cDNAs minus the signal peptides were also cloned into the pFLAG-ATS expression vector in the same way but using *XhoI* and *BglII* restriction sites and enzymes (Sigma, UK). The primers used are shown in AppendixI. The final constructs shown in Appendix II were introduced into the *E. coli* BL21, Origami and OrigamiB (Novagene, UK) and DH10B and DH5α (Invitrogen, UK) strains by transformation.

### 2.7.2. Protein expression and fraction purification

Overnight cultures were inoculated into 1 litre fresh L Broth media (as above) containing ampicillin 50 μg/ml, and incubated at 37°C. On reaching $OD_{600}$=0.6 isopropyl-l-thio-3-D-galactoside (IPTG) was added to 0.1 mM to induce expression and growth was switched to 30°C for 4 hours. Cells were harvested by centrifugation at 10,000 g for 10 mins. The pellet was resuspended in BugBuster (Novagen, UK) 5 ml per g cells. Insoluble debris was removed by centrifugation at 16,000 g for 20 min at 4°C. This soluble fraction was retained for SDS PAGE analysis.

### 2.7.3. HisBind / FLAG resin protein purification

Once the systems were optimised so that most protein is present in the soluble fraction, this fraction was purified as above. The protein was then purified using the His or FLAG tag by running the soluble fraction through a column using either HisBind resin (Novagen, UK) or FLAG resin (Sigma, UK) according to the manufacturer's instructions.

## 2.8. SDS PAGE

Samples were boiled for 10 minutes with SDS PAGE loading buffer (60mM Tris-Cl, 24%glycerol, 2% SDS, 14.4mM mercaptoethanol, 1% bromophenol blue) and separated on a 10 % SDS page gel (Sambrook et al., 1989) at 120V for 2 hours along with the Broad Range Protein Marker (NEB, UK). Gels were stained with Coomassie blue in order to check for successful induction of cathepsin B protein expression.

## 2.9. Western blot analysis

Western blots were performed as described by Sambrook et al. (1989). Proteins were blotted onto a PVDF membrane (Amersham, UK) by electrotransfer for 1 hour. Blocking was performed in antibody dilution buffer (10xTBS=12 g of Tris and 40 g of $NaCl_2$ per litre, adjusted to pH 7.6, 0.1% Tween and 5% BSA) for 1 hour. The blot was then incubated with a primary anti-His tag (Novagen, UK) or FLAG M2 (Sigma, UK) antibody at the appropriate dilution (1:2000) in antibody dilution buffer overnight at 4°C with agitation. The secondary anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (NEB, UK) was then incubated (1:5000) with the blot in antibody dilution buffer for 1 hour at room temperature. Protein detection was performed using a chemi-luminescent Photo-HRP kit (NEB, UK) according to manufacturer's instructions. The blot was exposed to X-ray film (Kodak, USA) and developed using an X-ray developer. Westerns with the mRFP antibody were done at a 1:2000 dilution with a secondary anti-rabbit HRP-conjugated antibody at a dilution of 1:5000 but were otherwise performed as above.

## 2.10. Transgenic line construction

### 2.10.1. Double and triple mutant generation

Double *cathepsin B* knockout lines were generated by crossing *cathb3-1* with either *cathb1-1* or *2-1* and selecting recombinants with genotyping PCR as above. A triple mutant could not be obtained by crossing so an RNAi hairpin construct was cloned according to Pawloski et al. (2005) and cloned into both constitutive (pGreen 0229-35s) (Hellens et al., 2000) and inducible (pER8) vectors (Zuo et al., 2000) using standard molecular biology techniques (Sambrook et al., 1989). Cloning primers are shown in Appendix I. Wildtype and *cathepsin B* double mutant *Arabidopsis* were transformed by floral dipping (Clough and Bent, 1998). Transgenic plants (Table 2.6) were selected using the herbicide Basta 150 µg/ml (pGreen) or hygromycin 20 mg/l (pER8).

### 2.10.2. Overexpressor line construction

Overexpressor lines (Table 2.6) were generated by cloning full length *cathepsin B 2* and *3* cDNA's into pGreen 0049-35s (Hellens et al., 2000) using standard molecular biology techniques (Sambrook et al., 1989). Cloning primers are shown in Appendix I. Wildtype *Arabidopsis* were transformed by floral dipping (Clough and Bent, 1998). Transgenic plants were selected using kanamycin (50 mg/l) and further screened for luciferase marker gene expression and then by Northern blot and quantitative RT-PCR.

**Table 2.6. Transgenic cathepsin B lines generated**

| Line | Gene(s) | Function/ Phenotype |
|---|---|---|
| *cathb1-1:3-1* | At1g02300, At4g01610 | Double knockout |
| *cathb2-1:3-1* | At1g02305, At4g01610 | Double knockout |
| *cathb2RNAi* | At1g02305 | RNAi line |
| *cathb 62-5* | At1g02300, At1g02305, At4g01610 | Triple knockout |
| *cathb 57-1* | At1g02300, At1g02305, At4g01610 | Triple knockout |
| *OX2-17* | At1g02305 | Overexpressor line |
| *OX2-12* | At1g02305 | Overexpressor line |
| *OX3-18* | At4g01610 | Overexpressor line |
| *OX3-3* | At4g01610 | Overexpressor line |

### 2.10.3. Real time *in planta* imaging of luciferase (LUC) activity

Before checking the LUC activity, luciferin (10 μM) was brushed on the leaf surfaces, and then plants were placed in the dark for 30 minutes in order to allow the luciferin to dry and to minimise background bioluminescence. All *in planta* LUC imaging was detected by using an ultra low light imaging camera system (Berthold, Redbourn, UK).

### 2.10.4. Localisation cloning

*NbCathB* cDNA was PCR amplified and cloned into pGEM-T vector (Promega, UK). The coding region was PCR amplified from a sequence-confirmed clone using primers designed to introduce an *Asc*I site at the 5' end and a *Not*I site at the 3' end while removing the stop codon. This PCR fragment was cloned into a version of pENTER 1A (Invitrogen) modified to contain *Asc*I and *Not*I restriction sites in the multiple cloning region. The *NbCathB* sequence was recombined with a derivative of the Gateway vector

pMDC84 (Curtis and Grossniklaus, 2003) in which the mgfp6 coding sequence had been replaced by *mRFP* (Campbell et al., 2002) then the cathepsin B-mRFP fusion construct was electroporated into *Agrobacterium tumefaciens* strain LBA4404 (Gilroy et al., 2007). Constructs are shown in Appendix II.

### 2.10.5. Transient PVX EPIC Expression

Green fluorescent protein (GFP) and extracellular protease inhibitors cystatin-like (EPIC) protease inhibitors in *A. tumefaciens* pGRAB potato virus x (PVX) expression vectors were obtained from Prof. S Kamoun. These were grown up in overnight cultures of L Broth supplemented with 50 mg $l^{-1}$ kanamycin, spun down and resuspended in 5 mM MES with 200 μM acetosyringone to an $OD_{600}$=1 and left in the dark for 2 hrs. This was then pressure infiltrated into the two largest leaves of small 4 leaf stage *N. benthamiana* plants which were then left for several weeks to allow systemic expression of the constructs.

## 2.11. Confocal microscopy

*A. tumefaciens* containing the *cathepsin B-mRFP* fusion construct was pressure infiltrated into leaves of 4-week-old *N. benthamiana*. A 1:1 mixture of *A. tumefaciens* containing the *EGFP-LT16b* and *cathepsin B-mRFP* constructs was also infiltrated for co-localisation. Cells expressing fluorescent protein fusions were observed using a Leica TCS-SP2 AOBS confocal microscope between one and five days post-infiltration. Images were obtained using an HCX APO 63x/0.9w water dipping lens. mRFP was imaged using an excitation wavelength of 568 nm from a 'lime' diode laser with emissions collected between 600 and 630 nm. GFP was imaged using 488 nm excitation from an argon laser, with emissions collected between 500 and 530 nm. Chlorophyll-associated autofluorescence was also obtained after excitation at 488 nm and the emissions collected between 650 and 700 nm. Brefeldin A treatment was with 10 μg $ml^{-1}$

(in water), infiltrated 24 h after agroinfiltration of the *cathepsin B-mRFP* construct and leaves were observed under the confocal microscope 6 h later (Gilroy et al., 2007).

## 2.12. Plant protein extraction and activity assays

### 2.12.1. Total protein extraction

Frozen leaves were finely ground and mixed with 300 µl of extraction buffer (50 mM monobasic, 50 mM dibasic potassium phosphate, pH6.8 and 1 mM 1,4-dithiothreitol). Samples were incubated on ice for 15 min and spun at maximum speed (16,000g) for 15 min to pellet cell debris. Supernatant was kept on ice or frozen at -70°C.

### 2.12.2. Apoplastic extraction

Ice-cold sterile distilled water (SDW) was vacuum infiltrated into ~60 *N. benthamiana* leaves 48 hrs after initiation of expression of CathB-mRFP or control contructs. Apoplastic fluid was then obtained by centrifuging (at 1250g for 10 min at 10°C) these leaves rolled in miracloth inside 30 ml syringes with fluid collected in 50 ml Falcon tubes. Proteins were concentrated using Amicon Ultra-15 filter devices (Millipore, UK) according to manufacturer's instructions.

### 2.12.3. Cathepsin B substrate assay

Assays were performed using clear 96-well microtitre plates (Fisher Scientific) and a DIAS plate reader (Dynatech Laboratories). Bradford reagent (Bio-Rad) was diluted 1:5 with SDW, aliquoted (199 µl) to each well of a 96-well microtitre plate and 1 µl of protein extract was added and mixed. The reaction was left for 30 min at room temperature 21°C and optical density was measured at 595 nm. The protein concentration was calculated using a formula derived from a bovine serum albumin dilution series. Cathepsin B colorimetric substrate Z-Arg-Arg-pNA. 2HCl (Calbiochem) was diluted to 2 mM in SDW. Assay buffer was prepared as recommended by

Calbiochem. Assay buffer and colorimetric substrate were mixed 1:1, then mixed with 1.8·volume of SDW. 140 µl of buffer/substrate solution was aliquoted into each well of a 96-well microtitre plate and 10 µl of protein extract added, mixed and incubated at room temperature and measured at 15 min intervals until readings level off. Optical density was read at 410 nm. Enzyme activity was divided by total protein (mg/ml) in crude extracts calculated using the Bradford assay (Gilroy et al., 2007).

### 2.12.4. DCG04 assay

*N. benthamiana* leaf protein extracts 17 ug total protein were incubated for 5 hours with (50 mM NaAc, 120 µg/ml L-cysteine, 1 µg/ml DCG-04) and competed with 16 µM of E-64, z-FA-fmk, Ca-074-Me, Ac-LVK-cho or *Avr2* as described previously (van der Hoorn et al., 2004). After labelling, biotinylated proteins were purified and detected on a protein blot using streptavidin-HRP, as described previously (van der Hoorn et al., 2004).

### 2.12.5. Mass spectroscopy

Isolated proteins were separated on a 10% SDS polyacrylamide gel and stained with colloidal Coomassie blue. The 30 kDa protein band was excised from the gel, treated with trypsin, and eluted peptides were analysed by MS/ MS as described previously (van der Hoorn et al., 2004).

## 2.13. Senescence assays

2 week old leaves were harvested and incubated in 3 mM MES pH 5.8 in Petri dishes in the dark (Oh et al., 1997) with samples taken at timepoints for qRT-PCR analysis. Total chlorophyll content was measured as a percentage of untreated samples. Chlorophyll from 100 mg tissue was dissolved in 5 ml dimethyl sulphoxide (DMSO) by heating, the absorbance of the resulting solution was measured at 663 and 645 nm and quantity was calculated according to Arnon (1949).

# 3. Isolation and Construction of Transgenic *Arabidopsis* Lines

## 3.1. *Arabidopsis thaliana*: the model plant

*Arabidopsis thaliana* or thale cress is a small "weed-like" plant which was first used by Laibach in the course of scientific studies in the 1940's. Since those early days *Arabidopsis* has been widely adopted by the plant scientist community as a model system to investigate areas as diverse as plant growth and development (Meyerowitz and Somerville, 1994) and disease resistance (Buell, 1998). With many ecotypes or varieties found throughout the world, *Arabidopsis* possesses many qualities which make it a useful model species, including its small size, easy cultivation, short lifecycle, the production of many seeds and the ability to be transformed without the need for tedious tissue culture regeneration (Meinke et al., 1998). With the formation of the *Arabidopsis* Genome Initiative (AGI) in the mid 90's, which led to the complete sequencing and annotation of the genome (AGI, 2000), the first for any plant species, both knowledge and the available resources have greatly improved. *Arabidopsis* is now known to possess 27235 genes on five chromosomes after the latest annotation (TAIR8 Release April 2008). This is the result of a complete genome duplication event, as well as many other gene duplications, losses and apparent diversifications (AGI, 2000). Approximately 30% of these genes encode products which have no identified function or indeed homology to any other known proteins (Wiseman and Ohlrogge, 2000) so the annotation of the genome is only the beginning.

## 3.2. Research tools

Among the tools which are now in use to investigate this wealth of genetic knowledge is gene chip technology where the expression of thousands of genes can be profiled in a

single experiment. Tissue specific and/ or various treatment responsive expression patterns can give clues to the involvement of a gene in a given process (Wiseman and Ohlrogge, 2000). Collections of DNA markers and polymorphisms such as CAPs and SSLPs are available, allowing existing mutant phenotypes to be mapped to their corresponding genes using simple PCR based techniques (Lukowitz et al., 2000). Large scale projects have also been developed to generate and catalogue pools of T-DNA insertion lines with the purpose of obtaining loss of function mutants for every gene by the Salk Institute for Genomic Analysis Laboratory (SIGnAL) (Alonso, 2003). With the explosion of data and resources generated, new web interfaces such as The Arabidopsis Information Resource (TAIR) and stock centres holding germplasm and mutant seed collections like the Arabidopsis Biological Resource Centre (ABRC) and the Nottingham Arabidopsis Stock Centre (NASC) were developed to disseminate the wealth of information and resources (Scholl et al., 2000; Huala et al., 2001; Garcia-Hernandez et al., 2002). It is now possible to take a gene of interest, search the genome for homologues using the basic local alignment search tool (BLAST), check the expression patterns and order T-DNA knockout mutant seed all in a day's work.

## 3.3. Isolation of T-DNA insert *cathepsin B* knockout lines

### 3.3.1. Homologue identification

The cathepsin-like protease *StCathB*, isolated in an SSH screen in potato for genes involved in the HR and defence against *Phytophthora infestans* (Avrova et al., 2004) were blasted against the *Arabidopsis* genome. This revealed that *Arabidopsis* possesses three *cathepsin B*-like genes with homology (2e-23) to *StCathB*: *At1g02300, At1g02305* and *At4g01610* hereafter referred to as *AtCathB1, 2* and *3* respectively. The proteins encoded by these genes have 74% identity and 83% positives with *St*CathB protein according to Blast2 analysis (Tatusova and Madden, 1999). These genes have been selected for further investigation here.

### 3.3.2. Genotyping

Two individual SALK T-DNA insertion lines were ordered from NASC for each *Arabidopsis CathB* gene in order to obtain knockout lines (Fig 3.1). Inserts which are present in an exon have more chance of effectively blocking gene expression than those present in introns or UTRs. The seeds which arrive are the progeny of a heterozygous individual so the plants require genotyping to identify those which are homozygous for the T-DNA insert. This was done by designing primers, using the SIGnAL website, which flank the site of the insert; amplification of an 800 bp product by PCR is possible only in the absence of the insert. Another primer specific for the T-DNA left border is used in a three primer reaction to yield a 400 bp product in the presence of the insert (Fig 3.2) Seed was collected from plants homozygous for the T-DNA inserts and the next generation of plants was genotyped to verify the presence of two copies of the T-DNA. When genotyping *cathb3-2* no T-DNA inserts were detected in two individual orders of seed, so a homozygous line was not obtained.

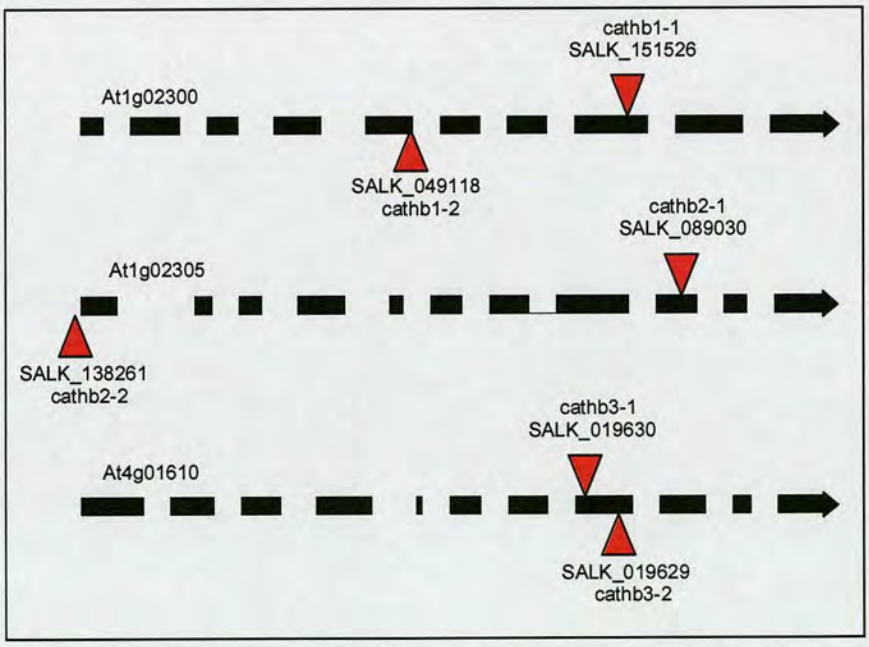


**Figure 3.1. Position of T-DNA inserts.** A schematic diagram of the three Arabidopsis cathepsin B genes with triangles showing the position of the SALK T-DNA insertions. Exons are black, introns are white.

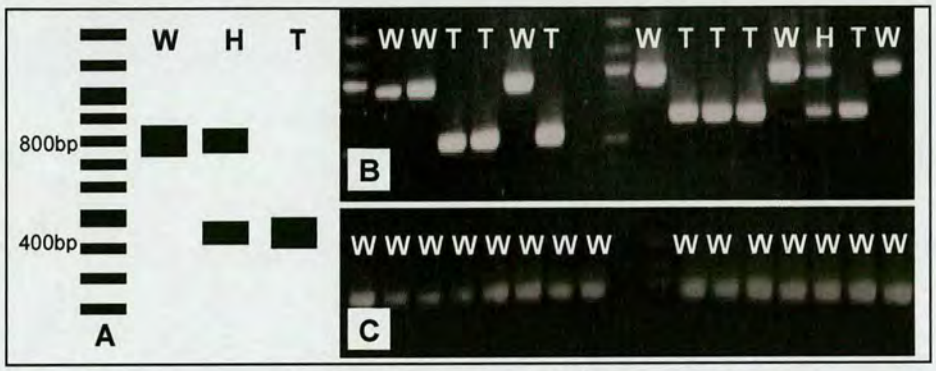


**Figure 3.2: Examples of T-DNA Genotyping using PCR.** (A) Shows the expected results for a genotyping PCR reaction using 3 primers. One primer specific for the Left border of the T-DNA insert and a pair of primers designed to flank the region containing the insert. In the absence of the T-DNA a band of ~800bp is formed, if the insert is present a band of ~400bp is also produced; plants heterozygous for the T-DNA show both bands. (B) Shows the genotyping of the progeny of plants heterozygous for the insert. (C) Shows a line in which no T-DNA insert was detected. W= wild type, no insert, H= heterozygous for the insert and T= homozygous for the T-DNA insert.

### 3.3.3. Knockout confirmation

In order to determine if the presence of homozygous copies of the T-DNA insert was sufficient to knockout the transcription of each *Arabidopsis CathB* gene RT-PCR was carried out to detect cathepsin B transcripts. Although the three genes possess extensive homology; it was possible to distinguish between them using PCR based methods but not by traditional Northern blotting hybridisation based methods. The RT-PCR results (Fig. 3.3a) show that out of the five insert lines tested only the three lines which were designated *cathb1-1, cathb2-1* and *cathb3-1* are true gene knockouts. It is interesting to note that the presence of the T-DNA insert in *cathb2-1* has a slight effect on the expression of *CathB1* in this mutant but not on *CathB3*. This may be due to genes *1* and *2* being in close proximity to each other. *CathB1* also appears to form two distinct transcripts which can be resolved by electrophoresis but are not annotated in the genome. Although, it was intended to obtain two independent knockout alleles for each gene one homozygous knockout line was isolated for each *AtCathB* gene.

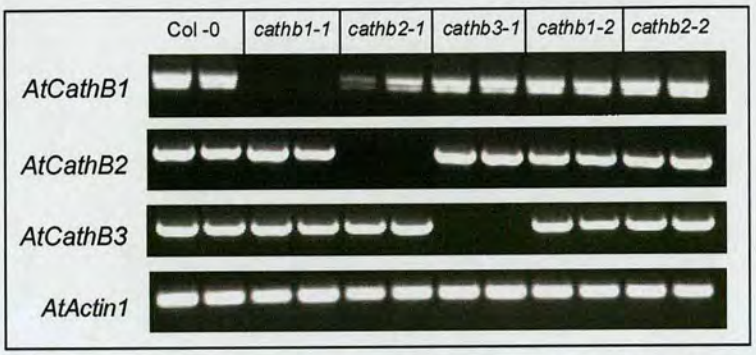


**Figure 3.3. RT-PCR gene knockout confirmation.** (A) One T-DNA insert knockout line was obtained per cathepsin B gene, the inserts in *cathb1-2* and *2-2* lines do not affect gene expression. Two independent samples are shown per insert line and Col-0 wildtype, actin is used as a loading control.

### 3.4. Double mutant generation and selection

As the *Arabidopsis cathepsin B* genes are highly similar, some functional redundancy may be expected to mask either partially or completely any phenotypes due to the absence of one or more of the genes. In anticipation of any compensation effects of the three genes; double and eventually triple mutants were to be generated. The *cathb3-1* line was crossed to both *cathb1-1* and *cathb2-1* in order to generate double knockout mutant lines as part of this ongoing process. Each line was used both as pollen donor and recipient in two reciprocal crosses in case there were any problems with fertility. All crosses produced viable seed, however, and the $F_1$ was left to self. The next generation underwent the same PCR based genotyping to identify the double mutants and RT-PCR was again used to verify the abolition of transcripts (Fig 3.4). As At1g02300 and At1g02305 are tightly adjacent on chromosome 1, separated by only 100 bp *cathb1-1* and *2-1* lines were not crossed as the recombination frequency to recover double mutants would be prohibitively low.

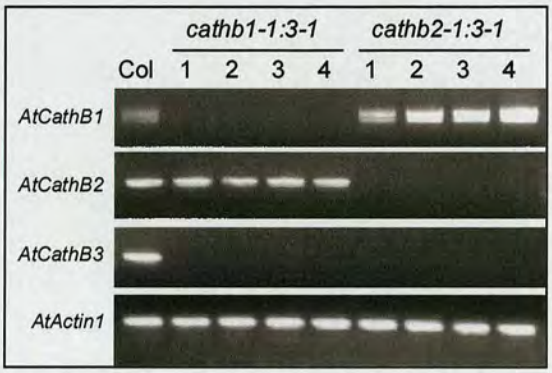


**Figure 3.4. RT-PCR showing double knockout mutants.** Double knockouts were obtained between *cathb1* and *3* and *cathb2* and *3*. Four independent samples are shown for each line along with a wildtype Col-0 control; actin is used as a loading control.

## 3.5. Triple mutant generation and selection

### 3.5.1. Gene silencing using RNA interference

As *cathb1* and *2* could not be crossed to obtain a triple knockout line another approach was taken. RNAi is a relatively new technique used to reduce gene transcript levels, which works on the basis that double stranded RNA is recognised by cells and then degraded by an RNAse type III protein called Dicer (Ketting et al., 2001; Knight and Bass, 2001). This is thought to have developed as a defence strategy to recognise and destroy viral replication intermediates (Ratcliff et al., 1997). Small fragments of the degraded RNA are then taken up by the RNA induced silencing complex (RISC) mediated by Argonaute (Ago) proteins and used to target further destruction of RNA with the same sequence (Baulcombe, 2004; Miska and Ahringer, 2007). However, employing RNAi technology is seldom likely to completely knockout a gene, in contrast to a T-DNA insertion, but good knockdowns of 70-90% silencing of transcripts is routinely achievable (Pawloski et al., 2005). Based on this principle a construct was designed to target *CathB2* using a region with high homology between the three *Arabidopsis* genes. 200 bp fragments of *CathB2* were cloned in sense and antisense

orientation separated by an intron, so that when transcribed a hairpin structure of double stranded *CathB2* RNA will be produced and target this gene's mRNA for degradation. As it is possible *CathB* genes are in some way necessary for *Arabidopsis* growth and development this construct was introduced to both constitutive and inducible expression vectors in case a triple mutant proved lethal (Fig 3.5). Using an inducible system has the advantage that plants are able to grow and develop normally up to the desired point when expression of the transgene can be stimulated by application of a chemical inducer, in this case estradiol. Both constructs were introduced into *cathb1-1:3-1* double mutants via *Agrobacterium*-mediated transformation to obtain a triple mutant.

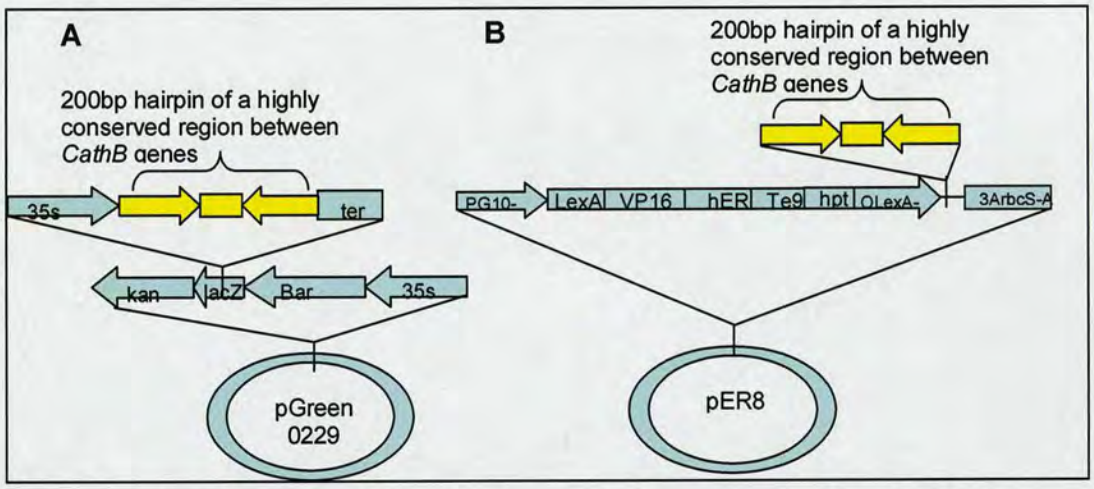


**Figure 3.5. Schematic diagrams of RNAi Constructs.** (A) Constitutive, modified pGreen (Hellens et al., 2000) and (B) Estradiol Inducible (Zuo et al., 2000) constructs were made.

### 3.5.2. RNAi line specificity

The constitutive construct was also introduced into wild-type *Arabidopsis* to investigate the specificity of the RNAi construct. Previous work was done silencing *CathB* in *N. benthamiana* and *Solanum tuberosum* where the number of *cathepsin B* genes present in the genome is unknown as these species have not yet been sequenced. So it would be useful to find if an RNAi construct designed for one *CathB* gene is capable of affecting the transcripts of closely homologous genes. Although the RNAi transgene was

designed primarily to knockdown *CathB2* transcripts, Figure 3.6 shows that the levels of *CathB3* and to a lesser extent *CathB1* transcripts are also affected.

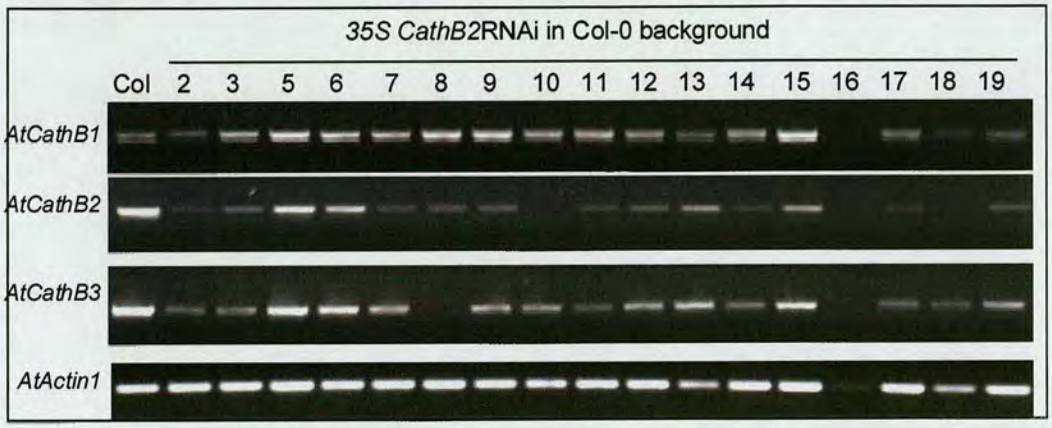


**Figure 3.6. RNAi construct specificity.** The expression levels of all three *CathB* genes were checked using RT-PCR for 19 $T_1$ basta resistant plants expressing 35SRNAi*CTB2* in a wildtype Col-0 background. Actin was used as a loading control and shows low cDNA levels in line 16.

### 3.5.3. Triple mutant screening

Approximately 200 basta resistant plants in the $T_1$ of pGreen0229-35SRNAi*CathB2* in the *cathb1-1:3-1* background were screened for their *CathB2* expression by RT-PCR. Of these, 11 plants were found to have lower expression and were left to self. In the $T_2$ the basta segregation was checked to determine how many copies of the transgene had integrated. Only two lines in the $T_2$ were found to have maintained the low expression of *CathB2*, #57 and #62, the latter having the lowest expression and 1 copy of the transgene. The $T_3$ generation was basta treated to check which $T_2$ individuals were homozygous for the transgene and the expression levels of CathB2 were quantified in homozygous individuals using Taqman analysis and it was determined that >90% of gene silencing was affected by the RNAi transgene (Fig3.7). As a good triple candidate was isolated with the constitutive construct, no further work was carried out on the inducible RNAi lines other than $T_2$ transformant selection (not shown).

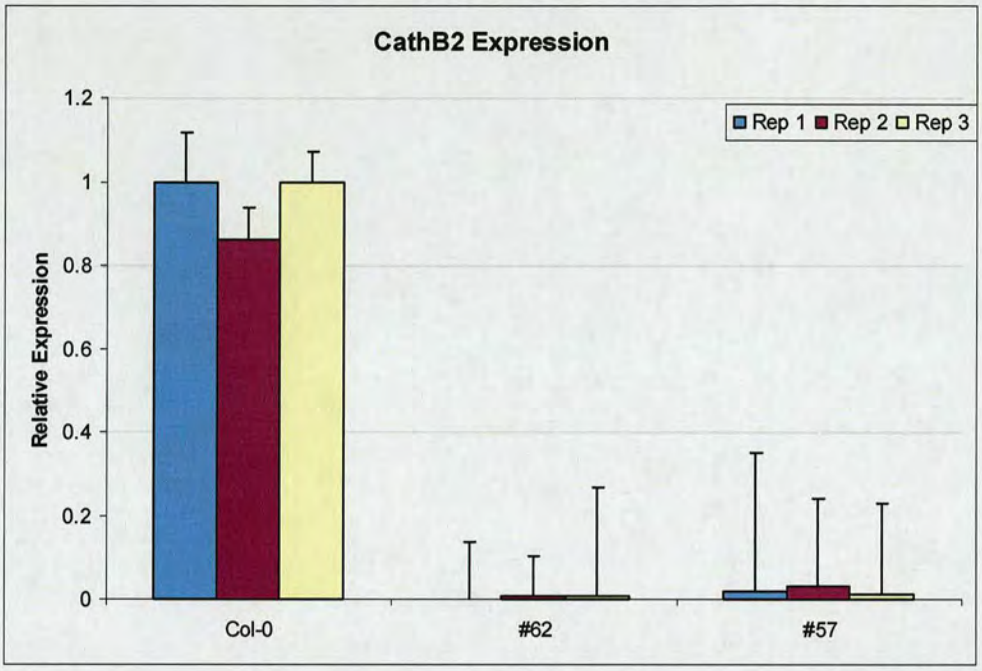


**Figure 3.7. Quantification of *CathB2* expression levels in homozygous #62 and #57 plants.** The values are normalised to actin expression. Each sample is shown relative to Col-0 expression at 0 dpi. Error bars are SE of three samples within one biological replicate. This experiment is shown for three independent biological replicates with similar results.

## 3.6. Overexpressor line construction and selection

### 3.6.1. Splicing of *CathB1*

Another way to overcome problems where functional redundancy can mask gene phenotypes is to overexpress a given gene (Weigel et al., 2000). The overexpression of the *Arabidopsis cathepsin B* genes was attempted but first the two alternate splice forms of *CathB1* were investigated. The two spliceforms designated *B1a* and *B1b* (Fig 3.8a) were therefore cloned and sequenced. There seems to be some problem with the splice sites between exons 4 and 5 as it was found that *B1a* is missing most of exon 5 whereas *B1b* has incorporated a large chunk of sequence from intron four (Fig 3.8b). These

transcripts were translated into protein sequences and found in both cases to encode truncated proteins due to the incorporation of stop codons prematurely due to changes in the reading frames caused by the mis-splicing.

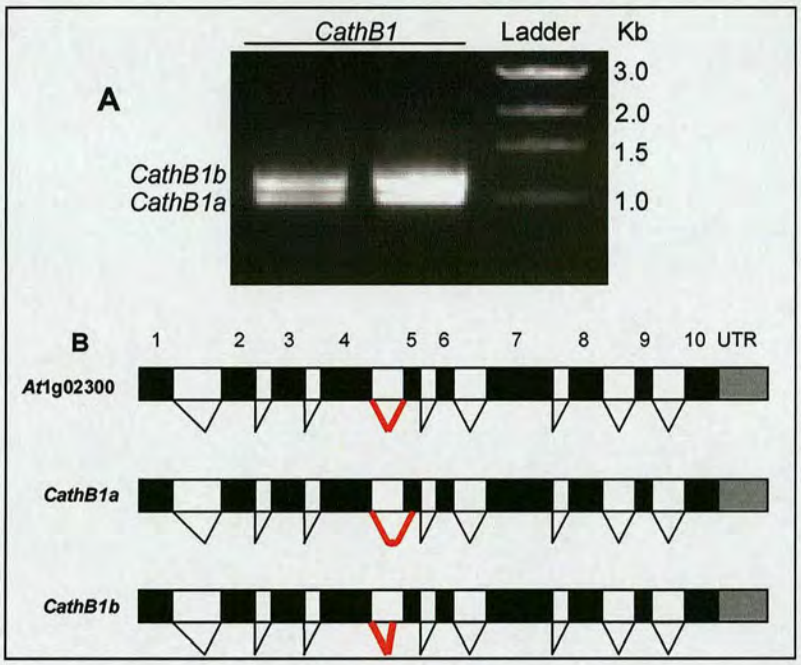


**Figure 3.8. Alternative Splicing of At1g02300.** (A) Two splice-forms of approximately 1kb of At1g02300: *CathB1a* and *B1b* are isolated after RT-PCR on Col-0 RNA. (B) This diagram shows the splicing of the TAIR predicted mRNA (At1g02300) and after sequencing the two splice-forms cloned. *B1a* is missing part of exon 5 while *B1b* has incorporated part of intron 4. Exons = black, introns = white, 3' UTR = grey, splicing is indicated with lines below the genes, the altered splicing is shown in bold.

### 3.6.2. *CathB2* and *3* overexpression

As *CathB1* encodes truncated proteins it was decided to focus on the overexpression of *CathB2* and *3*. The cDNAs used to clone these genes were obtained from Riken, Japan (Sakurai et al., 2005) and the full length coding sequence was cloned into an *Arabidopsis* expression vector containing a luciferase reporter gene and a kanamycin resistance gene for selection *in planta* (Fig 3.9a). $T_1$ plants were screened on plates for resistance to kanamycin and then put through a secondary screen for luciferase

expression to determine whether the transgenes had integrated into sites conducive to high levels of gene expression (Fig3.9b-d). $T_1$ plants were left to self and the $T_2$ seedlings were again screened on kanamycin plates for those lines with a 3:1 resistant: susceptible ratio which indicates just one copy of the T-DNA integrated into the genome (Fig. 3.9e-f).

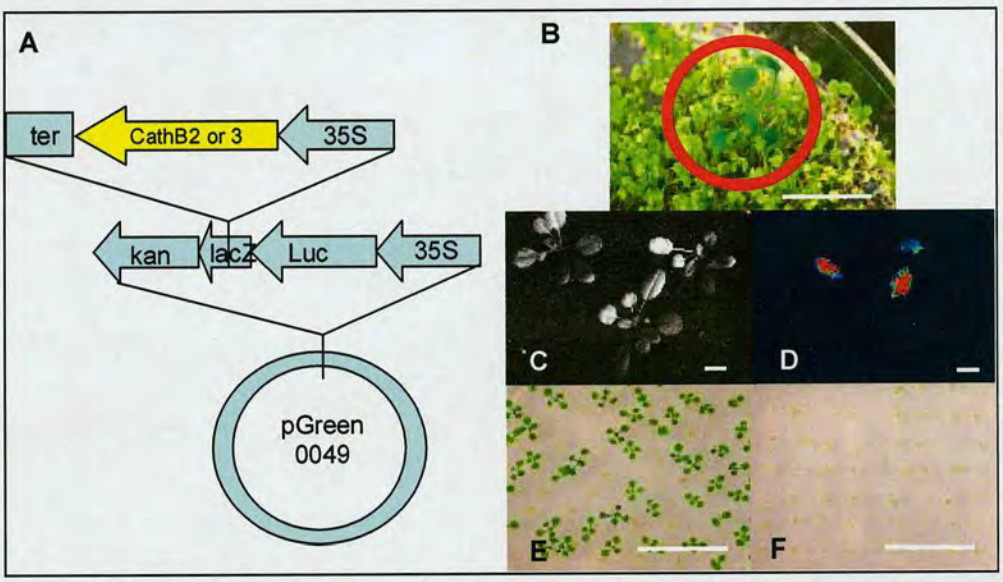


**Figure 3.9: OX line construction and selection.** (A) Schematic of transgene construction. (B) Selection of transformants on kanamycin plates. $T_1$ plants were screened for Luciferase activity using low light imaging under (C) light and (D) dark. Transgene segregation on kanamycin plates to check the insert number of (E) *OXCathB2* and (F) Col-0 control, scale bars are 2cm.

Seven individual $T_2$ lines were selected for each overexpressor construct bearing in mind transgene copy number and luciferase activity and these plants were left to self. $T_3$ seeds were subjected to kanamycin selection to identify those which were homozygous for the construct. RNA was isolated from several individuals for these homozygous lines and Northern blot analysis was performed using a full length *CathB* cDNA as a probe; this is known to cross-react to all three genes. The lines 2-12 and 2-17 were found to strongly overexpress *CathB2* and lines 3-3, 3-7 and 3-18 strongly overexpress *CathB3* (Fig. 3.10).

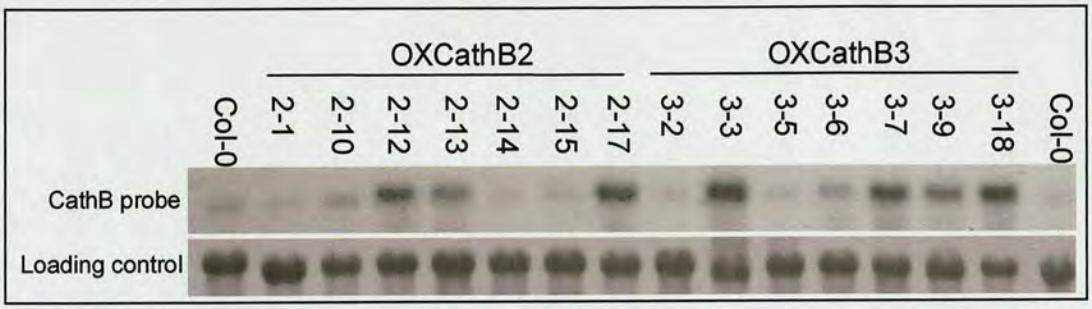


**Figure 3.10. Northern blot showing CathB expression in putative overexpressor lines.** Total RNA from $T_3$ plants containing each of the 35S::*CathB* transgenes was northern blotted and probed with a full length CathB3 cDNA which cross reacts with both genes. Total RNA was stained with methylene blue as a loading control.

## 3.7. Discussion

Homologues of the cysteine proteases which were laboriously isolated and cloned in the crop plant Potato, *Solanum tuberosum*, were easily identified using simple blast analysis in the model plant species *Arabidopsis thaliana*, for which the genome has been sequenced. Although these genes were predicted to be involved in incompatible interactions against the major pathogen *P. infestans*, an economically important field of study, working on potato genetics is very difficult and time consuming as there is little sequence information, transformation is difficult and there are fewer tools available to manipulate gene expression. However, by working with homologous genes in an organism such as *Arabidopsis* where this situation is the opposite, as more is known and the plant is more easily manipulated, it may be possible to implicate these genes in a known pathway and identify other candidates for use in Potato.

*Arabidopsis* has three *CathB* genes with ~75% similarity to *StCathB*. Individual knockout lines have been obtained for each gene and double mutants were generated by crossing. A triple mutant was generated using RNAi technology. It was discovered that the RNAi construct designed for *AtCathB2* was also able to affect the transcript

accumulation of the other two *cathepsin B* genes. This has implications for using silencing in other species such as *S. tuberosum* and *N. benthamiana* where the number of a given type of gene is not known. Providing there is enough sequence conservation it may be possible to knockout all members of the family. In this case the *35SRNAi::CathB2* transgene was able to knockout *CathB2-1* to a very low level in a *cathb1-1:3-1* background; this is effectively a triple knockout.

The constitutive RNAi construct was successful in producing a triple mutant so experiments to select lines from the inducible construct were discontinued at the T2 level. Inducible transgenes have many advantages if the gene which you are trying to knockout or overexpress has lethal consequences for plant growth and development, as the expression can be induced transiently for short periods of time after the necessary stages of development have passed. However, as with any system there are also drawbacks, such as the reproducibility of transgene induction and perturbation of the system under investigation by the chemical inducer. The pER8 vector used here has an estradiol-inducible construct, although no estrogen receptor has been identified in plants thus far. The inducible construct was employed in this instance in the eventuality that a constitutively generated triple knockout of *cathepsin B* genes was lethal, which is now known not to be the case.

The two apparently aberrant splice forms of *AtCathB1* discovered here to encode short truncated proteins is also interesting. These predicted proteins would contain a signal peptide, the pro-protein domain and the N terminus of the protease domain stopping just short of the crucial active cysteine residue. If this is the case then this gene is likely to encode a non-functional protein and therefore may be a pseudogene. However, the TAIR database shows 11 ESTs for this gene and recently a putative full length cDNA was isolated (Gene bank Accession AK221536) but the encoded protein they predict (BAD94873) only consists of the last 183aa of CathB1 and is missing the N-terminus of the protease domain including the critical active cysteine residue (Seki et al., 2002).

As well as knockout mutants the overexpressor mutants *OXCathB2* and *3* were isolated. It will be interesting to examine these mutants as any phenotypes would be predicted to be the opposite of the knockout mutants. One point to note is that although the genes have been demonstrated to be highly overexpressed by Northern blot, this may not translate into overexpressed active protein. Regulation of these proteins is thought to be under tight control as unrestrained protease activity could be extremely hazardous. Therefore, cysteine proteases are synthesised as inactive precursors or zymogens which require cleavage of a propeptide to activate them (Turk et al., 2001).

# 4. Cathepsin B in Basal and *R* Gene-Mediated Resistance

## 4.1. *Arabidopsis: Pseudomonas* model pathosystem

In the early 1990's research was published showing data obtained following the screening of multiple *Pseudomonas syringae* stains on the established model plant *Arabidopsis*, demonstrating the first laboratory recorded case of a pathogen infecting and causing disease symptoms on this plant (Dong et al., 1991; Whalen et al., 1991; Katagiri et al., 2002). *P. syringae* (DC3000) pathovar *tomato* (*Pst*), a gram negative bacterial pathogen, has become one of the most widely used laboratory strains, leading to the sequencing of its genome (Buell et al., 2003), thus, facilitating a model pathosystem where both host and pathogen are genetically tractable. This pathogen is thought to grow epiphytically on leaf surfaces before invading the plant's intercellular spaces where it subsequently multiplies and engages the release of water and nutrients into the apoplast by some unknown mechanism (Hirano and Upper, 2000; Katagiri et al., 2002).

Some early advances made with this pathosystem include the discovery that it adheres to the gene-for-gene model proposed by Flor in the 1970's, with the cloning of the *AvrRps2/RPS2* pair in the mid 90's (Mindrinos et al., 1994). Subsequently, different classes of *Resistance* (*R*) genes have been defined (Dangl and Jones, 2001) and key genes isolated which appear to regulate defence signalling pathways *NDR1*, *EDS1*, *PAD4*, *GSNOR1* and *NPR1* (Cao et al., 1994; Glazebrook et al., 1996; Century et al., 1997; Zhou et al., 1998; Feechan et al., 2005). Various forms of systemic immunity such as SAR and ISR (Pieterse et al., 1998; Delaney, 2000) and the hormonal regulators SA, JA and ET (Delaney et al., 1994; Penninckx et al., 1998) have also been uncovered through studies with this system, as have a series of lesion mimic mutants which highlight genes involved in the regulation of the HR, a form of PCD (Lorrain et al.,

2003). The identification and functional characterisation of *Pst* effectors such as AvrRpt2 and HopU1 and how they may manipulate their host targets RIN4 and GRP7 in order to suppress basal defence responses is a current hot topic (Mackey et al., 2003; Kim et al., 2005; Nomura et al., 2005; Fu et al., 2007).

Despite the wealth of knowledge accumulated from these studies, there is some debate about the validity of this pathosystem, as artificial methods of inoculation such as use of surfactants or syringe infiltration are required for *Pst* to infect *Arabidopsis* (Katagiri et al., 2002). Nevertheless, avirulence genes recognised by *Arabidopsis* are sufficient to convert virulent strains of *P. syringae* to avirulent strains on their crop plant hosts, indicative of conservation of defence signalling mechanisms (Whalen et al., 1991; Innes et al., 1993; Quirino and Bent, 2003).

### 4.2. Forward and reverse genetics

Most of the key genes involved in *Arabidopsis: P. syringae* interactions identified to date, have been isolated through the use of forward genetic approaches. This involves large scale phenotypic screening of mutagenised *Arabidopsis* plants for individuals with an altered response to the pathogen employed. The gene responsible for the phenotype can usually be subsequently identified by the tedious process of map-based cloning. Essential genes such as *EDS1, PAD4* and *NDR1* involved in both basal and *R* gene-mediated resistance (Glazebrook et al., 1996; Parker et al., 1996; Century et al., 1997) have been identified using this type of forward genetics approach, proving its usefulness, but there are also drawbacks. Screens of this type, while isolating important genes, are not able to detect those genes which may play a smaller or redundant role in the interaction. Moreover, continued screening has tended to re-isolate mutations in previously known genes, as in the case of *NPR1* and *NIM1* which have mutations in the

same gene and were identified by different groups simultaneously (Cao et al., 1994; Delaney et al., 1995).

Reverse genetics on the other hand, has been made possible on the sequencing of both model plant (AGI, 2000) and model pathogen (Buell et al., 2003) genomes, and entails the selection and functional characterisation of a gene suspected to be involved in a particular process. Reverse genetics approaches, such as those used here, have the advantage of being able to look at genes with modest effects on the interaction or the process under investigation, which would undoubtedly be missed using traditional forward genetic methods.

## 4.3. Cathepsin B in basal defence

### 4.3.1. *Cathepsin B* transcription is affected by virulent *Pst*

The expression of *CathB2* and *3* was investigated in 4-week-old wild-type *Arabidopsis* plants in response to inoculation with *P. syringae* pv. *tomato* DC3000 (hereafter *Pst*) which is known to trigger basal resistance. The expression of *CathB2* and *3* (Fig 4.1) is induced by approximately 3 and 3.5-fold respectively by 24 hours post infection with virulent *Pst*. More interestingly, the expression of both genes at 2 hours post infection appears to be repressed; the expression of *CathB2* remains repressed at 6 and 12 hour time-points whereas *CathB3* expression returns to its base level at these times.

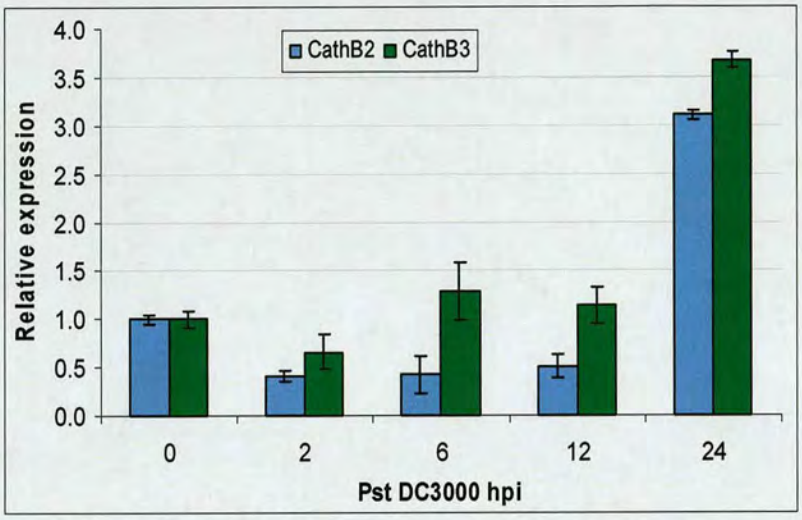


**Figure 4.1. *Cathepsin B* expression in Col-0 in response to challenge with virulent *Pst* DC3000.** The values are normalised to *Actin* expression. Each sample is shown relative to expression at 0 hpi. Error bars are SE of 3 samples within one biological replicate.

### 4.3.2. *Cathepsin B* single and double mutants are not susceptible to *Pst*

Colony count experiments carried out on the single and double *cathb* T-DNA insert knockout lines and cathb over-expressing lines has revealed that none of these mutants allow an increase in *Pst* DC3000 growth compared to wild-type Col-0 plants (Fig. 4.2). Neither Col-0 or *cathb* single, double or over-expresser mutant lines exhibit disease symptoms when inoculated with a low dose ($10^5$cfu/ml) of pathogen, whereas established susceptible mutants, *nahG* and *Atgsnor1-3* (Friedrich et al., 1995; Feechan et al., 2005), show extensive chlorosis.

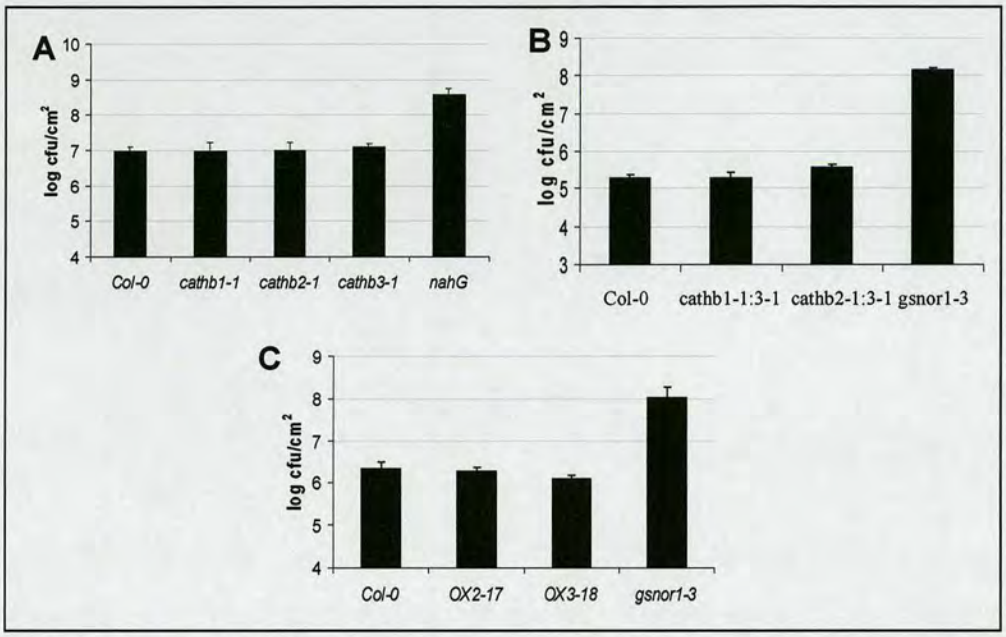


**Figure 4.2. *Pst* DC3000 colony counts with *cathb* transgenic lines.** (A) *Cathepsin B* single knockout lines. (B) Double knockout lines. (C) Over-expresser lines. Graphs show mean colony forming units recovered from each line at 4dpi. Error bars represent SE of four or more samples pooled from 10 individuals in one biological replicate. Wild-type Col-0 and susceptible mutants *nahG* and *gsnor1-3* are used as controls. Multiple biological replicates showed similar results.

### 4.3.3. *Cathepsin B* triple knockouts are more susceptible to *Pst*

In contrast to the single and double *CathB* knockout lines the triple knockouts *cathb#62* and *#57* permit significantly more growth of virulent *Pst* than wild-type Col-0 plants (Fig. 4.3). Mild symptom development can be observed on *#62 and #57* mutants as chlorosis or yellowing (Fig. 4.3) which is absent on Col-0 plants at the low dose ($10^5$cfu/ml) inoculated. However, these plants are clearly not as hyper-susceptible as established mutants such as *gsnor1-3* (Feechan et al., 2005). Nevertheless, this suggests that the three *cathepsin B* genes are acting redundantly during basal resistance to limit *Pst* growth, as the increased susceptibility in the triple mutants is masked in the double and single knockouts.

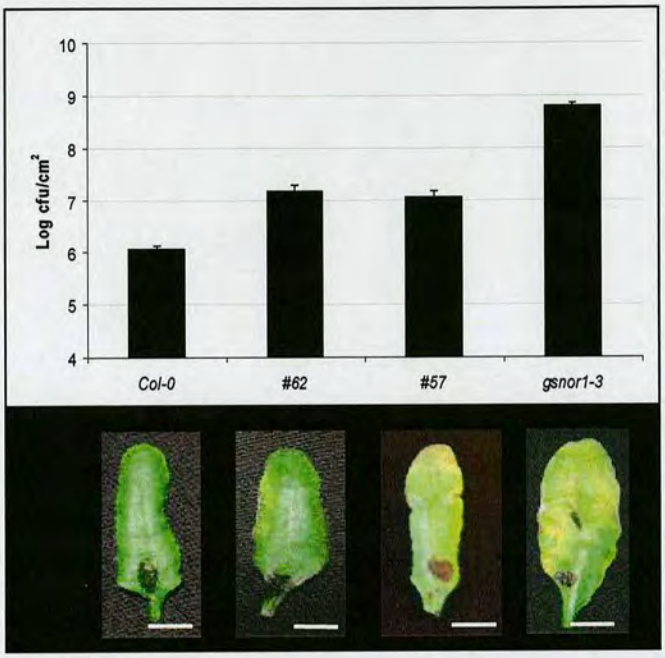


**Figure 4.3. *Pst* DC3000 colony counts with triple knockouts.** Graph shows mean colony forming units recovered from each line at 4dpi. Error bars represent SE of four samples pooled from 10 individuals in one biological replicate. Wild-type Col-0 and susceptible mutant *gsnor1-3* are used as controls. Typical symptoms observed are displayed below the graph for each line, scale bars are 1cm. Multiple biological replicates were performed with similar results.

### 4.3.4. Cell death to *Pst*

In order to examine cell death in response to virulent *Pst*, half leaves from four week-old wild-type and *cathb* double and triple mutant *Arabidopsis* were infiltrated with $10^6$ cfu/ml of *Pst*. Samples were taken at an early time-point of 19 hpi during the biotrophic phase in order to avoid the necrotic lesions formed at later stages of the interaction. A reduced amount of cell death was observed on both double mutants when compared with wild-type Col-0 plants (Fig. 4.4). In contrast, the cell death observed in the triple mutants appeared less than on wild-type but it also showed a different pattern of spatial localisation. In the triple mutants most cell death occurred in spongy mesophyll cells surrounding the leaf veins but in wild-type and double mutants cell death was found in palisade mesophyll cells and was not associated with the plant vascular system.

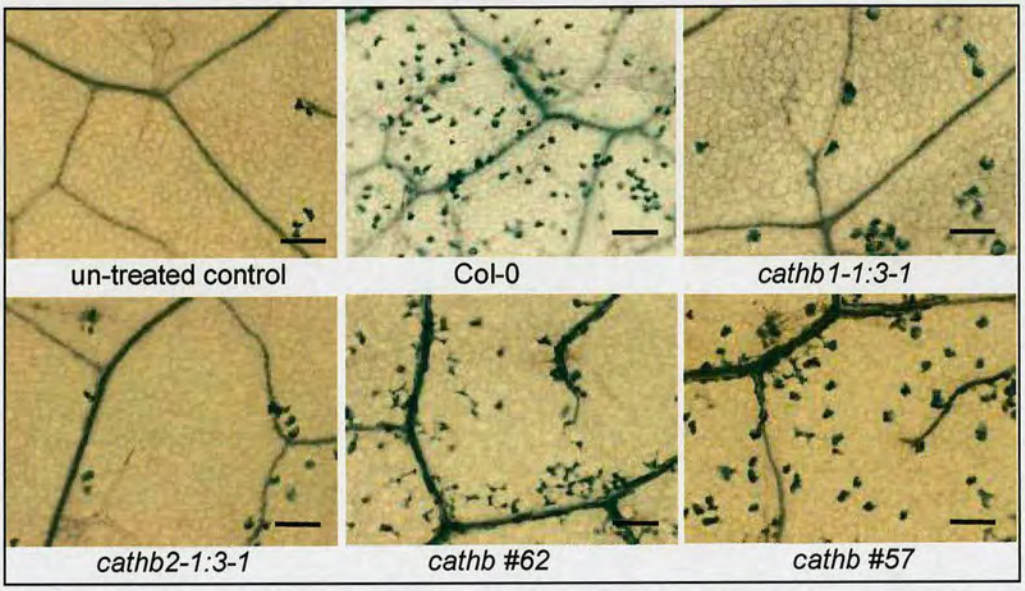


**Figure 4.4. Cell death post-inoculation with *Pst*.** Trypan Blue staining for cell death was performed at 19 hours post inoculation with $10^6$ cfu/ ml of *Pst* DC3000. Images were taken with the x4 objective using a light microscope, scale bars are 250μm.

## 4.4. Cathepsin B in *R* gene-mediated defence

### 4.4.1. *Cathepsin B* expression is affected by *Pst AvrB*

Wild-type Col-0 plants were infected with *Pst* DC3000 carrying the avirulence gene *AvrB* on a plasmid. This gene is recognised by the plant *RPM1* gene which triggers *R* gene-mediated resistance in *Arabidopsis* ecotypes carrying this gene. Transcription of *CathB3* was found to increase steadily in response to *Pst AvrB* challenge from 2hpi to 24hpi where it showed greater than a 10-fold increase in expression as measured by qRT-PCR (Fig. 4.5). In contrast *CathB2* was only observed to be 7-fold up-regulated at the 24hpi time-point with no change in expression at prior time-points. This is a greater increase in *cathepsin B* expression than the 3.5 fold increase observed in response to virulent *Pst* DC3000.

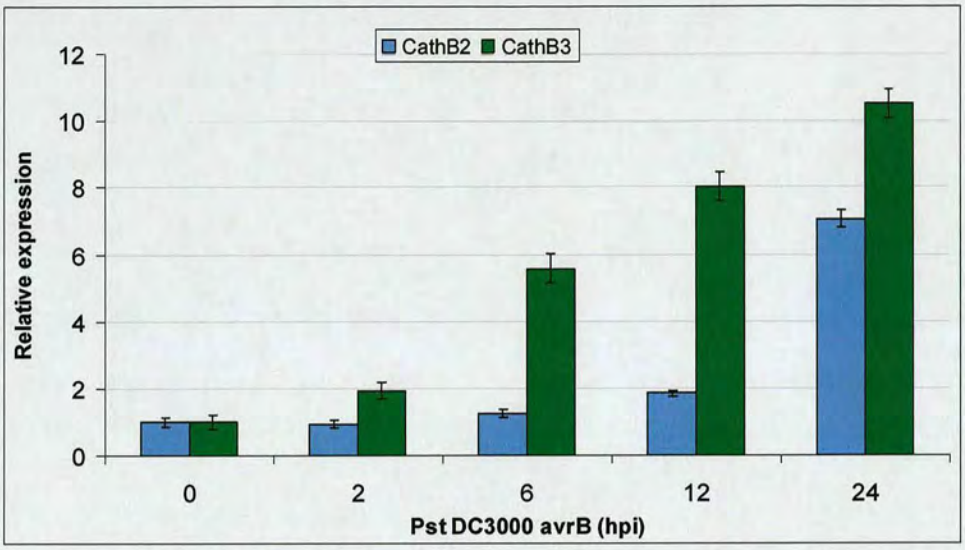


**Figure 4.5. *Cathepsin B* expression in Col-0 in response to challenge with *Pst* DC3000 *AvrB*.** The values are normalised to *Actin* expression. Each sample was measured in triplicate and is shown relative to expression at 0hpi. Error bars are SE of 3 samples within one biological replicate

### 4.4.2. *cathb* mutants do not enhance susceptibility to *Pst AvrB*

In order to determine if the increase in *CathB* expression in response to *Pst AvrB* indicated a role in resistance to this pathogen, assays were carried out to examine pathogen growth on *cathb* knockout lines. However, in spite of the enhanced expression of *CathB* genes when challenged with this pathogen, none of the transgenic *cathepsin B* lines showed a reproducibly significant increase in susceptibility to *Pst AvrB* as measured by colony count assays (Fig. 4.6). This indicates that if these genes do play a role in this interaction it does not seem to affect the growth of the pathogen.

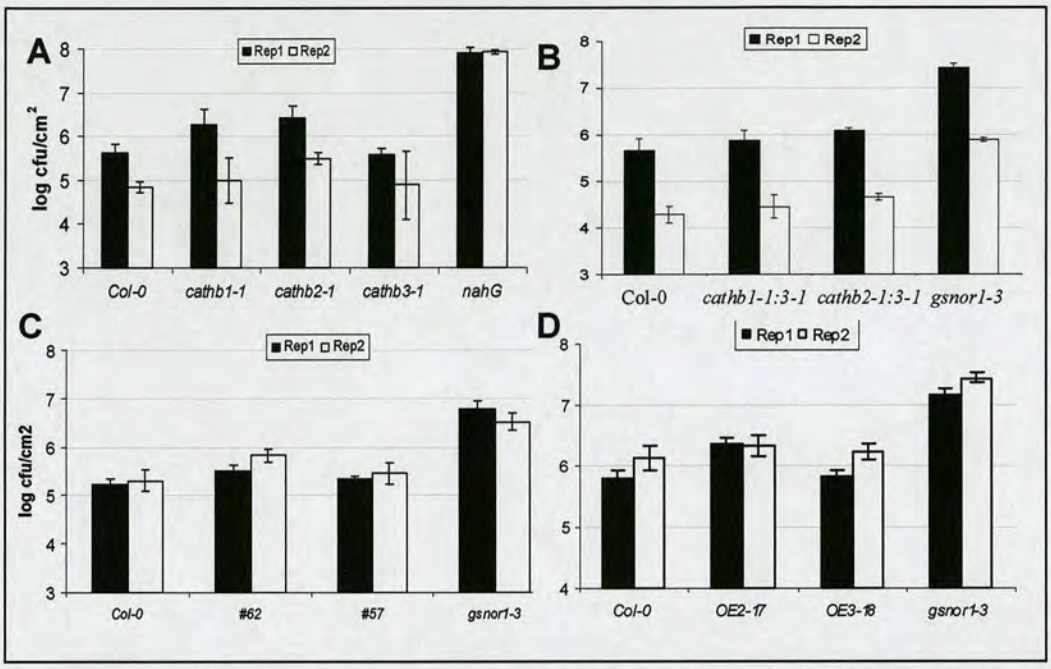


**Figure 4.6. Colony counts with *Pst AvrB*.** (A) single knockouts, (B) double knockouts, (C) triple mutants and (D) overexpressors. Graphs show mean colony forming units recovered from each line at 4dpi for two independent biological replicates. Error bars represent SE of four or more samples pooled from 10 individuals in one biological replicate. Wild-type Col-0 and susceptible mutants *gsnor1-3* or *nahG* are used as controls.

## 4.5. Cathepsin B attenuates the HR to AvrB

As *AvrB* is recognised by *Arabidopsis* accessions carrying the *R* gene *RPM1*, infiltration of *Pst* expressing this protein triggers a HR in leaf tissue. *N. benthamiana* plants where *CathB* was silenced exhibited a reduced HR to bacterial pathogens *E. amylovora* and *Pst* DC3000 (Gilroy et al., 2007). Therefore, the occurrence and severity of HR in response to *Pst AvrB* was investigated in the *cathb* transgenic *Arabidopsis* lines.

Inoculation of a high dose of bacteria ($10^8$cfu/ml) was not observed to result in any appreciable difference in the severity or timing of HR in any of the lines. However,

inoculation with a lower dose ($10^6$cfu/ml) of pathogen resulted in a modest decrease in cell death in double and triple *cathb* lines compared to Col-0 (Fig. 4.7) as observed using TB staining of infected leaf samples. No difference in cell death was detected between the OX lines and wild-type (data not shown). This reduction in cell death triggered by infiltration of *Pst* harbouring *AvrB* in *cathb* knockout lines may suggest a positive role in cell death regulation for these genes in wild-type *Arabidopsis*.

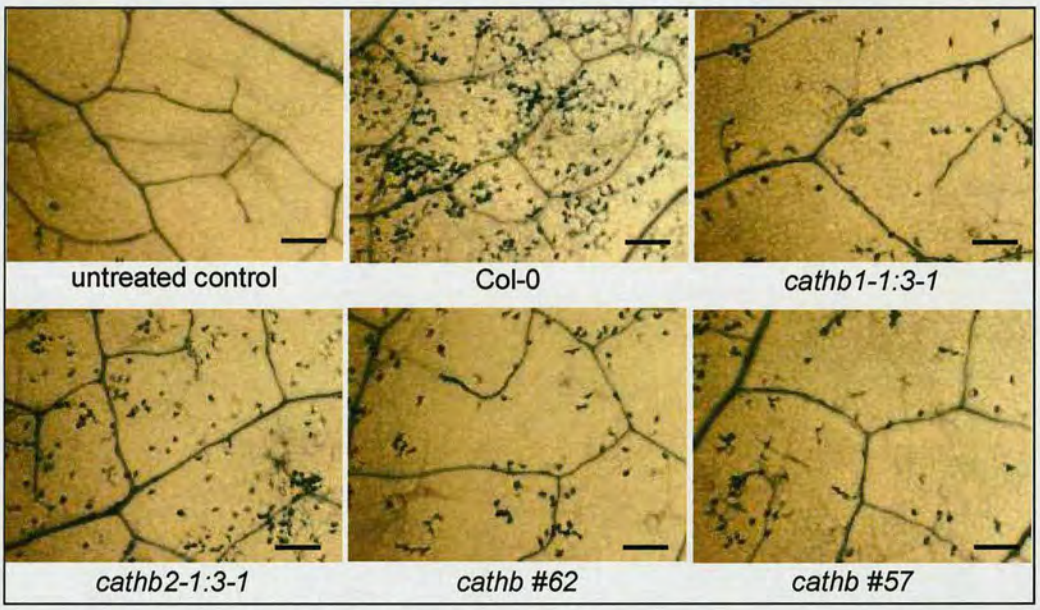


**Figure 4.7. Cell death post-inoculation with *Pst* AvrB.** Trypan Blue staining for cell death was performed at 19 hours post inoculation with $10^6$ cfu/ ml of *Pst* DC3000 *AvrB*. Images were taken with the x4 objective using a light microscope, scale bars are 250μm.

## 4.6. Discussion

The reverse genetics approach used here has made possible the discovery that the three *cathepsin B* genes in *Arabidopsis* are able to act redundantly to positively influence basal defence in response to a virulent strain of *P. syringae*: as demonstrated by an increase in bacterial numbers of 1 log of this pathogen in *cathb* triple mutants but not in

single or double knockouts. This suggests that a single *CathB* gene is both necessary and sufficient to restrict the growth of this pathogen *in planta*. This effect could not have been discovered using conventional forward genetic screens as all three *AtCathB* genes would have been required to be knocked out simultaneously.

In contrast to the new-found involvement of cathepsin B in basal defence, there was no consistently significant increase in susceptibility observed in any of the *cathb* mutants to *Pst* in the *R* gene-mediated resistance triggered by an *AvrB-RPM1* interaction. However, there was a substantial reduction in cell death (CD) triggered by this recognition event in *cathb* mutants compared to wild-type Col-0 as revealed by TB staining. This dissociation of CD with pathogen susceptibility is not unique as *atrbohD/F* double mutants exhibit a reduction in the HR to *AvrRpm1* but do not affect *Pst* growth (Torres et al., 2002). In contrast, *defence, no death1 (dnd1)* mutants show increased resistance to avirulent *P. syringae* in the absence of HR (Yu et al., 1998). It is possible that cathepsin B may be required for resistance mediated by other *Avr-R* gene interactions, as *NbCathB* has been previously demonstrated to be required for the HR triggered by *AvrR3a-R3a* but not by *Avr4-Cf4* (Gilroy et al., 2007). However, this work was necessarily carried out using expression constructs rather than by pathogen challenge so it is unknown whether the reduction in HR in *NbCathB* silenced plants would concomitantly affect pathogen growth as is seen with non-host bacterial pathogens (Gilroy et al., 2007) or if it would be unlinked, as seems to be the case for the *AvrB*-triggered HR in *Atcathb* mutants.

In general there was a higher density of CD in wild-type leaves infiltrated with *Pst* carrying *AvrB* compared to virulent *Pst*. This fits with the model of *Avr-R gene* recognition leading to a rapid and enhanced induction of the HR which is not a typical feature of a virulent interaction. Although a modest reduction in the amount of CD was observed in *cathb* mutants relative to wild-type plants in both compatible and incompatible interactions, it is uncertain if the CD observed after virulent *Pst* treatment

was due to low level pathogen recognition, wounding or disease symptoms. Other mutants which are perturbed in the HR, displaying runaway cell death (RCD) and lesion mimic phenotypes, such as *lesion simulating disease resistance response 1 (lsd1)* and *constitutive expresser of PR 5 (cpr5)* show increased basal resistance but this resistance has been demonstrated to be independent of RCD and of SA and *NPR1*-mediated defences in some instances (Bowling et al., 1997; Aviv et al., 2002; Lorrain et al., 2003). Mutants such as *lsd1* and *cpr5* are thought to be negative regulators of cell death and basal resistance, whereas cathepsin B is likely to be a positive regulator of these two processes as *cathb* knockout mutants redundantly display decreased cell death and increased susceptibility to virulent *Pst*. The transcription factor bZIP10 appears to be a positive regulator of both processes like cathepsin B and its function is mediated through interactions with LSD1 which is able to retain it in the cytoplasm (Kaminaka et al., 2006). It is conceivable that regulation of cathepsin B function may be achieved by interactions with other negative regulators of CD and basal resistance. With this in mind, it is interesting to note that each of the three *AtCathB* genes are up-regulated in the *cpr5* mutant, which exhibits enhanced basal defence against *Pst* DC3000, as revealed by microarray data collated in the Genevestigator database (Zimmermann et al., 2004; Zimmermann et al., 2005).

# 5. Cathepsin B Involvement in Non-Host Resistance

## 5.1. *Blumeria graminis* powdery mildews

Powdery mildews are obligate biotrophic plant pathogens which are able to complete their lifecycle on the leaf surface of their hosts. These pathogens can penetrate epidermal cells where they hijack plant nutrients by means of feeding structures called haustoria. *Blumeria graminis* sp. are economically important monocot powdery mildew pathogens and each *formae specialis* (f. sp.) or sub-species has a very specific host range. For instance *B. graminis* f. sp. *tritici* (*Bgt*) is a host pathogen on wheat and non-host on the closely related barley, whose host pathogen is *Blumeria graminis* f. sp. *hordei* (*Bgh*) and *vice versa* (Schweizer, 2007; Eichmann and Huckelhoven, 2008). Both *Bgt* and *Bgh* are non-host pathogens on the model plant *Arabidopsis* and these systems have been used to identify many previously unknown components of non-host resistance (NHR) (Collins et al., 2003; Yun et al., 2003; Lipka et al., 2005; Stein et al., 2006; Zhang et al., 2007).

*Bgt* spores or conidia land on the host leaf surface where they germinate to form a primary germ tube and an appressorial germ tube. The appresorium allows penetration directly through the intact leaf via a penetration peg and once inside the epidermal cell a feeding structure termed a haustorium is formed which is bilateral and possesses many fingered projections. Once the haustorium is established it provides the fungus with enough energy to produce secondary hyphae allowing subsequent penetration and haustorial formation in neighbouring cells and the growth of a micro colony. These colonies support the formation of chains of conidia able to re-infect other hosts, thus completing the lifecycle (Fig. 5.1) (Yun et al., 2003; Cui, 2007; Eichmann and Huckelhoven, 2008). On non-host plants these pathogens seldom get beyond the attempted penetration of epidermal cells.

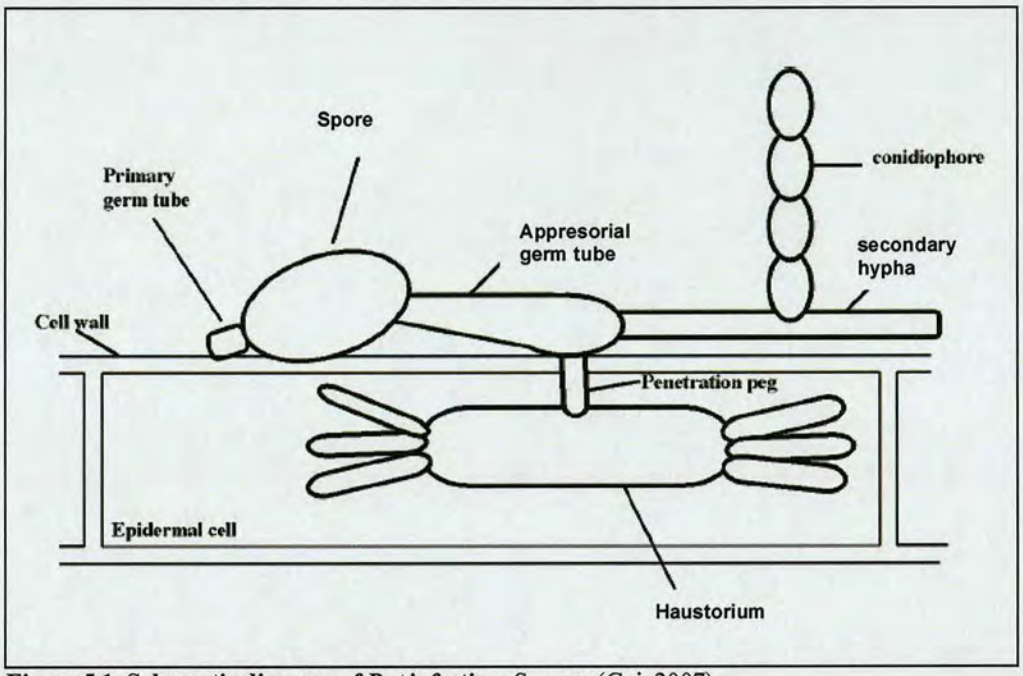


**Figure 5.1. Schematic diagram of *Bgt* infection.** Source: (Cui, 2007).

## 5.2. Non-host resistance to powdery mildews

Non-host or species resistance is defined as the resistance of an entire plant species to a particular pathogen: this resistance is both broad spectrum and durable (Heath, 2000). With respect to powdery mildew non-host resistance, two different models have been put forward by Schweizer (2007): 1) in the absence of targeted fungal effectors (*Avr* genes), recognition is mediated by PAMP receptors, and 2) multiple plant *R* gene products recognise multiple Avr proteins causing redundancy. For either the result would be durable resistance, but which, if any or indeed both, is the case?

In a non-host reaction to *B. graminis* challenge there are two categories of resistance which have been uncovered in mutant studies with *Bgt* and *Bgh* in *Arabidopsis*. The first is pre-invasion resistance, which comprises: preformed physical barriers, formation of cell wall appositions and phytoalexin accumulation in the area under the site of attempted fungal penetration. Three additional genes which prevent invasion in

*Arabidopsis* were uncovered in a screen using *Bgh*. These *Penetration* (*PEN*) genes encode: *PEN1* a syntaxin involved in vesicle membrane fusion (Collins et al., 2003); *PEN2,* a glucosyl hydrolase thought to process an unknown phytoalexin (Lipka et al., 2005); and *PEN3,* an ATP-binding transporter (Stein et al., 2006). In those rare occasions where the pre-invasion resistance is circumvented, a second category of post-invasion defences spring into action. These have been demonstrated to involve the *EDS1* gene involved in basal and *TIR-NB-LRR* defence, *PR* gene induction, ROS generation and epidermal cell death (Yun et al., 2003).

## 5.3. Previous work and aims

Virus Induced Gene Silencing (VIGS) of cathepsin B in *N. benthamiana* was shown to lead to a reduction in the HR and subsequent increase in susceptibility to two non-host bacterial pathogens of *N. benthamiana, Erwinia amylovora* and *Pseudomonas syringae* DC3000 (Gilroy et al., 2007). In this chapter, the response of *Arabidopsis* cathepsin B transgenic lines to the non-host fungal pathogen *B. graminis* f. sp. *tritici* race Sav GH135 (*Bgt1*) is investigated. This pathosystem is particularly useful as it has been studied previously and several factors involved in non-host resistance to *Bgt* in *Arabidopsis* have already been uncovered (Yun et al., 2003).

## 5.4. *Cathepsin B* knockout lines are more susceptible to *Bgt*

### 5.4.1. *Bgt* development is more advanced on *cathepsin B* double mutants

As the *cathepsin B* single knockout lines isolated for each gene did not show an altered response to *Pst* as discussed in the previous chapter, two *cathepsin B* double knockouts which were obtained by crossing were treated with the biotrophic fungal pathogen *Bgt*. Plants in which the *EDS1* gene was knocked out were used as a susceptible control. In

most cases, *Bgt* development on Col-0 plants was limited to spore germination, formation of the primary germ tube and appressoria (Fig. 5.2a) although very rarely a deformed unilateral haustorium was observed. Both *cathb1-1:3-1* and *2-1:3-1* mutants, like *eds1*, were found to support more fungal growth than the wild-type Col-0 control (Fig. 5.2b, c). However, the haustoria formed on the *cathepsin B* double mutants did not appear to be as fully developed as those on *eds1* (Fig. 5.2d). Moreover, the haustoria formed on the double mutants were also surrounded by a membrane which appeared to be absent on *eds1* plants. This membrane is presumably a plant-derived structure and may be a component of the defence response mounted to contain fungal growth.

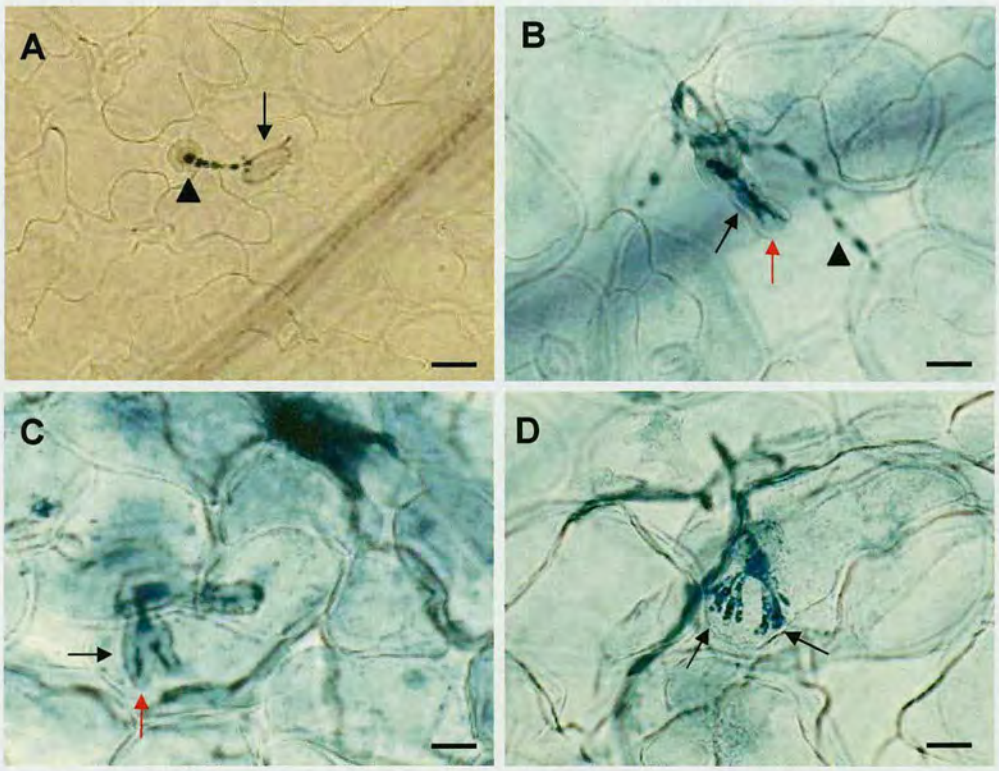


**Figure 5.2. *Bgt* fungal development on *Arabidopsis*.** (A) Germinated spore (arrow) and appressorium (arrowhead) on Col-0. (B) Haustorial (arrow) and secondary hyphal (arrowhead) development on *cathb1-1:3-1*. (C) Haustorium (arrow) on *cathb2-1:3-1*. (D) Clear development of the fingered protrusions (arrows) on both hands of a haustorium on *eds1*. A membrane surrounds the haustoria in B and C (red arrows) but is absent in D. Images were taken with a x20 objective, scale bars are 10μm.

### 5.4.2. Quantification of fungal development

The amount of secondary fungal growth i.e. both haustorial and secondary hyphal development which occurred on each *Arabidopsis* line was quantified and normalised against the number of spores which germinated. Both *cathb1-1:3-1* and *2-1:3-1* mutants were found to possess a small but significantly higher amount of haustorial formation than wild-type, although not as much as the *eds1* susceptible control (Fig. 5.3).

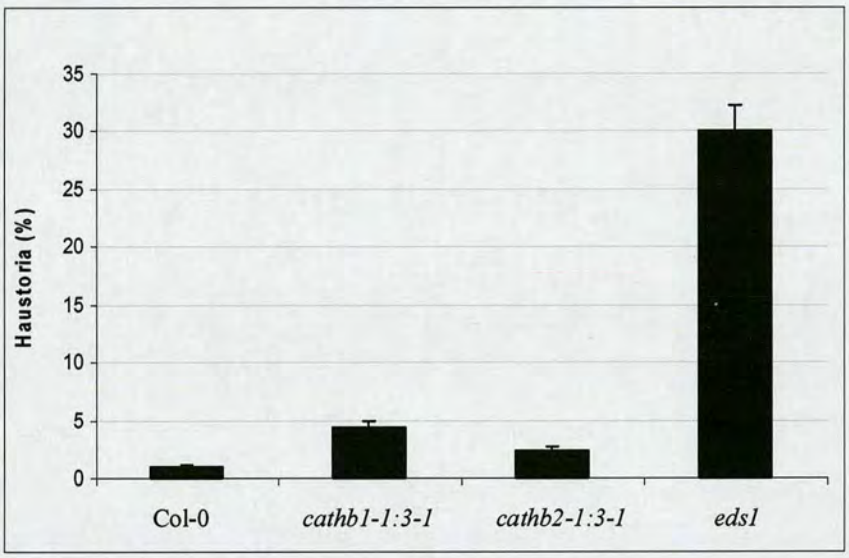


**Figure 5.3. Quantification of *Bgt* development.**. Error bars are SE of 10 samples within one biological replicate.

## 5.5. Epidermal cell death

Epidermal cell death appears to be an important marker of compatible or incompatible reactions between *Bgt* and its natural host plant wheat (Yun et al., 2003) with resistant varieties showing high epidermal cell death and susceptible varieties showing very little. Therefore, cell death or HR in response to *Bgt* inoculation was examined in the cathepsin B mutants using samples stained with trypan blue. The *cathb1-1:3-1* and

*cathb2-1:3-1* mutants showed a 3-fold or 4-fold reduction respectively in the amount of epidermal cell death to *Bgt* compared to Col-0. This is compared to an 8-fold reduction in epidermal cell death in *eds1* mutants (Fig. 5.4).

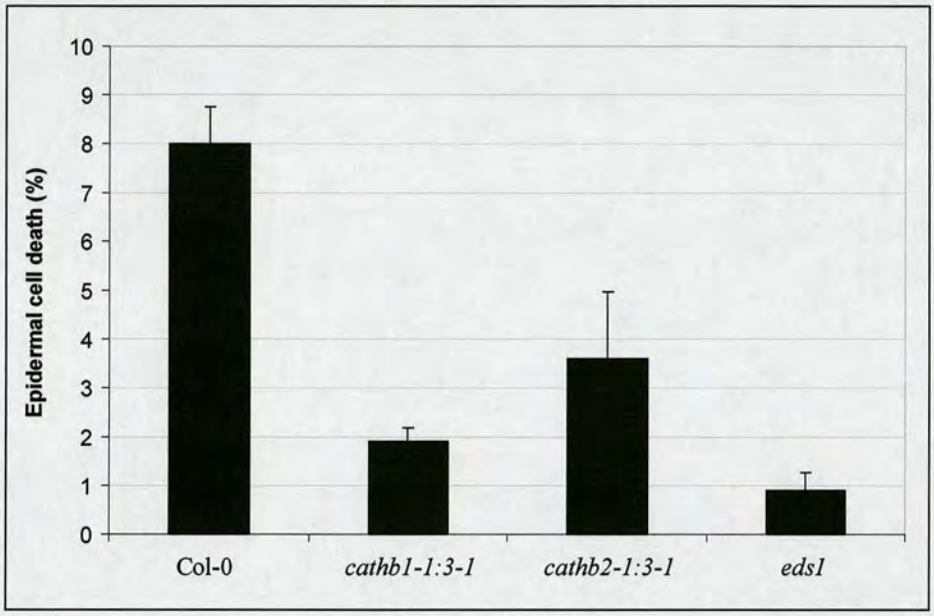


**Figure 5.4. Percentage Epidermal cell death to *Bgt*.** Error bars are SE of 10 samples within one biological replicate.

## 5.6. Defence gene expression

It has already been demonstrated that the defence associated genes *PR1* and *GST1* are up-regulated in wild-type *Arabidopsis* at 1 and 2 days post-infection in response to *Bgt* (Yun et al., 2003). Consequently, the expression of these marker genes was examined in the *cathepsin B* mutants at these timepoints using Northern blotting (Fig. 5.5). Surprisingly, it was found that both *PR1* and *GST1* were expressed with accelerated kinetics in *cathb1-1:3-1* and *2-1:3-1* mutants compared to wild-type Col-0. This would seem to be counterintuitive as there is no detection of the expression of these marker

genes in the susceptible control *eds1*. Increased defence gene expression would be expected to correlate with increased resistance to the pathogen. However, enhanced *PR1* expression may simply be a result of more cells being penetrated by the pathogen in the more susceptible *cathb* mutants. This is not observed for *eds1* mutants as *EDS1* is required for *PR1* expression

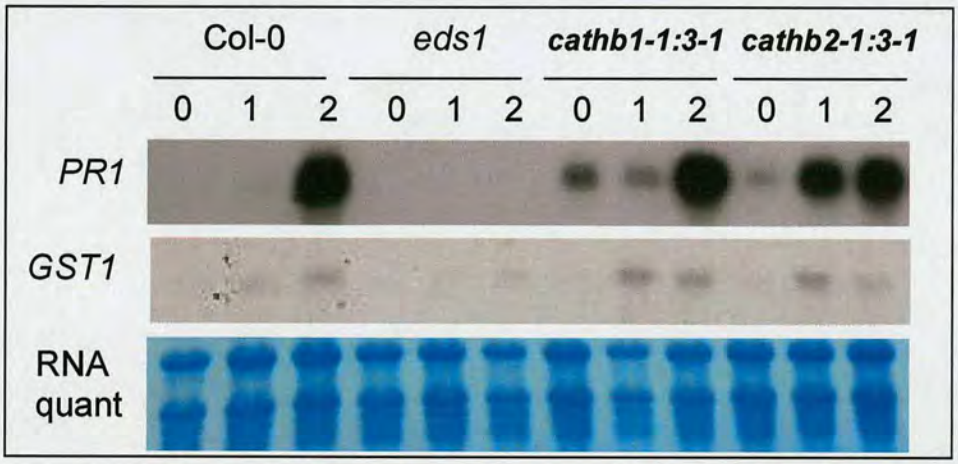


**Figure 5.5. Defence gene expression to *Bgt*.** Expression of *PR1* and *GST1* is shown at 0, 1 and 2 days post-infection with *Bgt*. RNA was stained with methylene blue to show equal loading and transfer. This was repeated twice with the same result.

## 5.7. *Cathepsin B* expression in response to *Bgt*

In order to further investigate this peculiar result the expression of *cathepsin B* genes was analysed in both wild-type and *eds1* mutants in response to *Bgt* inoculation. Quantitative RT-PCR experiments (Fig. 5.6) reveal that both *cathepsin B 2* and *3* are up-regulated ~1.9-fold and 2.8-fold respectively at 2 days post-infection of *Bgt* in wild-type *Arabidopsis* consistent with a role for this gene in NHR. Intriguingly, *cathb* transcripts do not accumulate in *eds1* plants following *Bgt* challenge. This would suggest that *EDS1* is a positive regulator of *cathb* expression.

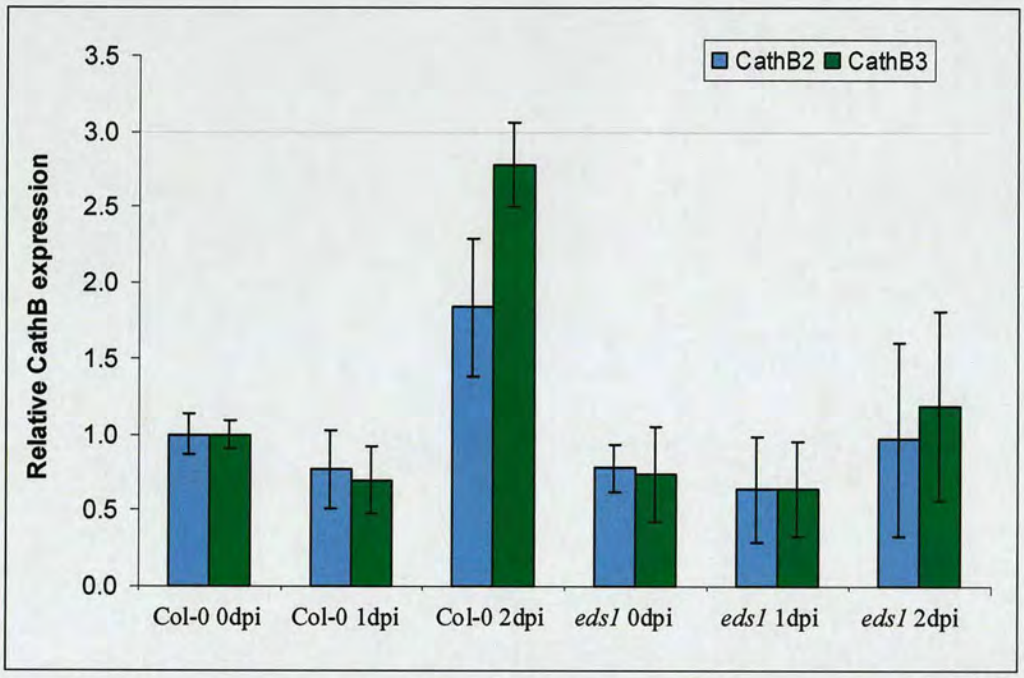


**Figure 5.6. *Cathepsin B* expression in Col-0 and *eds1* in response to *Bgt* challenge.** The values are normalised to actin expression. Each sample was measured in triplicate and is shown relative to Col-0 expression at 0dpi. This experiment was repeated with two independent biological replicates with similar results. Error bars are SE of 3 samples within one biological replicate

## 4.8. Discussion

Despite a huge increase in attention and research over the last seven years, many of the mechanisms responsible for non-host resistance still remain to be uncovered. This may be due to the fact that this form of resistance has been predicted to comprise multiple genes with overlapping functions (Heath, 2000). Nevertheless, there have been several recent advances in the field including the discovery that plants have the ability to recognise and respond to Pathogen Associated Molecular Patterns (PAMPs) with the first receptor, FLS2, being cloned (Gomez-Gomez and Boller, 2000, 2002; Montesano et al., 2003; Nurnberger et al., 2004). This recognition of non-self molecules is analogous to the mammalian innate immune system.

Several genes involved in non-host responses to specific pathogens have also been identified through extensive work involving the model plant *Arabidopsis*. These genes include *PEN1, 2* and *3*, which are involved in preventing penetration by non-host fungus *B. graminis* (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006; Zhang et al., 2007). Resistance to this pathogen in *Arabidopsis* is known to involve both pre- and post-invasion defences and that if these are simultaneously overcome due to mutation then *Arabidopsis* is effectively transformed from a non-host to a host for this pathogen (Yun et al., 2003; Lipka et al., 2005). Post-invasion responses to this pathogen are regulated by known basal and *R* gene signallers *EDS1* and *PAD4* and also involve HR and *PR1* induction (Yun et al., 2003; Lipka et al., 2005; Wiermer et al., 2005; Stein et al., 2006).

The occurrence of HR or epidermal cell death in response to this pathogen seems to be an important determinant of resistance to this fungus, even on resistant host plants where attenuation of HR to *B. graminis* renders them susceptible (Yun et al., 2003). In this study mutants in which expression of *cathepsin B* genes had been knocked out were shown to exhibit less epidermal HR and concomitant increased susceptibility. This follows the same pattern as the *N. benthamiana cathepsin B* silenced plants to non-host bacterial pathogens: reduced HR and increased pathogen growth (Gilroy et al., 2007). It is possible that cathepsin B is a conserved regulator of cell death in response to non-host pathogens. The increased fungal growth on these *cathepsin B* knockouts can probably be accounted for by the absence of HR.

*EDS1* is a component of post-invasion non-host resistance to *Bgt* in *Arabidopsis*; *eds1* mutants also show reduced HR and increased *Bgt* growth (Yun et al., 2003; Lipka et al., 2005), although more so than *cathb* double mutants. The expression levels of *cathepsin B* genes were found to increase in response to *Bgt* in wild-type plants but not in *eds1* mutants, suggesting that *CathB* acts somewhere downstream of *EDS1* in post-invasion defence signalling. Double mutant analysis could be undertaken to determine if *EDS1*

regulates epidermal cell death through *CathB* or via an independent pathway. It is also possible that the triple mutants could have a similar level of susceptibility to *eds1* given the stronger phenotype of the triple mutants compared to the double mutants in basal resistance (Ch 4) although the triple mutants were not tested with *Bgt* due to time constraints.

The fact that *eds1* mutants allow more *Bgt* development than *cathb* mutants may be due to the fact that *PR1* and *GST1* expression is practically abolished in the *eds1* mutant but *cathb* mutants show both increased and hastened expression of these genes compared to wild-type plants. This suggests that wild-type *EDS1* function positively regulates *PR1* and *GST1,* whereas *CathB* may negatively regulate them. Alternatively, the increased rate of haustorial formation in *cathb* mutants suggests more cells are challenged by the pathogen, leading to enhanced *PR1* expression. A similar phenomenon has been observed for NHR in *Arabidopsis* against the wheat rust pathogen *Puccinia graminis* (Shafiei et al., 2007). In contrast, *eds1* plants support more *Bgt* growth than wild-type but *eds1* is also a regulator of *PR1* expression and thus *PR1* transcript accumulation is reduced in these plants following attempted *Bgt* infection. However, it is possible that wild-type *CathB* is involved in some kind of negative feedback with respect to *PR1*.

The following model (Fig. 5.7) summarises some of the known factors involved in non-host resistance in *Arabidopsis* and attempts to integrate cathepsin B genes based on the data in this study. The *PEN* genes, together with the cytoskeleton, constitute a barrier to non-host fungal penetration. *EDS1* is important for post-invasion defence which positively regulates *PR1* and *GST1* expression as well as the HR. It is shown here that *EDS1* also positively regulates *CathB* transcription in response to *Bgt*. It is unclear if the reduction in the HR due to *EDS1* mutation is solely mediated by *CathB* or if this also involves additional pathways. Negative regulation of *PR1* and *GST1* is still a possibility although the enhanced expression of these genes in *cathb* mutants may be explained by heightened exposure to the pathogen as mentioned above.

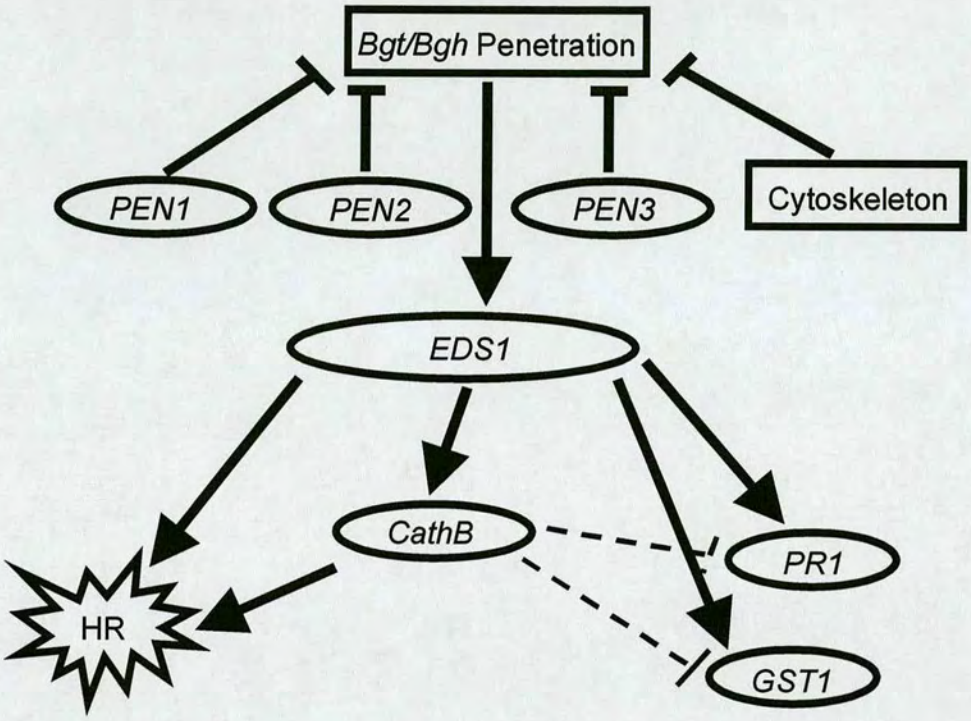


**Figure 5.7. A model integrating *cathepsin B* into known components of non-host resistance to *Blumeria graminis* in *Arabidopsis*.** Arrows indicate positive regulation and lines with cross-bars indicate negative regulation. Dashed lines indicate possible regulation.

# 6. Cathepsin B Involvement in Senescence

## 6.1. Senescence: developmental PCD

Senescence in plants is considered to be a developmental form of PCD undergone in organs at the end of their development (Orzaez and Granell, 2004). This is an active process involving the remobilization of nutrients into other parts of the plant and requires energy, which is supplied by the process itself, as well as *de novo* transcription and translation (Yoshida, 1962; Cuello et al., 1984; Buchanan-Wollaston, 1997).

Early studies examining morphological features in senescence observed chloroplast degradation and vacuolization of cytoplasm (Hurkman et al., 1979; Inada et al., 1998; Orzaez and Granell, 2004) although more recent studies have also identified chromatin condensation and DNA laddering in some cases (Inada et al., 1998; Kawai and Uchimiya, 2000; Simeonova et al., 2000). It has since been suggested by Orzaez and Granell (2004) that morphologically senescence loosely resembles Type 2 autophagic PCD in animals, Type 1 being classical apoptosis or physiological cell death and Type 3 lysosomal PCD, as reviewed by Kitanaka and Kuchino (1999). Moreover, genes involved in autophagy (APG) in yeast have homologues in *Arabidopsis* and are up-regulated at the latter stages of senescence, with some APG *Arabidopsis* mutants exhibiting altered senescence phenotypes (Doelling et al., 2002).

## 6.2. Diverse factors are implicated in senescence

Some of the multiple factors implicated in senescence include up-regulated genes presumed to be involved in nutrient salvage, including proteases, nucleases and many of the enzymes in glycolysis and gluconeogenesis (Buchanan-Wollaston, 1997).

Detoxifying enzymes such as catalase and glutathione-*S*-transferases are also up-regulated, apparently to protect cells from hazardous ROS until salvage is complete (Buchanan-Wollaston and Ainsworth, 1997). Transcription factors belonging to WRKY, homeobox, Myb and bZIP families have also been implicated in senescence (Buchanan-Wollaston et al., 2003; Guo et al., 2004). Plant hormones cytokinin and ethylene are additionally thought to be involved, with cytokinin found to cause a delay in senescence, and ethylene hastening the process (Gan and Amasino, 1995; Grbic and Bleecker, 1995; Oh et al., 1997).

However, there have been so many different experimental systems used to study senescence, sometimes with contradictory results between different species or organs or even within the same tissue, that there is as yet no comprehensive model which can incorporate all that is known (Orzaez and Granell, 2004). Nevertheless, the complexity suggested by experimental studies seems to indicate that senescence may involve multiple redundant branching pathways, so that blockages in one avenue results in diversions arriving at the same endpoint.

### 6.3. Proteases involved in senescence

Genes encoding proteases represent one of the most highly up-regulated groups during senescence (Guo et al., 2004). The cysteine protease, *senescence associated gene 12* (*SAG12*), first identified in the early 90s (Lohman et al., 1994) in a study looking for genes differentially expressed during senescence, has subsequently been shown to possess one of the highest EST counts in a senescence transcriptome study in *Arabidopsis* (Guo et al., 2004). Moreover, three out of the top eleven most highly expressed genes are cysteine proteases, with this gene family highly represented in ESTs (Guo et al., 2004).

Many of these proteases are predicted to localize to the vacuole and are thought to be involved in protein degradation. However, a recent study has identified new organelles which are exclusive to chlorophyll containing cells undergoing senescence. These organelles, named senescence associated vacuoles (SAVs), have been found to possess high cysteine protease activity and furthermore SAG12 has been localized to SAVs using GFP protein fusions (Otegui et al., 2005). In spite of this, homozygous *SAG12* mutant *Arabidopsis* plants show no delay in senescence and are still able to form SAVs with cysteine protease activity, so the role of this protein remains unclear (Otegui et al., 2005). Some cysteine proteases, including cathepsin B, are thought to play a more regulatory role in some forms of mammalian PCD rather than just wholesale protein degradation (Vancompernolle et al., 1998). As cathepsin B genes have also been found to be highly up-regulated during senescence in some studies (Guo et al., 2004) it is possible that these genes may play a more regulatory role in this plant developmental process.

### 6.4. Cathepsin B mutants show a slight delay in senescence

Information obtained from the Genevestigator database (Zimmermann et al., 2004; Zimmermann et al., 2005), which summarises a collection of microarray experimental data, has indicated that all three *Arabidopsis cathepsin B* genes are up-regulated during senescence. In order to examine if the transgenic *Arabidopsis cathepsin B* lines used throughout this project show a senescence-related phenotype, a dark-induced senescence assay was employed, as used by Oh et al. (1997). This found that the cathepsin B knockout mutants showed an observable delay in dark-induced senescence compared to wildtype Col-0 control plants (Fig 6.1), which was quantified using percentage chlorophyll content (Fig. 6.2). However this delay, although significant, is not as marked as that for the *ethylene insensitive 2* (*ein2*) control mutant already known to display delayed senescence.


**Figure 6.1. Dark-induced senescence phenotype in Arabidopsis lines.** (A) single knockouts, (B) double knockouts and (C) triple mutants. Pictures were taken at 5 or 6 days after beginning dark-induction treatment, scale bars are 4cm. Multiple biological replicates were carried out with similar results.

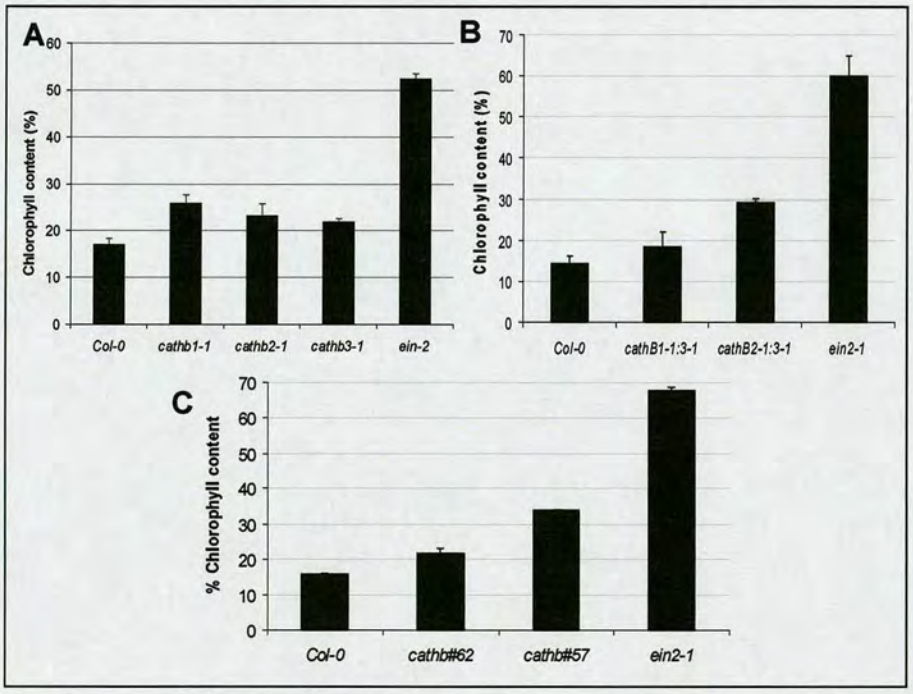


**Figure 6.2. Progression of dark-induced senescence quantified using percentage chlorophyll content.** (A) single knockouts, (B) double knockouts and (C) triple mutants. Total chlorophyll content was measured according to Arnon (1949) at 5 or 6 days after beginning dark-induction treatment and was expressed as a percentage of chlorophyll content at day 0. Multiple biological replicates were carried out with similar results. Error bars are SE of 3 or more samples within one biological replicate.

## 6.5. *Cathepsin B* expression in dark-induced senescence.

A dark-induction of senescence was employed here to synchronise the process in the different mutant and control *Arabidopsis* lines used. However, it is known that during dark-induced senescence there are both similarities and differences in gene expression compared to developmentally induced senescence (Buchanan-Wollaston et al., 2005). Therefore, the expression of *cathepsin B* genes was examined during dark-induced senescence, although it is known that they are highly up-regulated during developmental senescence. Quantitative real-time RT-PCR (qRT-PCR) analysis was used to examine the expression of *cathepsin B 2* and *3* in wildtype Col-0 and *ein2-1 Arabidopsis*. *CathB1* expression was not examined as working primers could not be designed to strict qRT-PCR specifications. Only *CathB3* is strongly induced (16-fold) in wildtype Col-0 at 4 days post-dark-induced senescence treatment (Fig. 6.3). *CathB2* is only very slightly induced in this assay. Interestingly, the expression of both *CathB* genes appears to be highly induced in the *ein2-1* mutant at many time-points. As *ein2-1* mutants accumulate more ethylene than wild-type but are completely insensitive to it (Guzman and Ecker, 1990) this suggests that either ethylene may have a negative regulatory effect on these genes under these conditions or alternatively *CathB* could be positively regulated by ethylene in an *EIN2*-independent manner.

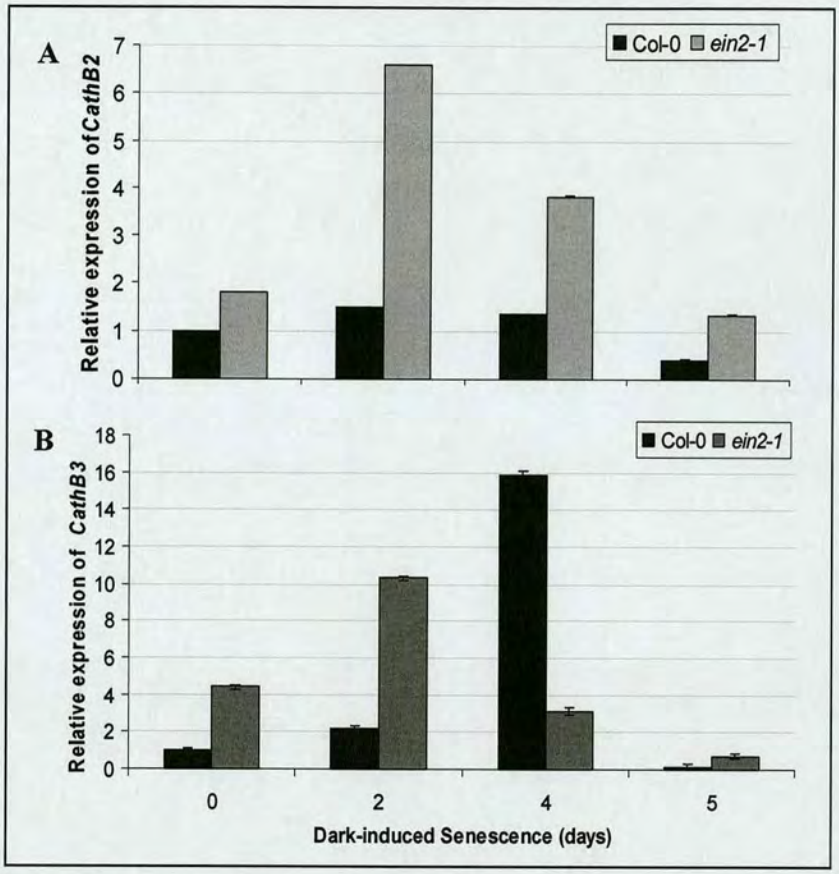


**Figure 6.3. Relative Expression of cathepsin B genes in dark-induced senescence in wildtype and *ein2-1 Arabidopsis*.** (A) *CathB2* and (B) *CathB3* expression. The values are normalised to *Actin* expression. Each sample was measured in triplicate and shown relative to Col-0 expression at day 0. Error bars are SE of 3 samples within one biological replicate.

## 6.6. *SAG12* is down-regulated in *cathepsin B* mutants

*SAG12* is a highly expressed marker gene of senescence (Lohman et al., 1994; Guo et al., 2004) which encodes a papain-like cysteine protease. In order to determine if *cathepsin B* genes may have a role in regulation of senescence the expression of *SAG12* was investigated in wildtype Col-0 and *cathb* triple mutants *#62* and *#57*. qRT-PCR analysis shows quite clearly (Fig. 6.4) that there is a large decrease of expression of

*SAG12* in both triple mutants at 4 days post dark-induction treatment, which correlates with the peak of *CathB3* expression in wildtype plants. This suggests that cathepsin B is upstream of SAG12 and may directly or indirectly regulate the expression of this gene.

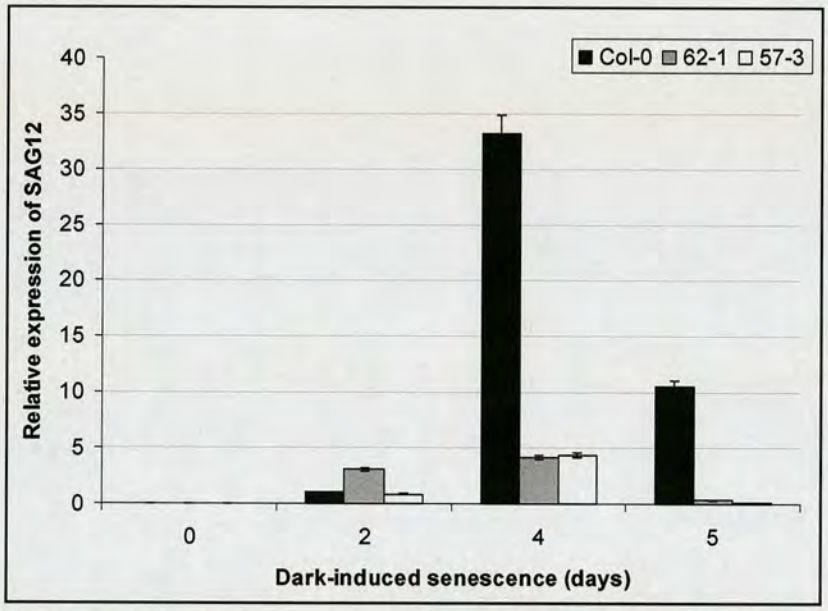


**Figure 6.4. *SAG12* expression in wildtype and *cathb* triple mutants during dark-induced senescence.** The values are normalised to *Actin* expression. Each sample was measured in triplicate and shown relative to Col-0 expression at 2 days. Error bars are SE of 3 samples within one biological replicate.

## 6.7. Off-target silencing is not possible between *cathepsin B2* and *SAG12*

As cathepsin B is also a papain-like cysteine protease, the *CathB2* gene used to design the silencing construct to generate the triple mutant was compared to *SAG12* to investigate the possibility of off-target silencing between *cathepsin B* and *SAG12*. Alignments were attempted between these two genes to confirm that there is not sufficient homology between these genes at the nucleotide level to trigger non-specific gene silencing. However, Blastn (bl2seq) pair-wise comparisons (Tatusova and Madden, 1999) were not possible as there is not sufficient similarity between the two sequences. In fact there is only 25 % identity at the amino acid level, suggesting these

two genes are evolutionarily unrelated. An alignment was produced using the MultAlin tool (Corpet, 1988), which showed that the largest stretch of conserved nucleotides only spanned 7 bps: as 21 bp long fragments are required for silencing it was concluded that off-target silencing should not occur in this case.

## 6.8. Discussion

To facilitate insight into the mechanisms of senescence, a developmental form of programmed cell death in plants, many studies have employed microarrays or comparative expression analysis to identify genes up-regulated during senescence, thus having a putative role in the process (Lohman et al., 1994; Weaver et al., 1998; Guo et al., 2004; Buchanan-Wollaston et al., 2005). One such study taking a more novel approach utilised enhancer trap lines to isolate genes expressed in senescing but not in non-senescing leaves (He et al., 2001). This has also enabled visualisation of the differential responses of these genes to various senescence enhancing treatments allowing a preliminary model network of signalling pathways to be proposed. However, as has been found in numerous studies, genetic mutants for many of these genes, including *SAG12*, seldom show any senescence-related phenotype (He et al., 2001; Otegui et al., 2005). This has been ascribed to the "plasticity of leaf senescence" (Gan and Amasino, 1997; He et al., 2001) and the fact that senescence is likely to be controlled by a regulatory network rather than any one single pathway (He et al., 2001).

Cathepsin B is a cysteine protease highly up-regulated in senescence for which genetic knockout mutants, as demonstrated here, display a slight but significant delay in dark-induced senescence. The data here also show a considerable reduction in *SAG12* expression in *cathb* triple mutants suggesting a putative regulatory role for cathepsin B. This contrasts with the accepted degradative role for proteases in senescence. Nonetheless, there is some precedent for cathepsin B-like cysteine proteases performing

a regulatory role in mammalian PCD via the activation of caspase enzymes (Vancompernolle et al., 1998). Moreover, a matrix metalloproteinase has been hypothesised to play a regulatory function in senescence either by continuous degradation or specific activation of a putative cell death stimuli-triggered receptor (Golldack et al., 2002).

Many defence genes are also up-regulated during senescence and this has been previously explained as a protective mechanism to allow complete mobilization of nutrients to occur in tissue highly susceptible to pathogen attack (Orzaez and Granell, 2004). Defence hormone SA is also implicated in regulation of senescence as SA pathway mutants have been shown to display delayed senescence (Buchanan-Wollaston et al., 2005). *Cathepsin B* genes belong to a growing number of genes up-regulated during senescence, as well as by pathogen challenge, and it is possible that some such genes may be involved with conserved mechanisms between different forms of plant PCD. Another such gene, a *metallothionein* has been shown to be up-regulated during senescence and in incompatible HR responses to *Pseudomonas syringae* and *Hyaloperonospora parasitica* as well as in necrosis induced during compatible interactions with virulent forms of these two pathogens (Butt et al., 1998). This points to conserved signalling in three different types of plant PCD: HR, necrosis and senescence.

Cathepsin B is a good candidate for a link between different types of plant PCD. These genes have been shown here, and in previous Chapters, to be up-regulated in senescence as well as in compatible, incompatible and non-host pathogen interactions. Furthermore, senescence is delayed in *cathb* mutants and there is a reduction in HR observed to non-host pathogen *Blumeria graminis* and to a compatible isolate of *Pseudomonas syringae*. Additionally, we have shown here that marker gene of senescence *SAG12* is down-regulated in *cathb* mutants whilst HR marker gene *HSR203J* has been previously shown

to be down-regulated in plants where *cathepsin B* has been silenced using VIGS (Gilroy et al., 2007).

The expression of *HSR203J* and a further HR marker gene *HIN1* has been shown to be specifically linked with PCD, as they are not induced by SA, unlike many defence genes (Gopalan et al., 1996; Pontier et al., 1998; Pontier et al., 1999). Interestingly *HIN1* but not *HSR203J* has been shown to be up-regulated in senescence, even as *SAG12* itself has been shown to be expressed weakly, late in the HR, in cells surrounding those that have undergone PCD (Pontier et al., 1999). Cathepsin B has been shown to regulate both *SAG12* and *HSR203J* but it is not yet known if it affects *HIN1* expression nor is it certain if *cathepsin B* is specifically expressed during PCD *per se* or if it is generally defence inducible.

# 7. Cathepsin B Localisation

## 7.1. Localisation

### 7.1.1. Where and why?

Using fluorescent protein tagging and confocal microscopy, it is possible to gather non-invasive information on where and when a protein is found in a particular organism, tissue, cell or sub-cellular organelle and in response to which stimuli. Data generated from these kinds of studies can shed light on the role of a particular protein and suggest pathways or processes it may be involved in or even confirm hypotheses generated from structure-function predictions (Heath, 2000b; Davis, 2004). Large-scale localisation approaches have also been taken recently to help with the annotation of the proteome in a post genomics era (Huh et al., 2003; Koroleva et al., 2005).

### 7.1.2. Tools and technology

One of the most common and useful methods of determining protein localisation is to make fusions with Green Fluorescent Protein (GFP), discovered from jellyfish *Aequorea victoria,* and its derivatives Yellow and Cyan Fluorescent Proteins (YFP and CFP respectively) (Lippincott-Schwartz and Patterson, 2003), and DsRed, discovered from coral *Discoma* spp., and its derivative monomeric Red Fluorescent Protein (mRFP) (Campbell et al., 2002). Fluorescent protein fusions have the advantage in investigation of protein localisation over other methods such as GUS protein fusions (Jefferson et al., 1987), antibodies (Bradbury et al., 2003) or aptamers (Stanlis and McIntosh, 2003) as this is the only method to date of looking at localisation and, indeed, re-localisation in living cells as it occurs.

Advances in Laser Scanning Confocal Microscopy mean that it is now possible to build up stacks of clear, unblurred 3D and 4D images of samples with the 4th dimension, of

course, being time (Paddock, 2000). Microscopic techniques such as Fluorescence Recovery after Photobleaching (FRAP) can be used to investigate the rates of protein trafficking and turnover, whereas Fluorescence Resonance Energy Transfer (FRET) can demonstrate protein-protein interactions (Lippincott-Schwartz and Patterson, 2003; Stephens and Allan, 2003; Shaw, 2006). All these things are increasing the possibilities for testing and evaluating our ideas about what a particular protein may be doing and why.

## 7.2. Mammalian cathepsin localisation

Mammalian cathepsin genes encode lysosomal cysteine proteases and were thought originally to be housekeeping proteins which functioned in wholesale protein degradation (Turk et al., 2000). Yet, it is now thought that these proteins have distinct roles after being localised in different cell types. For example cathepsin K is localised to osteoclasts and is involved in bone resorption (Chapman et al., 1997) and cathepsin S is involved in antigen presentation in B-cells (Riese et al., 1996). On the other hand, cathepsin B is widely expressed (Brix et al., 2007) and although it is one of the most concentrated proteases present in lysosomes making up to 20 % of the total protein (Xing et al., 1998) studies with specific inhibitors suggest that it is not particularly important for general protein degradation (Kominami et al., 1991; Turk et al., 2000).

Precise physiological roles for cathepsin B have yet to be clearly defined, but it has been implicated in apoptosis, hormone processing and extracellular matrix (ECM) remodelling (Vancompernolle et al., 1998; Linke et al., 2002; Brix et al., 2007; Buth et al., 2007). Cathepsin B is also widely associated with many pathological conditions such as cancer, osteoarthritis and Alzheimer's, and it was presumed that on exiting its natural lysosomal/ endosomal location it becomes a proteolytic 'time bomb'.

This is highlighted by the number of cases where cathepsin B expression and activity is found to be increased during cancer; there are reports of changes in its localization as the disease becomes malignant and of cathepsin B secretion by tumors undergoing metastasis (Podgorski and Sloane, 2003). A truncated splice form of cathepsin B missing the signal peptide and part of the prodomain appears to dominate in osteoarthritic cartilage and is mis-targeted to mitochondria in cells which subsequently die and exhibit nuclear fragmentation, a hallmark of apoptosis (Muntener et al., 2004).

However, it has been recently discovered that cathepsin B secretion is stimulated by thyroid stimulating hormone and it is able to degrade the prohormone thyroglobulin into the biologically active form thyroxin (Linke et al., 2002). Cathepsin B is also secreted by keratinocytes to degrade the ECM on regeneration after wounding (Buth et al., 2007). So, it would seem that there are physiological reasons and mechanisms to localize cathepsin B outside of its lysosomal home.

### 7.3. Plant protease localisation

There are roughly 500 proteases encoded by the Arabidopsis genome. These fall into 5 catalytically defined classes and are split further into clans and then families based on their active residues and sequence homologies (Fig. 7.1) (Beers et al., 2004; van der Hoorn and Jones, 2004). The three most abundant classes are the serine, aspartic and cysteine proteases. Bioinformatic analysis of these proteins using iPSORT was carried out to predict their localisation by analysing the proteins for the presence of sequences which may target them to the secretory pathway, chloroplasts or mitochondria (Beers et al., 2004). Many of these proteases are predicted to be secreted, that is to say they go into the endomembrane system, but very few have been confirmed experimentally (Beers et al., 2004).

CDR1 is an aspartic protease which has been found to accumulate in the apoplast in response to challenge by the pathogen *Pseudomonas syringae*. Constitutive expression of this protease causes both dwarfing and activated disease resistance, which are dependent on the presence of its catalytic amino acids (Xia et al., 2004). Serine proteases with caspase activity dubbed saspases were identified in oat. These are secreted into the apoplast upon treatment with the pathogen derived toxin, victorin, or following heat shock. It has been suggested that these proteases are involved in a proteolytic cascade which results in PCD (Coffeen and Wolpert, 2004).

The cysteine protease RD21 was found to accumulate in vesicles derived from the endoplasmic reticulum (ER) termed ER bodies. These are triggered by salt stress to fuse with the vacuole where these are assumed to become proteolytically active (Koizumi et al., 1993; Hayashi et al., 2001). A wound inducible vacuolar processing enzyme γVPE with a predicted role in the HR to TMV (Hatsugai et al., 2004) also appears to be localised to ER bodies before discharge to the vacuole (Hayashi et al., 2001). So it would seem that proteins predicted to be secreted by bioinformatic means do not always make it out of the cell.

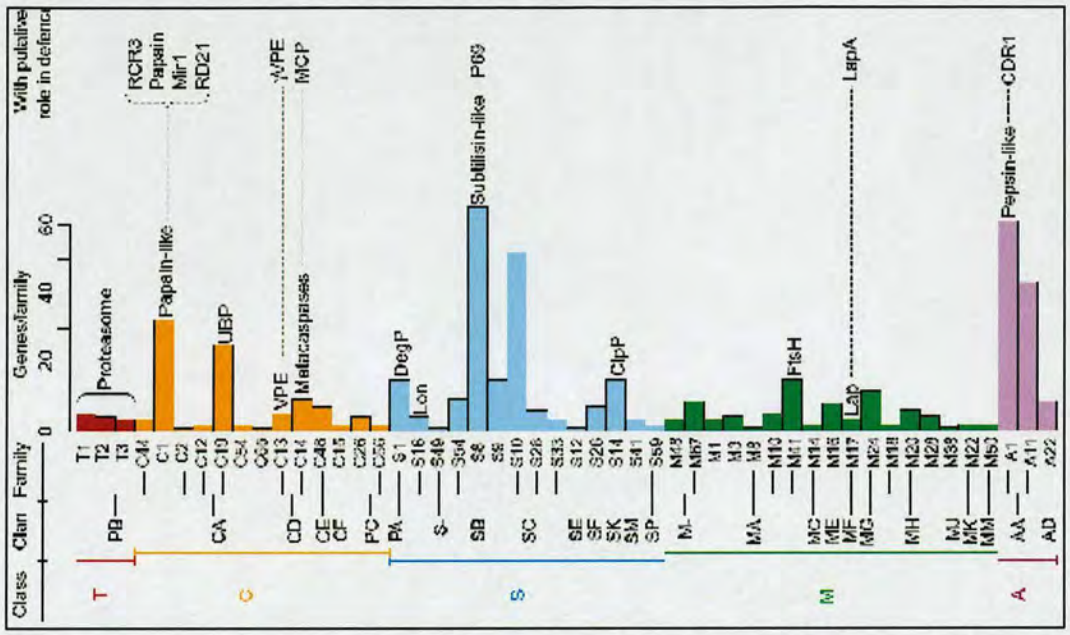


**Figure 7.1. Classification of Arabidopsis proteases.** Proteases can be subdivided into catalytic types on the basis of the residues used to cleave a peptide bond. The Arabidopsis genome encodes 198 serine (S), 112 aspartic (A), 95 cysteine (C), 80 metallo (M) and 12 threonine (T) proteases. Each protease class consists of several clans of proteases. Members of a single clan are believed, on the basis of their conserved tertiary structure and order and spacing of catalytic residues, to have a common evolutionary origin. Each clan of proteases consists of several families, which are identified by a number following the catalytic type (e.g. family C1 within clan CA). The number of proteases belonging to each family is indicated by bars. Source: Van der Hoorn and Jones (2004).

## 7.4. Localisation aims

The localisation of cathepsin B was investigated for NbCathB not AtCathB as transient expression studies are easier in *N. benthamiana* than in *Arabidopsis*. NbCathB protein was predicted to encode a signal peptide by program SignalP, indicating that this protein is targeted to somewhere in the endomembrane system and is possibly secreted. Moreover, a recent study in the closely related species *Nicotiana tabacum* identified a secreted cathepsin B-like homologue using a signal sequence trap system in yeast

(Hugot et al., 2004). Transgenic *N. benthamiana* plants, where the endogenous cathepsin B gene(s) has been silenced by VIGS, exhibit an attenuated HR in response to non-host bacteria and a potato–*P. infestans R* gene–*Avr* gene interaction (Gilroy et al., 2007). Therefore, it may be informative to determine where this protein acts as a way to shed light on the mechanism by which the HR is reduced. In order to achieve this, c-terminal fusion constructs were made with NbCathB and the fluorescent proteins GFP and mRFP under the control of the 35s promoter (Appendix II). These constructs were introduced into *Agrobacterium* expression vectors (Curtis and Grossniklaus, 2003) which mediate transient overexpression in *N. benthamiana.* The fluorescent proteins were fused to the C-terminus of cathepsin B as the propeptide on the N-terminus is known to be cleaved to generate active protein in mammalian systems (Rowan et al., 1992; Turk et al., 2001).

## 7.5. NbCathB is a secreted protein

Cloning of localisation constructs was done by Dr Petra Boevink. Confocal microscopy was done with the help and instruction of Dr Petra Boevink. Replicates of Westerns and protein profiling were done in collaboration with Dr Ingo Hein. Samples for Mass Spectroscopy analysis were sent to Dr Renier van der Hoorn.

### 7.5.1. NbCathB::GFP

When the *NbCathB::GFP* construct was expressed *in planta, N. benthamiana* cells were examined under the confocal microscope. Only a limited amount of GFP fluorescence could be observed in the endoplasmic reticulum (ER) (Fig. 7.2a). One possible explanation for this observation is that the fusion construct was being secreted into the plant apoplast. GFP is unstable in the apoplast (Satiat-Jeunemaitre et al., 1999; Batoko et al., 2000) and, therefore, no fluorescence could be detected in this region.

In order to examine this possibility, plants which were expressing the GFP construct were treated with either water or brefeldin A (BFA) which is an inhibitor of the secretory pathway (Klausner et al., 1992). Plants treated with the water control maintained the weak ER fluorescence observed previously, whereas plants treated with BFA accumulated many bright aggregates in the ER (Fig. 7.2b). This seems to agree with the theory that CathB is secreted into the apoplast. When secretion is inhibited by BFA the GFP fusion is retained in the ER, as it is unable to exit the cell. We went on to test this hypothesis with an mRFP fusion construct which should be stable in the apoplast.

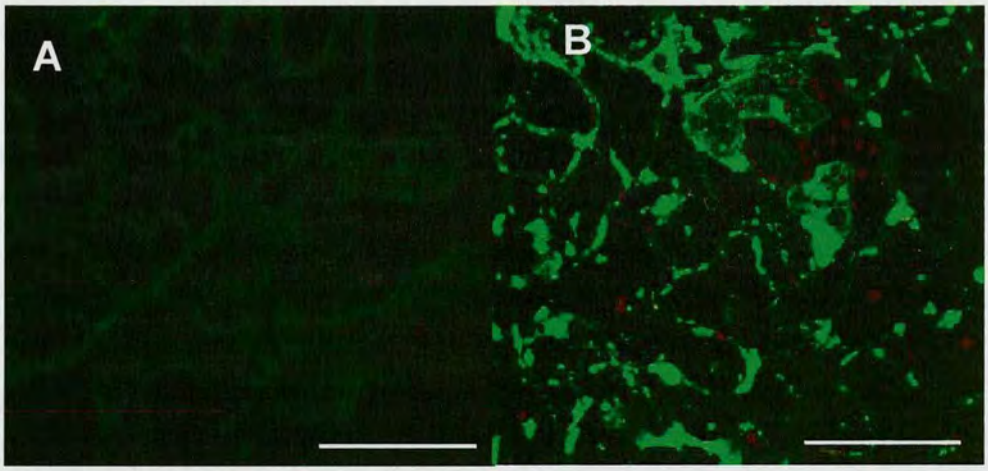


**Figure 7.2. The effect of BFA on NbCathB::GFP fluorescence.** (A) Weak GFP fluorescence in the ER. (B) After BFA treatment the fusion construct accumulated in aggregates in the ER being unable to leave the cell. Scale bars are 50μm.

### 7.5.2. mRFP localisation

An *NbCathB::mRFP* construct was cloned, expressed in *N. benthamiana* and examined under the confocal microscope. mRFP is stable and able to fluoresce outside plant cells and a clear pattern of apoplastic localisation was observed for this construct (Fig. 7.3a). In areas where strong apoplastic fluorescence is observed, mRFP fluorescence can also be seen in the ER within these cells (Fig. 7.4a). Again BFA treatment was used to inhibit secretion and again the fusion construct was found to accumulate in aggregates

inside the ER (Fig. 7.3b), whereas water treatment had no effect. To verify this pattern of apoplastic fluorescence a secreted mRFP (sec::mRFP) construct (Appendix II) which consists of mRFP fused to a signal peptide was employed and subjected to the same experiments. The localisation of this construct was observed to be very similar to that of the NbCathB::mRFP construct, apoplastic fluorescence with no change after the control treatment with water (Fig. 7.3c) and formation of aggregates in the ER following BFA treatment (Fig. 7.3.d).

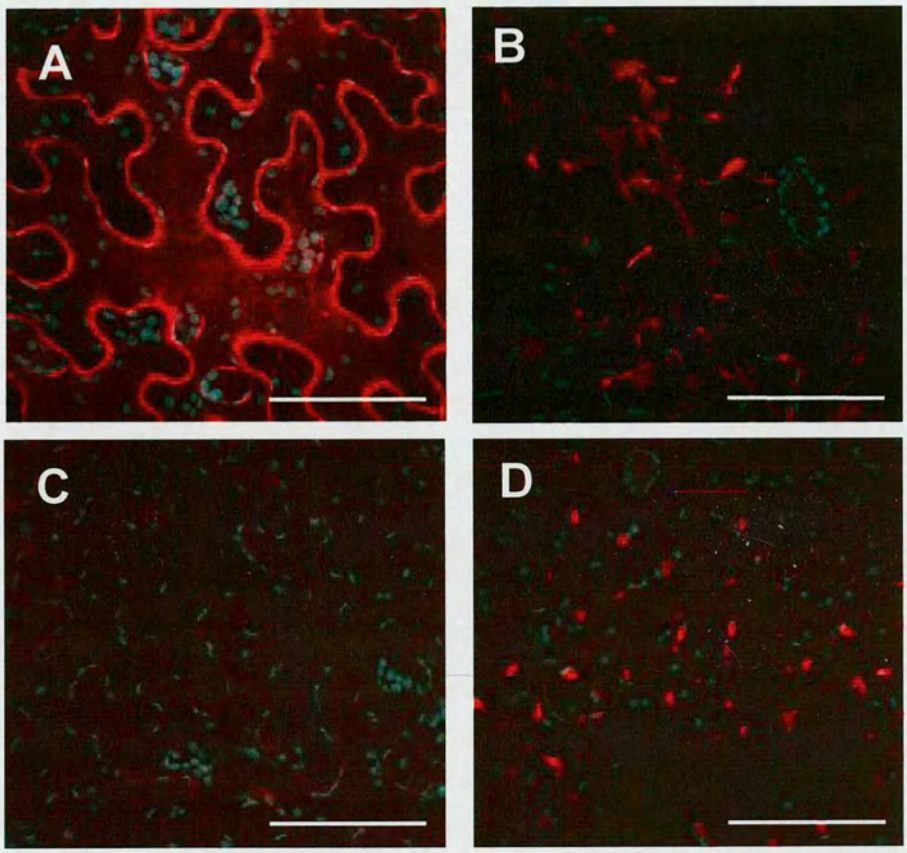


**Figure 7.3. The effect of BFA on mRFP fluorescence.** (A) Apoplastic localisation of NbCathB::mRFP is retained following treatment with water for 6 hr but (B) the protein formed aggregates within the cell when leaves were treated with brefeldin A (BFA), which inhibits secretion. The secreted mRFP control construct follows the same pattern of localisation to the CathB::mRFP construct with (C) $H_2O$ and (D) BFA treatment. Scale bars are 50μm.

### 7.5.3. Co-infiltration with a membrane marker

As a final confirmation to demonstrate that the localisation of NbCathB is indeed in the apoplast and not just to the area proximal to the plasma membrane our mRFP fusion construct was co-infiltrated into *N. benthamiana* with the plasma membrane tag EGFP-LT16b. In this way the cell membrane is labelled with GFP and mRFP localized to the apoplast can be easily observed external to this (Fig. 7.4b). The strongest fluorescence is seen at cell junctions.

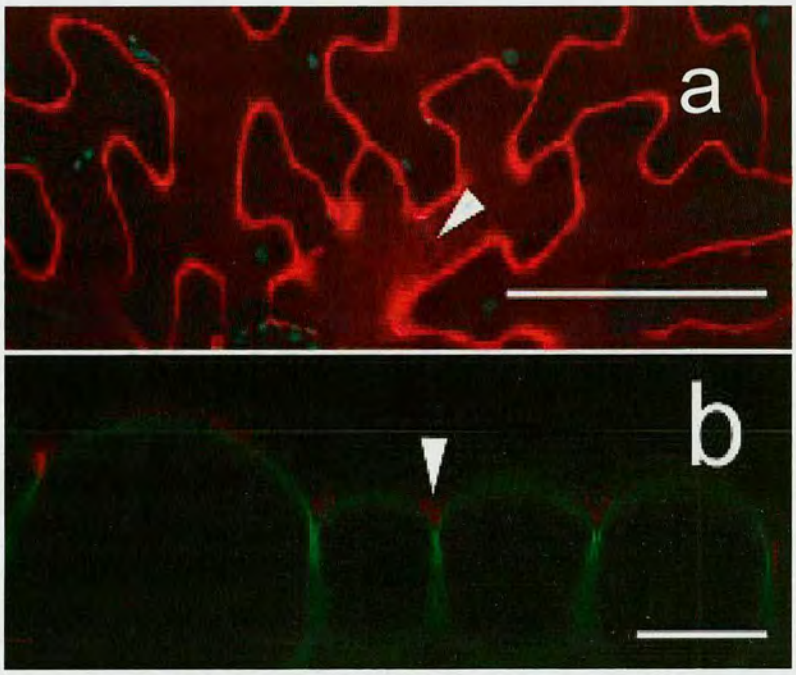


**Figure7.4: Cathepsin B is localised to the apoplast.** (**A**) Expression of the NbCathB::mRFP fusion in leaf epidermal cells resulted in largely apoplastic fluorescence; this image is a maximum projection of a stack of 20 confocal images covering 19 µm in depth. In highly over-expressing cells some fluorescence visible within the cell (arrowhead) appeared to be in the endoplasmic reticulum. Scale bar is 100 µm. (**B**) When co-expressed with the plasma membrane marker EGFP-LT16b, NbCathB::mRFP fluorescence was observed outside the GFP-LT16b-labelled plasma membrane of the abaxial epidermal cells. This image is a cross-section through a projected stack of 72 confocal images covering 43 µm in depth. mRFP fluorescence was particularly bright close to cell junctions (arrowhead). Scale bar is 20 µm. Source: (Gilroy et al. 2007)

### 7.6. mRFP is cleaved on secretion

We went on to check that the mRFP tag was fused to cathepsin B. Whole and apoplastic protein extracts of leaves expressing the NbCathB::mRFP construct (also called the OX construct) were run on SDS PAGE and Western blotted using an mRFP antibody as a probe (donated by Professor John Hancock, Institute for Molecular Bioscience, Brisbane, Australia). The full length NbCathB::mRFP fusion protein was exclusively detected in whole cell extracts and not in the apoplastic extracts (Fig. 7.5a). Another band of approximately 30 kDa was detected in both extracts; this corresponds to free mRFP which coincidently is the same size as the predicted active cathepsin B protein size. Also the accumulation of the 30 kDa band did not increase on BFA treatment whereas the amount of full length protein increased (Gilroy et al. 2007, Appendix III). This suggests that the mRFP fusion tag is cleaved on secretion.

In order to demonstrate that the cathepsin B is secreted along with the mRFP apoplastic extracts were prepared from control leaves and leaves infiltrated with the NbCathB::mRFP (OX) construct. Coomassie blue staining of these samples on SDS PAGE shows that a band of 30 kDa is present in the OX sample but not the control (Fig 7.5b). This band was extracted and sent away for Mass Spectroscopy analysis. This result (Fig. 7.6) confirmed the presence of peptides for both cathepsin B and mRFP. The peptides which were identified for cathepsin B belonged exclusively to the protease domain, suggesting the prepro domain was cleaved on secretion.

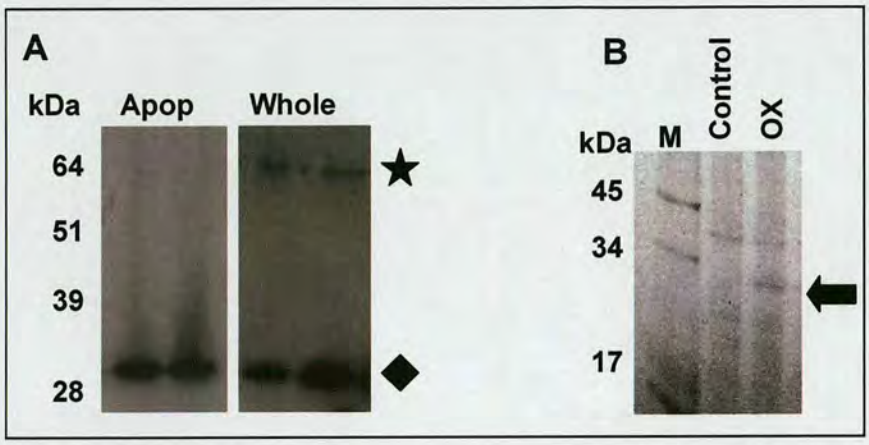


**Figure 7.5. mRFP is cleaved from cathepsin B on secretion.** (A) Western blots of apoplastic and whole cell protein extracts of leaves expressing NbCathB::mRFP probed with an mRFP antibody, full length fusion protein (star) is present only in the whole extract, free mRFP (diamond) is present in both extracts. (B) SDS PAGE of apoplastic extracts from leaves expressing control and OX constructs, CathB (arrow) is present only in OX sample, lane M is a ladder.

NbCathB:*MAMNHMSLVTFLLLIGASVLVLQVVAEQPISQAKAESAILQDSIVKQVNENEKAGWKAALNPRFSN*
*FTVSQFKRLLGVLPTRKGDLKGIPILTHPKLLELPQEFDARVAWPNCSTIGRILDQGHCGSCWAFGAVES*
LSDRFCIHYGLNISLSANDLLACCGFLCGDGCDGGYPLQAWKYFVRKGVVTDECDPYFDNEGCSHPGC
EPAYPTPKCHRKCVKQNLLWSKSKHFGVNAYMISSDPHSIMTELYKNGPVEVSFTVYEDFAHYKSGVY
KHVTGDVMGGHAVKLIGWGTSEDGEDYWLLANQWNRGWGDDGYFKIRRGTDECEIEDEVVAGLPS
ARNLNMELDVSDAFLDAAM::**mRFP**::AAAPVSRYLDPAFLYKVVDSLARLEMASSEDVIKEFMRFKV
RMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFQYGSKAYVKHPADIPDYL
KLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASTE
RMYPEDGALKGEIKMRLKLKDGGHYDAEVKTTYMAKKPVQLPGAYKTDIKLDITSHNEDYTIVEQ
YERAEGRHSTGA

| aa | XC | Identified sequence |
|---|---|---|
| 10 | 2.50 | HPADIPDYLK |
| 10 | 2.89 | KPVQLPGAYK |
| 18 | 5.31 | LDITSHNEDYTIVEQYER |
| 11 | 3.33 | LLELPQEFDAR |
| 10 | 2.97 | TMGWEASTER |
| 13 | 3.13 | GTNFPSDGPVM*QK |
| 13 | 3.27 | GTNFPSDGPVMQK |
| 9 | 2.58 | GWGDDGYFK |
| 11 | 3.18 | LEM*ASSEDVIK |
| 11 | 3.21 | LEMASSEDVIK |
| 12 | 3.42 | M*YPEDGALKGEIK |
| 19 | 5.49 | MEGSVNGHEFEIEGEGEGR |
| 13 | 2.53 | MYPEDGALKGEIK |

**Figure 7.6: Identification of cathepsin B peptides by Mass Spectroscopy.** Identified peptides from *N. benthamiana* cathepsin B and mRFP in the apoplast following overexpression of *NbCathB::mRFP*; **Bold**: identified peptides; *underlined italics*: signal peptide; *italics:* prepro domain; boxed: catalytic cysteine residue; underlined: putative N-glycosylation site; M* is oxidized methionine; XC: X-correlation value: >1.5 maybe; >2.5 good; >3.5 excellent; >4.5 no doubt. Red aa indicate possible digestion sites. Source: (Gilroy et al. 2007)

## 7.7. Secreted cathepsin B from the OX mRFP construct is active

Protease activity profiling (van der Hoorn et al., 2004) is another useful tool we employed to examine the whereabouts and activity of cathepsin B. This system utilises DCG04, a biotinylated derivative of the general inhibitor of all papain-like cysteine proteases, E64 (Greenbaum et al., 2000). DCG04 covalently binds to the critical cysteine residue in the substrate binding site in active cysteine proteases. These proteases can be purified on magnetic beads and then probed on Western blots with an anti-streptavidin antibody. In this way a profile of all the active proteases of this class can be obtained and can then be identified by Mass spectrometry (MS).

Experiments using this technique were carried out on whole and apoplastic protein extracts from control and OX localisation construct expressing *N. benthamiana* leaves 2-3 days after infiltration. The whole protein extracts (Fig. 7.7a) show the expected pattern of 3 bands of approximately 25, 30 and 38 kDa which have been previously identified to contain aleurins, cathepsin B, xylem cysteine proteases (XCPs), RD21 mature form, and RD21 plus granulin domain respectively (van der Hoorn et al., 2004). In contrast in the apoplastic extracts there appears to be very little cysteine protease activity in the control apoplast sample but in the OX apoplast sample a single clear band corresponding to cathepsin B generated by the *Agrobacterium* overexpression is present (Fig. 7.7a). These two apoplastic extracts were then further tested for cathepsin B activity against a commercial colorimetric substrate. The OX sample was found to possess activity for this substrate greater than 6-fold higher than the control (Fig. 7.7b) which had very little activity compared to buffer alone (data not shown).

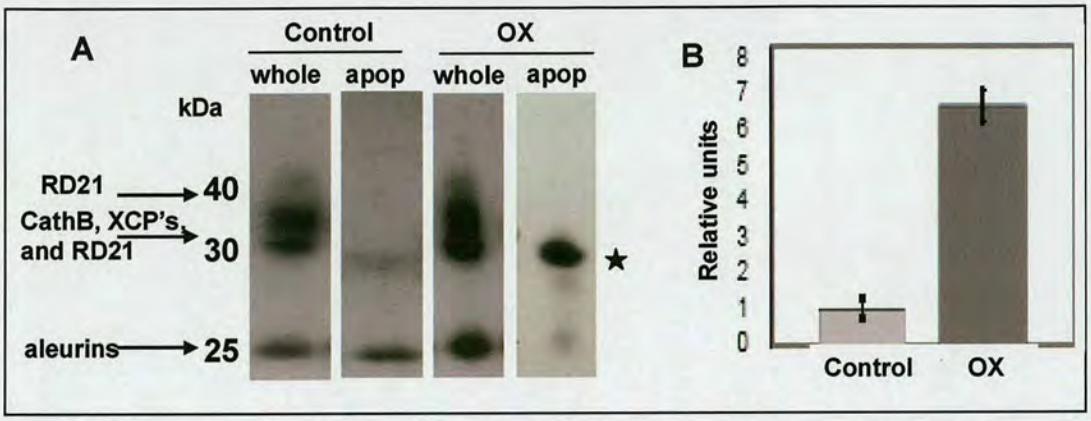


**Figure 7.7: Protease profiling and cathepsin B activity.** (A) Whole and apoplastic protein extracts from control and OX expressing leaves. Active proteases captured by DCG04, the identity of the bands as found by mass spec is shown on the left. A cathepsin B band (star) is only present in apoplastic extract from OX but not control plants. (B) Cathepsin B activity of OX and control total protein extracts on a commercial colorimetric substrate. Relative cathepsin B activity of OX apoplastic protein samples was >6-fold higher than control samples.

## 7.8. Discussion

It has been demonstrated here, and subsequently published in Gilroy et al. (2007), that NbCathB is localized to the plant apoplast and that it is cleaved into an active form on secretion. It could be suggested that the apoplastic localization observed is an artifact of the cathepsin B overexpression. This seems unlikely as the localization is in agreement with the predicted results of the computer program SignalP. The presence of a signal peptide targets proteins into the endomembrane system, where, in the absence of any other signalling motifs causing retention or redirection, the protein is secreted by default (Burgess and Kelly, 1987). Apoplastic localization also agrees with a previous study in a closely related tobacco species where a cathepsin B protein was identified as secreted in a Yeast-based screen (Hugot et al., 2004). However, these two methods of confirmation use either *in silico* or *in vitro* methods so we checked *in vivo* using a

tomato apoplast extract labeled with DCG04. A cathepsin B protease was indeed detected in this sample by MS (Fig. 7 in Gilroy *et al.* Appendix III).

Mammalian cathepsin B is a largely lysosomal enzyme with the stability and activity of the protein dependent on pH (Mort and Buttle, 1997; Turk et al., 2000). The acidic environment of the apoplast, which is similar to that of lysosomes, may be a prerequisite for cathepsin B stability and activity, assuming plant and mammalian enzymes possess similar properties based on their homology. The secretion of proteases such as cathepsin B may also be an important regulatory control measure for plants as proteolytic enzymes unrestrained inside cells could have adverse consequences. The localization of mammalian cathepsin B is certainly crucial for the regulation of its activity as changes from physiological to pathological conditions appear to be accompanied by aberrant changes in the location of cathepsin B, for example, in the development of cancer, Alzheimer's and Osteoarthritis (Vancompernolle et al., 1998; Podgorski and Sloane, 2003; Muntener et al., 2004). Nevertheless, there are physiological reasons and mechanisms for mammalian cathepsin B to be secreted (Linke et al., 2002; Buth et al., 2007).

Silencing or peptide inhibition of NbCathB has been demonstrated to attenuate the HR to the non-host bacterial pathogens *Erwinia amylovora* and *Pseudomonas syringae* (Gilroy et al., 2007). As an extracellular localization has now been demonstrated for NbCathB several mechanisms may explain how cathepsin B can effect a reduction of the HR to this pathogen.

One mechanism may be pathogen recognition. RCR3 is a secreted cysteine protease from tomato which is required for resistance to the pathogen *Cladosporium fulvum*. RCR3 is a target of the *C. fulvum* virulence factor Avr2 which binds and inhibits RCR3. This is thought to be an example of the guard hypothesis as the RCR3–Avr2 complex is recognized by the plant R protein Cf2 and this recognition triggers an HR (Rooney et al.,

2005). It is possible that cathepsin B may recognize or be targeted by an *E. amylovora* protein and the failure of this to occur in silenced plants fails to trigger an HR. However, this seems unlikely, as silencing attenuated the HR also to *P. syringae*, and during R3a–AVR3a interaction.

A second mechanism for cathepsin B action could be in mediating signalling cascades leading to PCD or HR. Two serine proteases with caspase like activity (saspases) were identified in oat to be secreted upon induction of PCD by the toxin victorin or heat shock. Based on work with different protease inhibitors and the timing of PCD the authors of this study hypothesize that these saspases function early on in a proteolytic cascade which leads to the HR, and contains a papain cysteine protease (Coffeen and Wolpert, 2004). Although none of the prospective protease targets of these enzymes have been identified yet, cathepsin B is a candidate to take part in this signalling in oat and other plants.

Additional mechanisms underpinning cathepsin B function, may include direct toxic effects towards the pathogen or the production of cleavage products which are recognized by the host and so trigger the HR. Several proteases are known to be secreted and accumulate in the manner of PR proteins after pathogen recognition and possibly possess antimicrobial activity. Such proteases include P69 (Tornero et al., 1996) and PIP1 (Tian et al., 2007) although these proteins would doubtless accumulate after the HR would have occurred (Tornero et al., 1997).

Another important question here is how an apoplastic cathepsin B would impact on intracellular recognition events, as was demonstrated with the R3a–Avr3a interaction in *NbCathB* silenced plants (Gilroy et al., 2007). Another example where this may occur is where the secreted aspartic protease CDR1 in *Arabidopsis* (Xia et al., 2004) mediates resistance to *Pseudomonas syringae*, a pathogen which typically injects intracellular effectors using a type III secretion system (Alfano and Collmer, 2004). It is also

possible that not all cathepsin B is secreted and that some remains inside the cell and functions there in recognition. The cysteine protease RD21 is found to be localized to both the apoplast in tomato (van der Hoorn, personal communication) and to ER bodies in *Arabidopsis* which subsequently deliver it to the vacuole (Hayashi et al., 2001). A lot may hinge on the cell type, developmental stage and abiotic/ biotic factors. While we observed faint GFP and mRFP cathepsin B fusion protein expression in the ER it is likely that this was just a snapshot of the protein on its way out of the cell. Nevertheless, we cannot rule out the presence of internal storage of cathepsin B especially in very small quantities as these would not have been observed microscopically using our detection limits.

Although we have confirmed that NbCathB is secreted into the apoplast by various means using DCG04 assays and MS, the mRFP tag is unfortunately cleaved off upon secretion so we are unable to follow any re-localisation of the cathepsin B which may or may not occur following pathogen challenge. Other cases of tags being cleaved on secretion of the fusion proteins have been reported (van Esse et al., 2006) which complicates such studies.

An unexpected bonus to come out of this localization work is that overexpression of the fusion construct resulted in high cathepsin B activity. As this activity is present in the apoplast it is easily purified with few contaminating proteases. Control protein apoplastic extracts show little detectable cathepsin B activity by both DCG04 and colorimetric substrate assays compared to our construct. Therefore, this may be a useful system for testing different substrates and inhibitors of cathepsin B, to determine the range of the plant protease as compared to known specificities of the mammalian enzyme. We may also be able to investigate possible pathogen-derived inhibitors and interactors.

# 8. Cathepsin B Activity and Inhibitors

## 8.1. Use of inhibitors to infer protease involvement in plant defence

Proteases such as caspases play important roles in apoptosis, a form of PCD in animals (Earnshaw et al., 1999). Many studies have been carried out in plants investigating possible conserved action of these proteases in the HR, another form of PCD sharing some morphological features of apoptosis, reviewed in Danon *et. al.* (2000). These studies have employed various inhibitors and substrates known to work on mammalian proteases in an attempt to implicate and identify the corresponding proteases in the plant HR.

No plant gene showing a conserved evolutionary origin with animal caspases has yet been discovered. Nonetheless, a related group of metacaspases have been identified in *Arabidopsis* but these proteases possess differing substrate-specificity to true caspases (Vercammen et al., 2004; Vercammen et al., 2006). To date caspase activity has been reported for a vacuolar processing enzyme (VPE) required for virus induced HR (Hatsugai et al., 2004), an as yet un-identified HR-active protease with caspase 3 activity (Chichkova et al., 2004) and subtilisin-like serine proteases (saspases) involved in victorin-induced PCD (Coffeen and Wolpert, 2004). Moreover, there are many reports of inhibitor treatments which block or attenuate the plant HR which have been construed as proof of involvement of various classes of proteases (Levine et al., 1996; D'Silva et al., 1998; del Pozo and Lam, 1998; Yano et al., 1999). However, caution should be taken when interpreting inhibitor data as these inhibitors are based on what is known from mammalian systems and some have been found to target additional protease activities to those under investigation. For example methylketone-based caspase inhibitors can also target papain-like cysteine proteases (Rozman-Pungercar et al., 2003)

and homologues of mammalian PCD-implicated proteases of this class are known to be present in plants.

### 8.2. Pathogen protease inhibitors as effectors

Another circumstance which may lend credence to the theory that proteases are involved in the HR, and more broadly in plant defence, is the fact than some of the effectors and virulence factors produced by pathogens on attempted incursions into their plant hosts have been found to be various types of protease inhibitors. Both fungal and oomycete plant pathogens have been shown to secrete protease inhibitors into the plant apoplast upon infection. Furthermore these inhibitors have been shown to interact with and selectively inhibit several plant PR proteins including P69, RCR3 and PIP1 from tomato (Tian et al., 2004; Rooney et al., 2005; Tian et al., 2005; Tian et al., 2007).

### 8.3. Cathepsin B activity aims

Previous work carried out using commercial cysteine protease inhibitors in addition to VIGS has implied a role for cathepsin B as a positive player in the development of the HR in response to certain plant-pathogen interactions but not others (Gilroy et al., 2007). In order to demonstrate cathepsin B activity could account for this activity, although it does not prove it is solely responsible, the *N. benthamiana* cathepsin B implicated in this research and AtCathB2 and 3 from *Arabidopsis* were to be expressed as recombinant protein fusions in *Escherichia coli*. The purified proteins could subsequently be assayed for effects on cathepsin B activity in response to both commercial and pathogen-derived cysteine protease inhibitors.

## 8.4. Recombinant cathepsin B expression in *E. coli*

Initially, full length cDNA sequences for each gene were cloned into the pET32 His-tagged expression vector. After induction of expression no full length protein was ever observed under any of the induction conditions or in any of the specially developed cell lines employed. The only induced protein which could be detected using the appropriate antibody (Fig. 8.1) was a small 20 kDa His-tagged presumed degradation product of AtCathB2 which was purified and unsurprisingly found to possess no activity.

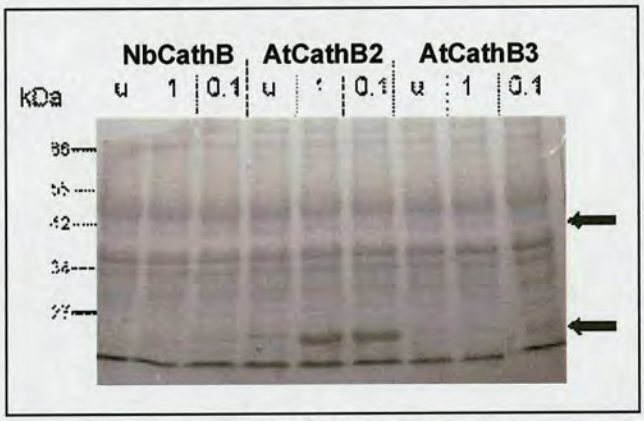


**Figure 8.1. SDS PAGE gels of crude cell culture extract.** No correctly sized bands are shown in the induced samples (1 and 0.1 mM IPTG) compared to the un-induced controls (u) at the predicted protein size of approximately 40 kDa (top arrow). A small protein of approximately 20 kDa (lower arrow) is shown in the induced samples for the pET-AtCathB2 construct (containing the At1g02305 cDNA).

Each cDNA was then re-cloned minus its natural signal peptide into a new FLAG-tagged expression vector. After induction protein bands of the expected size of 39 kDa were visualised on a western blot by means of an anti-FLAG tag antibody (Fig 8.2a) and subsequently purified with affinity resin columns (Fig. 8.2b). Each purified protein sample was observed to contain several smaller FLAG-tagged degradation products in addition to the full length proprotein.

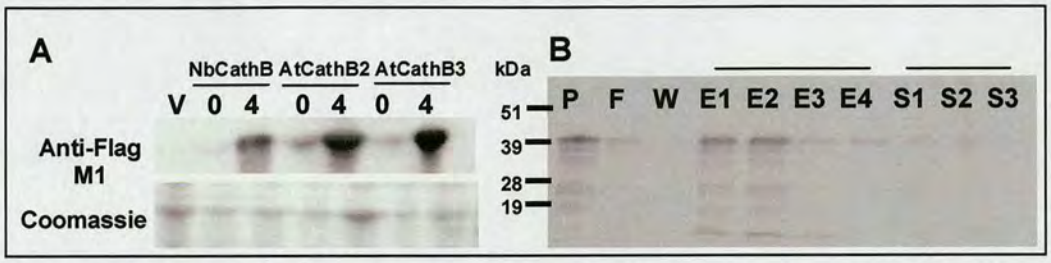


**Figure 8.2. FLAG tagged recombinant cathepsin B protein production and purification.** (**A**) Recombinant FLAG-tagged cathepsin B proprotein is expressed for each construct and is detected by a FLAG-specific antibody. No tagged protein is detected in the V= vector control and only small amount is detected at 0= prior to induction compared to 4=four hours post induction with IPTG. (**B**) The NbCathB construct was purified using a FLAG resin column and the western probed with an anti-FLAG antibody shows various fractions from the column. P=crude sample prior to purification, F=flow through of crude sample, W=column wash flow through, E=column eluate fractions 1-4, S=fractions 1-3 from flow through of the stripping buffer.

Purified protein samples were then subjected to two different cathepsin B activity assays. Neither the cathepsin B commercial substrate assay nor the DCG04 assay was able to detect any activity in these protein samples under a range of conditions or after various previously determined activating treatments (Kuhelj et al., 1995) (Fig 8.3). Therefore, it was not possible to produce active cathepsin B using an *E. coli* expression system and time constraints prohibited attempting these experiments with yeast or insect/ mammalian cell expression systems which have not been previously employed in the laboratory and which may or may not have proved successful.

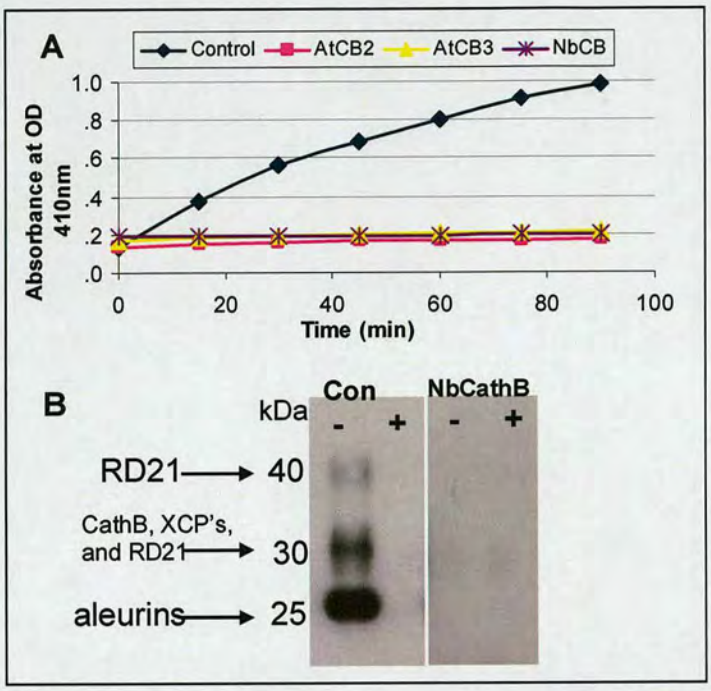


**Figure 8.3. FLAG tagged recombinant cathepsin B protein activity assays.** (**A**) The graph shows that protein extracts from the 3 recombinant cathepsin B constructs have no activity on a cathepsin B colorimetric substrate compared to a bovine cathepsin B control. (**B**) The purified NbCathB protein shows no activity can be detected using DCGO4 which binds active cysteine proteases, whereas the control *N. benthamiana* plant sample yields several active cysteine proteases.

## 8.5. Commercial cathepsin B inhibitors

Although it has not been possible to produce active cathepsin B using an *E. coli* expression system, we have been able to partially purify NbCathB in an active form using *Agrobacterium*-mediated expression *in planta* of our NbCathB-mRFP localisation construct as discussed in Chapter 7. We have already demonstrated that apoplastic protein extracts generated using this construct possess cathepsin B activity which is not detectable in control extracts (Fig. 7.7, Chapter 7).

Apoplastic protein samples produced using this OX construct were incubated with either general papain class cysteine protease inhibitor E64 or with mammalian cathepsin B specific inhibitors Ac-LVK-CHO, CA-074-Me and z-FA-fmk used in Gilroy et al. (2007), to determine if these inhibitors were able to inhibit plant cathepsin B activity. Using both the commercial substrate and DCG04 assays these inhibitors were able to effectively reduce the measured cathepsin B activity of this extract to background levels (Fig. 8.4). This suggests that, in addition to mammalian cathepsin B, plant cathepsin B is also inhibited by these inhibitors.

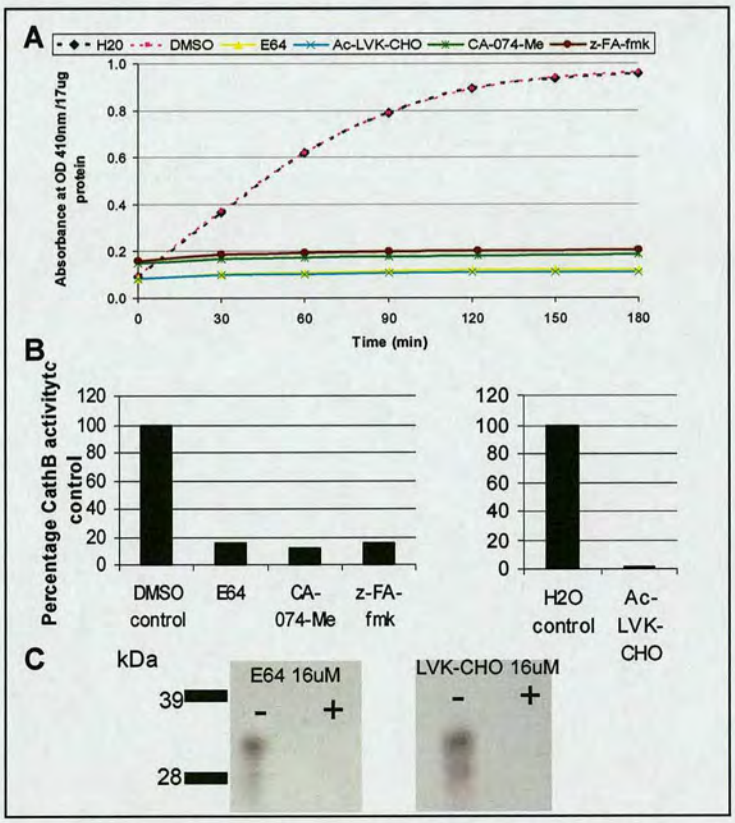


**Figure 8.4. Commercial Inhibitors target cathepsin B activity.** (A) Detection of CathB cleavage product over time after treatment with inhibitors E64, CA-074-Me, z-FA-fmk and Ac-LVK-CHO or water/ DMSO controls. (B) Percentage cathepsin B activity of the control OX samples with different treatments, calculated from the slope of the initial reaction rate. (C) Western Blots of the DCG04 assay showing no active CathB signal in the presence of the inhibitor (+) compared to no inhibitor (-).

## 8.6. Pathogen-derived cysteine protease inhibitors

### 8.6.1. Avr2 from *C. fulvum*

*Cladosporium fulvum* is a fungal pathogen of tomato. Among the many effectors produced by this pathogen is Avr2 which is recognised by the Cf2 *R* gene product in tomato (Luderer et al., 2002). Avr2 has been demonstrated to encode a cysteine protease inhibitor which is known to interact with tomato cysteine protease RCR3 (Rooney et al., 2005). In order to examine whether this pathogen effector is additionally able to target cathepsin B, purified Avr2 protein (courtesy of Prof. Pierre de Wit) was incubated with CathB OX apoplastic extracts. Avr2 does not effect any change in NbCathB activity as measured by the substrate assay (Fig. 8.5a and b). In contrast, there looks to be a slight decrease in CathB activity after incubation with Avr2 using the DCG04 assay method (Fig. 8.5c), although the same concentration of protein and inhibitor were used for both assays. However, previous experience of the use of the DCG04 system has indicated that it is qualitative rather than quantitative.

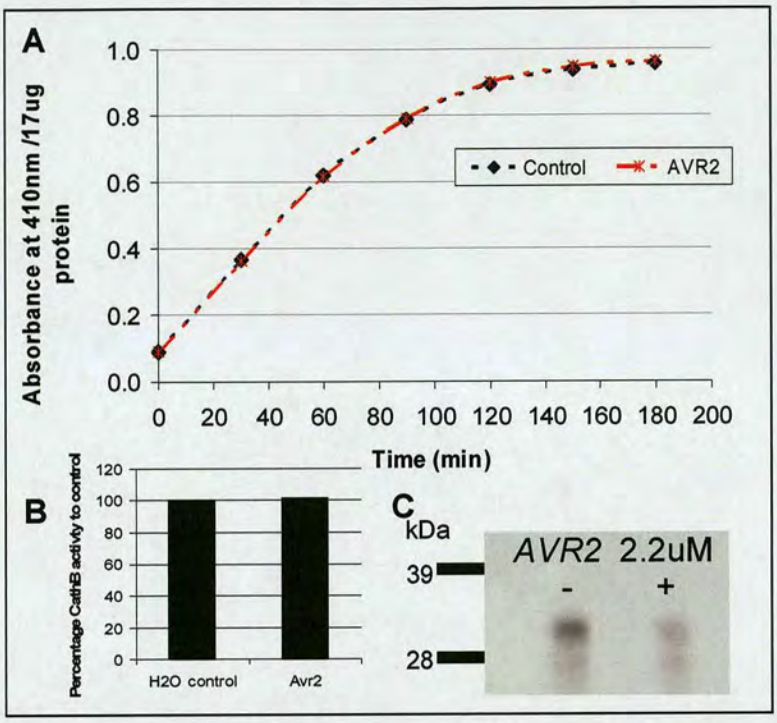


**Figure 8.5. Avr2 does not target cathepsin B activity.** (A) Detection of CathB cleavage product over time after treatment with Avr2 or water control. (B) Percentage cathepsin B activity of the control OX sample, calculated from the slope of the initial reaction rate. (C) Western blots of the DCG04 assay showing active CathB signal in the presence (+) or absence (-) of Avr2.

### 8.6.2. EPIC inhibitors from *P. infestans*

A pathogen of Solanaceous species, the oomycete *Phytophthora infestans,* is also known to produce various different protease inhibitors. These include KAZAL-like extracellular protease inhibitors (EPI) serine protease inhibitors of which tomato PR protein P69B is a known target (Tian et al., 2004; Tian et al., 2005) and extracellular protease inhibitors cytatin-like (EPIC) cysteine protease inhibitors which have been identified to target a second tomato PR protein PIP1 (Tian et al., 2007). Although cloning of several EPIC genes to produce and purify tagged protein fusions is underway, several experiments were carried out to look for an inferred interaction between EPICs and cathepsin B.

*N. benthamiana* plants transiently expressing potato virus X (PVX)-EPIC2B, 3 or 4 constructs under the control of the 35s promoter (courtesy of Prof. Sophien Kamoun, sequence information Tian et al. 2007) or a PVX-GFP construct were inoculated with *Erwinia amylovora* to examine the effect of inhibitors on the HR. A significant reduction in the percentage of HR was observed, particularly in plants expressing EPIC2 and 4 compared to the GFP control (Fig. 8.6). The strongest reduction in HR, ~70%, was found in plants expressing EPIC2. Similar experiments were done with the same pathogen in *N. benthamiana* plants silenced for *NbCathB*, which showed a similar decrease in HR (Gilroy et al., 2007).

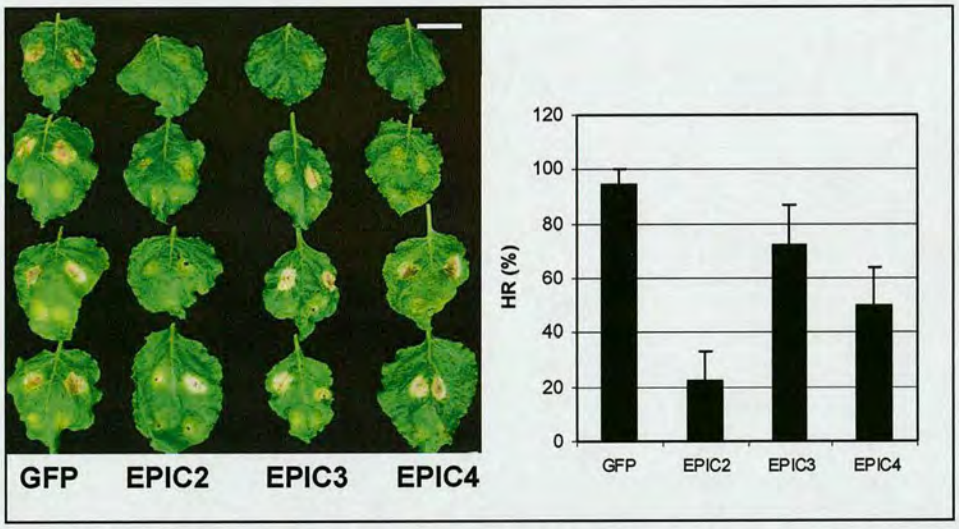


**Figure 8.6. Reduction in HR to *E. amylovora* in PVX-EPIC Expressing Plants.** The pictures on the left indicate the HR symptoms displayed in response to *E. amylovora* inoculation in plants expressing each of the PVX-EPIC or control constructs, scale bar is 6cm. The Bar graph on the left quantifies the percentage HR observed from three leaves on three individuals, Error bars are SE within one biological replicate.

Unfortunately, preliminary attempts to co-express the PVX-EPIC constructs with the CathB:mRFP OX construct were inconclusive about any effects of these inhibitors on cathepsin B activity, possibly due to the stress of the two pathogen over-expression systems employed.

## 8.7. Discussion

Although no true caspase genes, nor homologues of other caspase apoptotic pathway genes, such as *Bcl-2* and *Bax*, have yet been discovered in plants, many plant proteases have been implicated in PCD and plant defence (Watanabe and Lam, 2004). Some of these plant proteases have caspase activities, such as VPE and saspases (Coffeen and Wolpert, 2004; Hatsugai et al., 2004), and may be predicted to act on conserved caspase targets, as over-expression of some mammalian apoptosis factors can manipulate the plant HR (Lacomme and Santa Cruz, 1999; Mitsuhara et al., 1999). One of the most numerous types of protease present in plants belong to a group of papain-like cysteine proteases (PLCPs) (van der Hoorn and Jones, 2004). Proteins which belong in this family include cathepsin B which has homologues in mammals which have been shown to act in caspase-independent apoptosis and also to process caspases into their active forms (Vancompernolle et al., 1998; Foghsgaard et al., 2001).

Cathepsin B silencing using VIGS and inhibition using peptide inhibitors has been demonstrated to attenuate the HR in response to some plant-pathogen interactions but not to others (Gilroy et al., 2007). This suggests that there are multiple pathways which lead to PCD in plants, as is the case in mammals, and that cathepsin B is not involved in all of them. However, a protease cascade comprising different classes of proteases acting sequentially over time has been proposed to occur at least in one instance (Coffeen and Wolpert, 2004). Multiple inhibitor studies, reviewed in van der Hoorn and Jones (2004), provide sometimes conflicting evidence for protease involvement and seem to indicate more than one pathway leads to HR/ PCD. For example, caspases but not serine proteases are required for cell death in one study but the reverse is true for another (del Pozo and Lam, 1998; Yano et al., 1999). Moreover, some of the inhibitors employed have been found to be less specific than was previously thought (Rozman-Pungercar et al., 2003).

For these reasons, the expression and purification of recombinant cathepsin B was attempted to demonstrate if this plant protease had the same specificity for the inhibitors and substrates employed, to further implicate it in the HR. Unfortunately, although cathepsin B protein was successfully expressed it was found to be inactive and resisted attempts to activate it. Perhaps additional post-translational modifications are required for this plant protein not available in a bacterial expression system, although active human cathepsin B and papain itself have been expressed in *E. coli* (Taylor et al., 1992; Kuhelj et al., 1995).

However, additional evidence showing that several commercially available mammalian cathepsin B inhibitors are able to inhibit NbCathB has been shown using partially purified apoplastic extracts from CathB:mRFP OX plants, of which cathepsin B was the most abundant protein measurable with the detection limits employed here. This showed that these inhibitors had differing abilities to block plant cathepsin B activity. For example, general papain family inhibitor E64 and cathepsin B specific inhibitor Ac-LVK-CHO appeared to be slightly better at blocking plant cathepsin B activity than cathepsin B specific inhibitors CA-074-Me and z-FA-fmk.

Interestingly, some of the effectors produced by pathogens to populate the molecular battlefield in interactions with their plant hosts have been found to be protease inhibitors. Tomato plants under attack by diverse pathogens accumulate PR-protein P69B a subtilisin-like serine protease presumably as a defence mechanism (Tornero et al., 1997). Tomato pathogen *Phytophthora infestans* secretes various protease inhibitors, of these, both EPI1 andEPI10 bind to and inhibit P69B as a probable counter-defence mechanism (Tian et al., 2004; Tian et al., 2005). A similar interaction is thought to occur with tomato PIP1, another PR-protein induced in response to pathogens, and *P. infestans* EPIC2B, a cysteine protease inhibitor which has been demonstrated to target and inhibit this protein (Tian et al., 2007). Additionally, *Cladosporium fulvum* also secretes cysteine-rich protein Avr2 which binds to and

inhibits tomato cysteine protease RCR3. However, the plant has evolved a counter-counter-defence to recognise this interaction mediated by *R* gene product Cf-2 which leads to the HR and resistance to the detriment of the pathogen (Rooney et al., 2005). Moreover, bacterial pathogen *Pseudomonas syringae* secretes a cysteine protease via the TTSS which has been shown to suppress plant cell death during infection although the target is not known (Lopez-Solanilla et al., 2004). This suggests that protease-protease inhibitor interactions could be crucial in determining the success of a plant-pathogen interaction.

Here, Avr2 was tested to determine if it was able to target and inhibit another plant defence-related cysteine protease, NbCathB. However, Avr2 does not seem to be a likely inhibitor of cathepsin B, as it was completely unable to reduce the activity of cathepsin B to its colorimetric substrate in contrast to the commercial inhibitors used. Perhaps more promising is the finding that *P. infestans* EPIC inhibitors, particularly EPIC2B, is able to reduce the HR to *E. amylovora* by ~70% when expressed in *N. benthamiana*. Although this data is purely correlative the HR to this pathogen was similarly inhibited in cathepsin B silenced using VIGS in *N. benthamiana* plants, suggesting a possible target of EPICs may be cathepsin B. Further studies comprising assays with purified proteins and co-immunoprecipitation would be required to validate such an interaction. Determining whether cathepsin B is a *bona fide* target of pathogen effectors would be extremely interesting and its apoplastic localisation certainly lends itself to potential exposure to such secreted factors.

# 9. General Discussion and Future Work

Both plants and animals employ PCD to regulate many developmental and physiological processes as well as defensively to eliminate the spread of pathogenic microbes (Mittler and Lam, 1996; Meier et al., 2000; Heath, 2000a; Barber, 2001). Proteases such as caspases are known to play important roles in the regulation of apoptosis, a form of PCD in animals (Thornberry and Lazebnik, 1998; Degterev et al., 2003). Although there are no true caspase genes in plants, accumulating evidence suggests that other proteases may play important roles both in plant PCD and in disease resistance (Beers et al., 2000; van der Hoorn and Jones, 2004). Cathepsin B is a non-caspase cysteine protease whose involvement has been implicated in apoptosis (Vancompernolle et al., 1998; Foghsgaard et al., 2001; Kingham and Pocock, 2001; Cirman et al., 2004). *Cathepsin B*-like genes are conserved in plants and have been demonstrated to be up-regulated during plant PCD processes such as the HR and senescence (Avrova et al., 2004; Guo et al., 2004). Recent work in our laboratory has shown that silencing of *cathepsin B* in *N. benthamiana* abrogates the HR in response to non-host bacterial pathogens and in some *R–Avr* gene interactions (Gilroy et al., 2007). In this work, reverse genetics approaches have been used to investigate the possible involvement of *cathepsin B*-like genes in plant PCD and disease resistance in the model plant *Arabidopsis thaliana*.

## 9.1. Cathepsin B positively regulates PCD in plants

This work has provided several additional lines of evidence that suggests *cathepsin B* genes are involved in the positive regulation of PCD in plants. 1) *Atcathb* knockout mutants show a reduced HR in response to the incompatible bacterial pathogen *Pseudomonas syringae* pv. tomato (DC3000) (*Pst*) expressing the AvrB effector and also exhibit less non-HR cell death to a compatible isogenic strain of *Pst* lacking AvrB.

2) *Atcathb* double mutants are unable to sustain the normal levels of epidermal HR cell death in response to penetration of non-host fungal pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*). 3) Furthermore, *Atcathb* knockouts also show a modest but significant delay in the induction of dark-induced senescence, a developmental form of plant PCD. This suggests that cathepsin B may be a common element of both pathogen-inducible and developmental PCD signalling in plants.

Among the other genes also implicated in the involvement of different types of PCD in plants is the conserved autophagy gene *ATG6/ Beclin1*. Senescence has been suggested to proceed via type 2 autophagic cell death and many plant *atg* mutants including *atg6,* undergo early senescence (Orzaez and Granell, 2004; Patel and Dinesh-Kumar, 2008) Autophagy also seems to have a protective function in limiting the spread of pathogen-inducible cell death, as *atg6* mutants are unable to contain the spread of either the HR to avirulent *Pst* or disease-associated cell death to virulent *Pst*, although there is no apparent change in the amount of avirulent *Pst* growth (Patel and Dinesh-Kumar, 2008). *ATG6* and *CathB* genes are both up-regulated by virulent and avirulent *Pst* challenge but, in contrast to the enhanced HR in *atg6* mutants, *Atcathb* mutants display reduced HR to avirulent *Pst.* Nevertheless, intriguingly both changes in the amount of HR have no effect on avirulent *Pst* growth. It is tempting to speculate about a possible link between cathepsin B and autophagy in plant PCD regulation; as in animals, cleavage of the anti-apoptotic factor Bcl-2 by cathepsins positively regulates apoptosis whereas Bcl-2 binding to Beclin-1 (ATG6) can inhibit autophagy (Pattingre et al., 2005; Turk and Stoka, 2007). There is no homologue of Bcl-2 in plants but considering the apparent cell death protective function of *ATG* genes it would be interesting to test their expression in *Atcathb* mutants and *vice versa*.

LMM *cpr5* and its independently identified allelic mutant *hypersensecence 1* (*hys1*) are proposed to be involved in initiation of both pathogen-induced PCD and senescence and are negative regulators of PCD just as cathepsin B is a positive regulator. *CPR5/HYS1*

encodes a type IIIa transmembrane protein with a NLS (Yoshida et al., 2002) and a review of LMMs (Lorrain et al., 2003) placed CPR5 downstream of EDS1. Mutations in *EDS1* partially suppress *cpr5* lesion propagation but not cell death initiation although *EDS1* expression is up-regulated in *cpr5* mutants, possibly due to the enhanced SA accumulation in *cpr5* plants (Clarke et al., 2001). This is intriguing because EDS1 is also required for the pathogen-inducible expression of *CathB* to *Bgt* and, moreover, microarray data collated in the Genevestigator database shows each of the three *AtCathB* genes are up-regulated in the *cpr5* mutant (Zimmermann et al., 2004; Zimmermann et al., 2005).

EDS1 appears to be a key regulatory node in defence and has roles in non-host, basal and *R* gene-mediated resistance. It is also thought to positively regulate cell death and is required for cell death amplification via the transduction of ROS signals (Rusterucci et al., 2001). No role has yet been identified for EDS1 in senescence although *senescence associated gene 101* (*SAG101*) has recently been described as an EDS1 interactor in addition to known EDS1 interactor PAD4 (Feys et al., 2005). Furthermore, *pad4* mutants show reduced SAG expression and increased susceptibility in response to aphid challenge whereas hyper-senescent *cpr5* mutants were more resistant (Pegadaraju et al., 2005). There is overlap between senescence and disease resistance signalling as many defence genes are up-regulated during senescence and defence hormones SA/ JA and ET can positively regulate senescence (Orzaez and Granell, 2004). In addition, senescence-like yellowing is often observed following pathogen challenge and the senescence marker gene *SAG12* is expressed in the latter stages of the HR, whereas the HR marker gene *HIN1* but not *HSR203J* is expressed during senescence (Pontier et al., 1999). Furthermore, it has been shown here and in Gilroy et al. (2007) that *SAG12* and *HSR203J* expression is dependent on the presence of *CathB*. Thus it seems likely that there may be common regulatory elements between pathogen-inducible and developmental PCD signalling and, as CathB and CPR5 are involved in both processes, they could be proposed to act antagonistically downstream of EDS1-PAD4-SAG101

interactions in the following model (Fig. 9.1). In order to test and refine this model it would be necessary to determine if EDS1 (as well as PAD4 and SAG101) is required for *CathB* expression in response to cell death inducing stimuli other than *Bgt* infection. It will also be important to establish if the reduction in cell death in *eds1* mutants can be accounted for by *cathb* triple mutants or if EDS1 has an additional mechanism of regulating PCD. The increase in *CathB* expression in *cpr5* mutants indicated in the microarray data should be verified and then determined if this is solely due to the positive regulation of *EDS1* in these mutants or if secondary regulation is also employed. It would also be informative to check *CPR5* expression in *cathb* mutants as well as the expression of various defence marker genes.

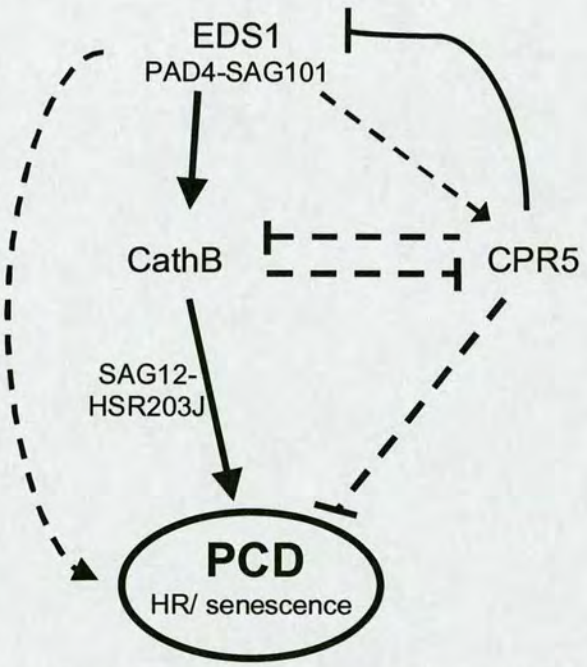


**Figure 9.1. A model proposing how PCD signalling may be regulated via CathB–EDS1-CPR5 interactions.** Arrows indicate positive regulation, lines with a crossbar indicate negative regulation, dashed lines indicate possible regulation.

## 9.2. Uncoupling of PCD and defence in *cathb* mutants.

The correlation between the occurrence of the HR and disease resistance seems to be very clear during non-host *Bgt–Arabidopsis* interactions. In a small number of instances the pre-penetration defences fail and the fungus can penetrate into epidermal cells. In normal circumstances this triggers the HR which prevents any further fungal growth. However, in *eds1* and *cathb* mutants there is a reduction in the HR in response to *Bgt* penetration and this leads to an increase in fungal growth and development. In spite of the clear requirement for the HR for the defence response to *Bgt*, a reduction in HR does not always permit a concomitant increase in pathogen susceptibility.

*Arabidopsis cathb* triple mutants exhibit a reduction in the HR in response to *Pst AvrB* challenge but, surprisingly, are not more susceptible to this pathogen and allow similar levels of bacterial growth as wildtype plants. As R–Avr recognition strongly induces a plethora of defence responses the HR in itself may not be required to restrict bacterial growth, although it is expected to amplify defence signalling. However, mutants, such as *defence, no death 1* (*dnd1*), which display fully competent resistance in the absence of cell death, indicate that the occurrence of the HR is not essential for defence signalling and can be uncoupled (Yu et al., 1998). Also, in *cathb* mutants, while there is a significant reduction in cell death it is not completely abolished, suggesting CathB is not necessary for all cell death. It may be useful to explore if there is a requirement for CathB in resistance/ HR in other *R* gene interactions as experiments carried out in *N. benthamiana* plants silencing *CathB* showed that the HR was blocked in some instances but not in others, although the system used did not allow monitoring of changes in *R–Avr* mediated susceptibility (Gilroy et al., 2007).

More interesting than the lack of requirement for CathB for resistance in an incompatible interaction, despite its effect on the HR, is the finding that CathB is a prerequisite for full basal resistance to compatible pathogen *Pst*. The requirement for

cathepsin B was only discovered after examining the growth of virulent *Pst* in the triple mutants, as no sign of increased susceptibility was observed in single or double knockout mutants. This suggests that each of the three *CathB* genes in *Arabidopsis* can act redundantly during this interaction and the presence of one is sufficient to compensate for the loss of the others and to prevent an increase in *Pst* growth. As HR cell death is not a feature of resistance to virulent *Pst,* it is likely that CathB may have an additional role in defence, independent of its ability to regulate PCD. Intriguingly, *lsd1* mutants which are involved in PCD regulation also have basal defence phenotypes independent of the ability to influence cell death. While SA and NPR1 are required for runaway cell death (RCD) in *lsd1* mutants, LSD1 acts independently of SA and NPR1 to negatively regulate basal defence (Aviv et al., 2002). In order to investigate how CathB is conferring basal resistance the expression of defence marker genes for the various known pathways could be examined in *cathb* triple mutants challenged with *Pst* and compared to wildtype. The SA levels in response to *Pst* infection could also be measured to determine if CathB acts upstream of SA accumulation. EDS1 which is required for the induction of *CathB* expression in response to *Bgt* is also involved in basal resistance to virulent oomycetes, fungi and bacteria as well as its role in non-host resistance (Wiermer et al., 2005). It may be informative to compare the levels of susceptibility to *Pst* in *eds1* and *cathb* triple mutants and to check if the induction of *CathB* expression in response to *Pst* is also EDS1-dependent. If this is the case then CathB may be a regular downstream component of EDS1 signalling.

### 9.3. Cathepsin B is an extracellular enzyme

Cathepsin B is demonstrated here and consequently published in Gilroy et al. (2007) to encode an apoplastically localised enzyme which is activated upon secretion even in the absence of pathogen challenge. This immediately raises questions about how it can conceivably regulate intracellular PCD and defence signalling. Several possible

mechanisms of cathepsin B action have already been proposed and considered in some detail in the discussion in Chapter 7 although several points are also worth brief examination. The fact that NbCathB was found to be secreted in a constitutively active form in the absence of the pathogen suggests several possibilities: 1) that this protein may have a housekeeping function independent of its defence and PCD-inducible phenotypes; 2) therefore, that its defence and PCD-inducible functions are dependent on the induction of additional proteins (including substrates that are targets of cathepsin B); and 3) that it is kept under tight regulation in plants and its induction following PCD stimuli reflects an amplification of its function in association with PCD. Some evidence that CathB is under tight regulation in plants is seen in the plants over-expresssing *CathB* which behave as wildtype and do not seem to show either enhanced resistance or PCD phenotypes in any of the experiments so far tested. As well as producing proteases plants also express many different classes of protease inhibitors. Cystatins are endogenous papain class cysteine protease inhibitors and should thus be able to inhibit cathepsin B. *Cystatin 1* (*cys1*) over-expression in *Arabidopsis* is sufficient to block pathogen-induced cell death (Belenghi et al., 2003) similar to the phenotype observed in *cathb* knockout mutants, so may represent a possible regulatory control mechanism for CathB activity. The ability of plant cystatins to inhibit CathB could be tested using purified cystatins and the apoplastic CathB over-expression assay systems used to test the commercial and pathogen-derived CathB inhibitors.

Protease localisation may be an important regulatory element as the re-localisation of several proteases has been noted in response to certain stimuli. Saspases are constitutively active like CathB but are re-localised from inside the cell to the apoplast upon victorin treatment which induces PCD (Coffeen and Wolpert, 2004). In seedlings the cysteine proteases RD21 and VPE are localised to organelles called ER bodies which fuse with the vacuole releasing their contents upon treatments which induce PCD (Hayashi et al., 2001). Although CathB was localised to the apoplast in this study it is worth remembering that the fluorescent mRFP tag was cleaved on secretion so any re-

localisation of CathB in response to pathogen challenge could not be observed. Due to fusion tag cleavage the availability of a sensitive antibody for plant cathepsin B would be required to enable any potential re-localisation to be examined in future experiments.

## 9.4. Conclusions

In the course of this work evidence has been produced which supports the role of cathepsin B-like protease in plant PCD and disease resistance. New developments include the demonstration of a possible regulatory role for these proteases in developmental PCD and that the three genes in *Arabidopsis* are able to maintain basal defences in a functionally redundant manner. There is also some evidence that CathB may mediate defence and cell death signalling downstream of EDS1, an important regulatory node in plant disease resistance. The following provides a summary of the main conclusions reached during this project:

- The three *cathepsin B* genes in *Arabidopsis* act redundantly to confer basal resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*).
- *R* gene-mediated resistance is not compromised to *Pst* carrying *AvrB* in *cathb* triple mutants. However, there is a reduction in the amount of HR cell death in these mutants compared to wildtype plants treated with this pathogen.
- *Cathepsin B* genes are also required for full non-host resistance to fungal pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*) as *cathb* mutants show increased post-invasive fungal development.
- *Cathepsin B* transcription is downstream of *EDS1* and *cathb* mutants show a reduction in epidermal cell death and an increase in *PR1* and *GST1* expression to *Bgt* challenge.

- *Cathepsin B* genes are also involved in senescence, a developmental PCD process, as *cathb* triple mutants show a modest but significant delay in senescence and the expression of senescence marker gene *SAG12* is compromised in these mutants.
- NbCathB is localised to the plant apoplast where it is active on secretion, in the absence of pathogen challenge.
- Plant cathepsin B enzymes may have similar characteristics to their mammalian homologues as semi-purified NbCathB is able to cleave a CathB substrate and is inhibited by a range of commercial cathepsin B inhibitors designed for the specificity of the mammalian enzyme.
- NbCathB is not inhibited by cysteine protease inhibitor Avr2 from *Cladosporium fulvum* and insufficient evidence was produced for its inhibition by EPIC cysteine protease inhibitors from *Phytophthora infestans*.

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Appendix I

Additional primer sequences

Appendix I. Additional Primer Sequences

| Gene | Primer name | Sequences | Enzyme | Product | Function |
|---|---|---|---|---|---|
| At1g02300 | At1g02300F | GGCCATGGCTGATAGTTGTTG | *Nco I* | 1148 bp | *CTB1* cDNA cloning |
| At1g02300 | At1g02300R | GACTCGAGACTGAGGAAACCAGA | *Xho I* | | *CTB1* cDNA cloning |
| At1g02305 | At1g02305F | AAAGATCCATGGCTGATAATGTAT | *Nco I* | 1094 bp | *CTB2* cDNA cloning |
| At1g02305 | At1g02305R | TAACTCGAGGAAACAAGAAGA | *Xho I* | | *CTB2* cDNA cloning |
| At4g01610 | At4g01610F | ACTCTGTTTCCATGGCTGTTT | *Nco I* | 1140 bp | *CTB3* cDNA cloning |
| At4g01610 | At4g01610R | TAACTCGAGGCAACCGGAA | *Xho I* | | *CTB3* cDNA cloning |
| At1g02305 | FLAG-CTB2F | AACTCGAGGAAAATCTTTCCAAG | *Xho I* | 999bp | FLAG vector cloning (- signal peptide) |
| At1g02305 | FLAG-CTB2R | CCAGATCTGAGGAAACAAGAAGA | *Bgl II* | | FLAG vector cloning (- signal peptide) |
| At4g01610 | FLAG-CTB3F | TACTCGAGGAAAGTCTTACCAAACAG | *Xho I* | 997bp | FLAG vector cloning (- signal peptide) |
| At4g01610 | FLAG-CTB3R | AAAGATCTTGCAACCGGAAGAT | *Bgl II* | | FLAG vector cloning (- signal peptide) |
| NbCathB | FLAG-NBCTBF | TACTCGAGGAACAACCAATATCCC | *Xho I* | 1020bp | FLAG vector cloning (- signal peptide) |
| NbCathB | FLAG-NBCTBR | TTAGATCTCATTCACATTGCGG | *Bgl II* | | FLAG vector cloning (- signal peptide) |
| At1g02305 | CTB2-NcoIF | ATCCATGGAAAATCTTTCCAAGC | *Nco I* | 996bp | pET32 vector cloning (- signal peptide) |
| At1g02305 | CTB2-XhoIR | TAACTCGAGGAAACAAGAAGATCA | *Xho I* | | pET32 vector cloning (- signal peptide) |
| At4g01610 | CTB3-NcoIF | TACCATGGAAAGTCTTACCAAACAG | *Nco I* | 996bp | pET32 vector cloning (- signal peptide) |
| At4g01610 | CTB3-XhoIR | TAACTCGAGGCAACCGGAA | *Xho I* | | pET32 vector cloning (- signal peptide) |
| NbCathB | NBCTB-NcoIF | TGCCATGGAACAACCAATATC | *Nco I* | 1005bp | pET32 vector cloning (- signal peptide) |
| NbCathB | NBCTB-XhoIR | TACTCGAGCATTGCGGCG | *Xho I* | | pET32 vector cloning (- signal peptide) |

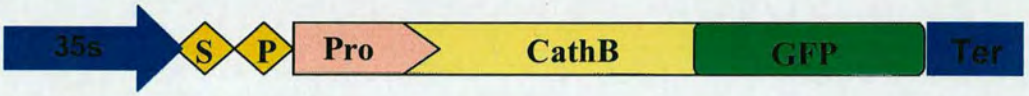
Appendix I cont. Additional Primer Sequences

| Gene | Primer name | Sequences | Enzyme | Product | Function |
|---|---|---|---|---|---|
| At1g02305 | OEctb2F | AAGCGGCCGCTAATCAGAAG | *Not I* | 1166bp | Overexpresser line construction |
| At1g02305 | OEctb2R | ATTGGATCCGGTAACAACAAC | *Bam HI* | | Overexpresser line construction |
| At4g01610 | Oectb3F | AAGCGGCCGCATACAAACA | *Not I* | 1168bp | Overexpresser line construction |
| At4g01610 | OEctb3R | TTGGATCCGATATTAAACCG | *Bam HI* | | Overexpresser line construction |
| At4g01610 | OECF | TAGCGGCCGCCTAAGAAGCAGACTC | *Not I* | 1121bp | Overexpresser line construction |
| At4g01610 | OECR | GCGGATCCAACATTTCCATCTTT | *Bam HI* | | Overexpresser line construction |
| At1g02305 | iasCTB2F | CCACTAGTCTTGTTGCAGGGTATTGCAG | *Spe I* | 926bp | Antisense line construction (inducible) |
| At1g02305 | iasCTB2R | ATCTCGAGCATGTTCAATGCCACATTC | *Xho I* | | Antisense line construction (inducible) |
| At1g02305 | casCTB2F | CCGAATTCTTGTTGCAGGGTATTGCAG | *Eco RI* | 926bp | Antisense line construction (35s) |
| At1g02305 | casCTB2R | ATGCGGCCGCATGTTCAATGCCACATTC | *Not I* | | Antisense line construction (35s) |
| GUS | GUS-S | CCGACGAAAACGGCAAGAAAAAGCTGT | | ~1000bp | RNAi line construction |
| GUS | GUS-A | CCAGAAGTTCTTTTTCCAGTACCT | | | RNAi line construction |
| | T1 | TGATAGTGATAGTGATAGTGA | | ~1.5kb | RNAi line construction |
| | T2 | AGCGTTAGCGTTAGCGTTAGC | | | RNAi line construction |
| At1g02305 | Specific S | CATTGGAGGTCATGCTGTTAAA | | 228bp | RNAi line construction |
| At1g02305 | Specific A | GAGGAAACAAGAAGATCATCTGAA | | | RNAi line construction |
| | ctb2-xhoIA | TGATAGTGATAGTGATAGTGActcgagGAGGAAACAAGAAGATCATCTGAA | *Xho I* | | RNAi line construction (inducible) |
| | ctb2-speIA | AGCGTTAGCGTTAGCGTTAGCactagtGAGGAAACAAGAAGATCATCTGAA | *Spe I* | | RNAi line construction (inducible) |
| | Gus1-ctb2S | tttcttgccgttttcgtcggCATTGGAGGTCATGCTGTTAAA | | | RNAi line construction |
| | Gus2-ctb2S | actggaaaaagaacttctggCATTGGAGGTCATGCTGTTAAA | | | RNAi line construction |
| | ctb2-notIA | TGATAGTGATAGTGATAGTGAgcggccgcGAGGAAACAAGAAGATCATCTGAA | *Not I* | | RNAi line construction (35s) |
| | ctb2-bamhIA | AGCGTTAGCGTTAGCGTTAGCggatccGAGGAAACAAGAAGATCATCTGAA | *Bam HI* | | RNAi line construction (35s) |

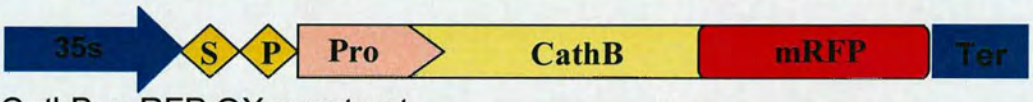
**Appendix II**

Schematic diagrams of localisation and protein expression constructs.


Sec::mRFP construct

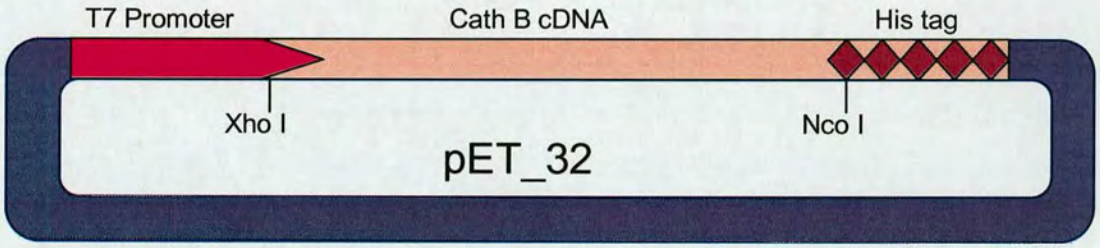



CathB::GFP construct

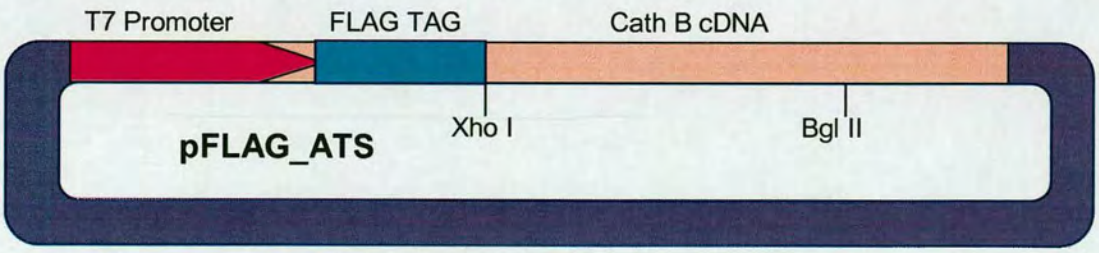



CathB::mRFP OX construct

SP is signal peptide, Pro is propeptide, Ter is NOS terminator, 35s is the 35s promoter.




His tag fusion construct




FLAG tag fusion construct

Appendix III

Gilroy et al., 2007

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# Involvement of cathepsin B in the plant disease resistance hypersensitive response

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**Summary**

**A diverse range of plant proteases are implicated in pathogen perception and in subsequent signalling and execution of disease resistance. We demonstrate, using protease inhibitors and virus-induced gene silencing (VIGS), that the plant papain cysteine protease cathepsin B is required for the disease resistance hypersensitive response (HR). VIGS of cathepsin B prevented programmed cell death (PCD) and compromised disease resistance induced by two distinct non-host bacterial pathogens. It also suppressed the HR triggered by transient co-expression of potato *R3a* and *Phytophthora infestans Avr3a* genes. However, VIGS of cathepsin B did not compromise HR following recognition of *Cladosporium fulvum* AVR4 by tomato Cf-4, indicating that plant PCD can be independent of cathepsin B. The non-host HR to *Erwinia amylovora* was accompanied by a transient increase in cathepsin B transcript level and enzymatic activity and induction of the HR marker gene *Hsr203*. VIGS of cathepsin B significantly reduced the induction of *Hsr203* following *E. amylovora* challenge, further demonstrating a role for this protease in PCD. Whereas cathepsin B is often relocalized from the lysosome to the cytosol during animal PCD, plant cathepsin B is secreted into the apoplast, and is activated upon secretion in the absence of pathogen challenge.**

**Keywords: papain, apoptosis, protease, non-host, gene-for-gene.**

## Introduction

Plants have pre-formed barriers and inducible innate immune systems that prevent infection by most pathogenic micro-organisms (Nürnberger and Lipka, 2005; Hye-Sook and Collmer, 2005). A component of disease resistance that is often induced is the hypersensitive response (HR), a form of localized programmed cell death (PCD). The HR may be triggered in non-host resistance, i.e. in plants that are challenged by micro-organisms that are pathogens of other plant species (Alfano and Collmer, 2004). In addition, pathogen recognition through interactions of plant resistance (*R*) gene products and corresponding pathogen avirulence (*Avr*) gene products may induce HR in what is known as a gene-for-gene interaction (Greenberg and Yao, 2004). Absence of corresponding alleles of the *R* gene in the host or the *Avr* gene in the pathogen leads to a compatible (susceptible) interaction.

Although little is understood about the regulatory and mechanistic processes underlying inducible disease resistance, extracellular and intracellular proteases play diverse, fundamental roles during pathogen recognition and induction of defences (Van der Hoorn and Jones, 2004). Indeed, some proteases may be suppressed by pathogen protease inhibitors during infection. Examples include an apoplastic papain-like protease from *Lycopersicon esculentum*, RCR3,

which is a putative virulence target of the protease inhibitor, AVR2, from the fungal pathogen *Cladosporum fulvum* (Rooney *et al.*, 2005). This molecular interaction is perceived by tomato R protein, Cf-2, leading to a HR. A subtilisin-like protease and pathogenesis related (PR) protein, P69B, is involved in proteolytic defence responses in the plant extracellular matrix (Tornero *et al.*, 1996, 1997). P69B is targeted directly by a Kazal-like extracellular serine protease inhibitor from the *Solanum tuberosum* late blight pathogen *Phytophthora infestans* (Tian *et al.*, 2004). Moreover, recently it has been demonstrated that a cystatin from *P. infestans*, EPIC2, interacts with and inhibits a novel papain-like cysteine protease, PIP1, which is a PR protein closely related to RCR3 (Tian *et al.*, 2007).

In animals, a form of PCD called apoptosis involves cysteine proteases called caspases that cleave a limited set of cellular protein substrates (Thornberry and Lazebnik, 1998). Caspase knockouts or caspase inhibitors counteract apoptosis in animals. Since the plant HR and apoptosis share many physiological and morphological features, it is reasonable to predict that protease components of PCD may be conserved between the kingdoms. Thus, caspase inhibitors and substrates from animal research were used to demonstrate that caspase-like activities are induced during HR and that caspase inhibitors block the HR (Del Pozo and Lam, 1998; Chichkova *et al.*, 2004; Woltering, 2004). However, plants lack homologues of caspase genes, and recent reports indicate that plant proteases with caspase-like activities belong to different families. Vacuolar processing enzyme (VPE) exhibiting caspase-1-like activity during Tobacco mosaic virus-mediated HR in tobacco (Hatsugai *et al.*, 2004) is a member of the C13 family, structurally related to caspases (C14), and subtilisin-like serine proteases (family S8) exhibiting caspase specificity (saspases) are activated during PCD induced by a plant pathogen-derived toxin, victorin (Coffeen and Wolpert, 2004). Nevertheless, protease inhibitors such as E64, AEBSF and TLCK, that do not inhibit plant caspase activities, can still block plant cell death, suggesting that additional proteases, such as papain-like proteases, are effectors or regulators of plant PCD (D'Silva *et al.*, 1998; Coffeen and Wolpert, 2004; Woltering, 2004).

Additional proteases shown to play a role in plant disease resistance include the extracellular aspartate protease, CDR1 (Xia *et al.*, 2004). Antisense *CDR1* plants were compromised in resistance to avirulent *Pseudomonas syringae*, whereas over-expression led to increased resistance to virulent *P. syringae*. CDR1 is structurally related to cathepsin D (Xia *et al.*, 2004). We have demonstrated that a potato cathepsin B-encoding gene, *StCathB*, is rapidly upregulated specifically by resistance (*R*) gene-mediated HRs elicited by *P. infestans* (Avrova *et al.*, 2004). Cathepsin B is structurally unrelated to cathepsin D, being a member of the papain family of cysteine proteases. Cathepsin B has been implicated in many diverse roles in animals, including PCD (Zeiss, 2003). In animals, cathepsin B can activate caspases (Kingham and Pocock, 2001; Vancompernolle *et al.*, 1998), and cathepsin B knockout mice fail to exhibit apoptosis (Guicciardi *et al.*, 2001; Zeiss, 2003). Nevertheless, cathepsin B can cause PCD independently of caspases (Foghsgaard *et al.*, 2001). It was thus reasonable to regard cathepsin B as a candidate cysteine protease involved in plant disease resistance.

We showed, using cathepsin B inhibitors and virus-induced gene silencing (VIGS), that cathepsin B plays a role in both host and non-host plant disease resistance. We determined that cathepsin B transcription and enzymatic activity are induced during the HR, and showed that suppression of the HR by silencing of cathepsin B significantly reduces the induction of the HR marker gene *HSR203*. Finally, we demonstrated, by using activity profiling (Van der Hoorn *et al.*, 2004) and C-terminal monomeric red fluorescent protein (mRFP) fusion, that cathepsin B is activated upon secretion into the plant apoplast, in the absence of pathogen challenge.

## Results

### *Cathepsin B inhibitors suppress non-host disease resistance*

*Nicotiana* species are non-hosts for the apple pathogen *Erwinia amylovora* (*Eam*), which elicits a rapid HR. To investigate the potential involvement of cathepsin B in *Eam*-mediated non-host disease resistance, we used inhibitors described in the animal literature as cathepsin B specific, z-FA-fmk, Ac-LVK-cho, CA-074-Me and the broad cathepsin B, S, L and papain inhibitor Z-FGNHO-Bz. Each inhibitor was infiltrated with $10^6$ colony forming units (cfu) $ml^{-1}$ of *Eam* into *Nicotiana benthamiana* leaves. In the absence of inhibitor, *Eam* elicited a visible HR by 24–48 h post-infiltration (hpi). In contrast, the HR was abolished or considerably reduced and delayed after infiltration of *Eam* with cathepsin B or general papain inhibitors (Figure 1a).

Two of the cathepsin B inhibitors (Ac-LVK-cho and Ca-074-Me) were selected to investigate whether they compromised disease resistance to *Eam* by measuring accumulation of viable *Eam* cells at 4 days post-infiltration (dpi). Each inhibitor resulted in approximately 10-fold increased recovery of viable bacterial cells (Figure 1b). When *Eam* was grown in King's broth in the presence or absence of cathepsin B inhibitors no significant differences in growth curves were observed (results not shown), excluding the possibility that the presence of the inhibitors had an effect on bacterial growth *in vitro*. Thus, cathepsin B inhibitors considerably reduced non-host disease resistance in *N. benthamiana* to *Eam* and resulted in a visible reduction in HR-like cell death.

To investigate the specificity of the cathepsin B inhibitors, we performed protease activity profiling. We used DCG-04, a biotinylated derivative of E-64, which inhibits papain-like proteases in an activity-dependent manner (Van der Hoorn

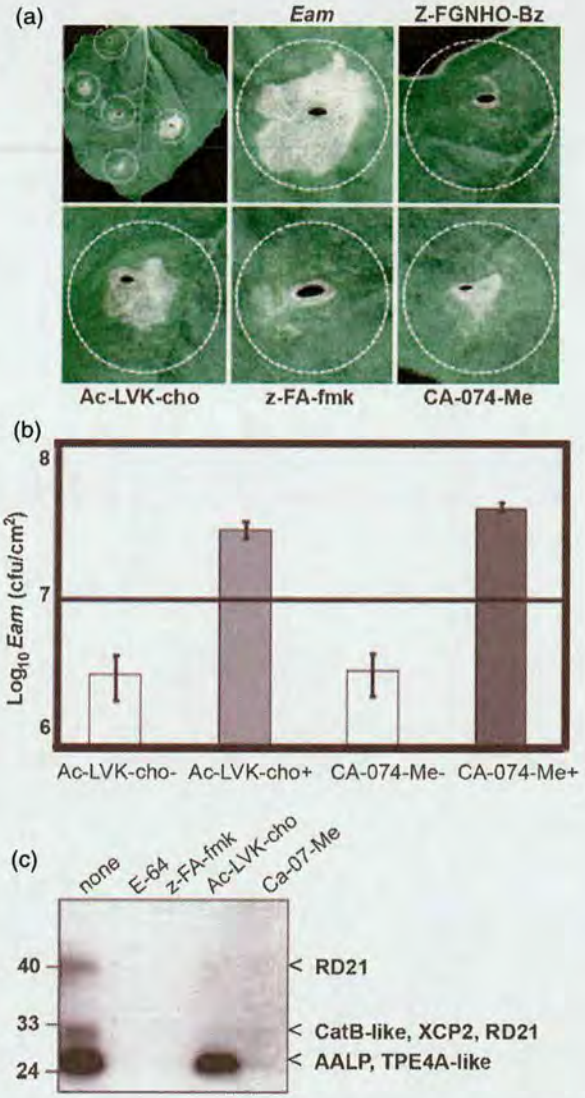



**Figure 1.** Cathepsin B inhibitors suppress disease resistance to *Erwinia amylovora* (*Eam*).
(a) The hypersensitive response induced by infiltration of $10^6$ colony-forming units (cfu) $ml^{-1}$ *Eam* was compromised by infiltration of *Eam* with 1 mM of the inhibitors Z-FGNHO-Bz, z-FA-fmk, Ac-LVK-cho and CA-074-Me. Zones of infiltration are indicated by dotted circles.
(b) Increases in viable *Eam* colonies recovered from leaves 4 days post-infiltration of *Eam* with 1 mM z-FA-fmk, Ac-LVK-cho or CA-074-Me, were observed relative to recovery from leaves infiltrated only with *Eam*. Results are each the mean ± SEM of three independent experiments each involving six replicate plants.
(c) Specificity of cathepsin B inhibitors was investigated by proteases activity profiling of Arabidopsis leaf extract with the biotinylated DCG-04 in the presence or absence of 0.4 mM inhibitors E-64, z-FA-fmk, Ac-LVK-cho and Ca-074-Me.

*et al.*, 2004). Incubation of this probe with Arabidopsis leaf extracts results in three major signals on a blot probed with streptavidin-horseradish peroxidase (HRP) (Figure 1c, first lane). These signals are known to represent six different papain-like cysteine proteases, including cathepsin B in the 30 kDa region (indicated on the right in Figure 1c) (Van der Hoorn *et al.*, 2004). We used this assay to investigate the specificity of cathepsin B inhibitors. The presence of E-64, z-FA-fmk and Ca-074-Me prohibited labelling of all six proteases, whereas inhibitor Ac-LVK-cho only prevented labelling of RD21, cathepsin B, and XCP2 (Figure 1c, lanes 2–5). This indicates that whilst inhibitors z-FA-fmk, Ac-LVK-cho and Ca-074-Me block cathepsin B activity, they also inhibit other papain-like cysteine proteases. Since the role of cathepsin B in non-host resistance was inconclusive from these inhibitor studies, we embarked on VIGS to interfere specifically with cathepsin B function.

### *Virus induced gene silencing of cathepsin B*

*NbCathB*, encoding cathepsin B, was cloned from *N. benthamiana*. The putative protein encoded by *NbCathB* was aligned with cathepsin B proteins from *Nicotiana rustica*, *Solanum tuberosum*, *Arabidopsis thaliana* and *Homo sapiens* and revealed that the expected peptidase C1 and propeptidase C1 cleavage domains were conserved (Supplementary Figure S1a). Publicly available expressed sequence tag (EST) databases contained, respectively, four and two independent contigs annotated as full-length cathepsin B in the closely related solanaceous species, potato (TC137447, TC145097, TC152070 and TC142279) and tomato (TC175119, TC182010). Indeed, there are three independent genes annotated as cathepsin B in *A. thaliana* [NM_100110 (*At1 g02300*); NM_100111 (*At1 g02305*); NM_116392 (*At4 g01610*)]. In each case, these genes are highly related (Supplementary Figure S1b), implying the involvement of gene families in the production of plant cathepsin B. The cathepsin B EST sequences from potato and tomato are highly similar (80–98% nucleotide (nt) identity), precluding the design of VIGS constructs that discriminate the sequences. Constructs were thus designed that were likely to silence the entire cathepsin B gene family in *N. benthamiana* whilst avoiding 'off-target' silencing of additional genes. Using the siRNA scan website (http://bioinfo2.noble.org/RNAiScan.htm; Xu *et al.*, 2006), two independent portions, of 364 and 247 bp (Supplementary Figure S1a), of *NbCathB* were screened against EST datasets from *N. benthamiana*, and from tomato, potato and tobacco to seek 22 nt stretches of homology with other genes and thus the potential for 'off-target' silencing. For both selected portions, hits were made only to sequences annotated as cathepsin B in each dataset [EST contig TC9934 (DQ492297) in *N. benthamiana*; NP917849 (AF359422) in tobacco; and all the EST contigs in potato and tomato]. It was thus unlikely that the sequences would silence genes other than cathepsin B. To further assess the potential for off-target silencing from a fully sequenced plant genome, equivalent

protein-coding portions (Supplementary Figure S1a) of each of the three *A. thaliana* genes annotated as cathepsin B were screened against all *A. thaliana* coding sequences. In each case, the query sequence showed matches only to itself and the other two cathepsin B family members, further confirming the unlikelihood of silencing genes other than cathepsin B, and indicating that the portions would silence all cathepsin B homologues in *A. thaliana*. The 364-bp and 247-bp portions of *NbCathB* were cloned in antisense orientation into a Tobacco rattle virus (TRV) vector for VIGS (construct TRV::*NbCathB-1* and TRV:*NbCathB-2* respectively) with the intention of, in each case, silencing all *NbCathB* homologues within *N. benthamiana*.

To assess the effect of silencing *NbCathB* genes on the HR, comparisons were made with plants inoculated with TRV either harbouring, as a negative control, *gfp* (TRV::*gfp*) or, as a positive control, a portion cloned in antisense of the *N. benthamiana sgt1b* cDNA (TRV::*Nbsgt1*), encoding a ubiquitin ligase-associated protein shown previously to be involved in both host and non-host plant disease resistance (Peart *et al.*, 2002).

*Nicotiana benthamiana* plants inoculated with TRV::*NbCathB-1*, TRV::*NbCathB-2* or TRV::*gfp* showed no discernible altered phenotype. In contrast, as observed previously (Peart *et al.*, 2002), *Nbsgt1*-silenced plants were shorter and more branched. Real-time RT-PCR revealed an approximately 90% reduction in transcript level of *NbCathB* in plants 21 days after inoculation (dai) with TRV::*NbCathB-1* or TRV::*NbCathB-2*, compared with levels 21 dai with TRV::*gfp* (Supplementary Figure S2a). Western blot analysis confirmed that *Nbsgt1* was silenced, as SGT1 protein was significantly less abundant in leaves from plants 21 dai with TRV::*sgt1b* than in plants 21 dai with TRV::*gfp* (Supplementary Figure S2b).

### *VIGS of cathepsin B confirms its role in non-host disease resistance*

*Nicotiana benthamiana* plants inoculated with the TRV constructs were infiltrated with either $10^6$ or $10^7$ cfu $ml^{-1}$ of *Eam*. At 24 to 48 hpi a clear HR-like cell death was visible on plants harbouring TRV::*gfp* (Figure 2a), as witnessed for non-TRV-inoculated plants (Figure 1a). However, in plants inoculated with TRV::*NbCathB-1* the *Eam*-mediated HR was abolished at the lower concentration ($10^6$ cfu $ml^{-1}$), and weak or delayed until 72 hpi at the higher concentration ($10^7$ cfu $ml^{-1}$) of bacteria (Figure 2a). Suppression of *Eam*-mediated HR by silencing *NbCathB* was similar to that observed after silencing *Nbsgt1* (results not shown).

Accumulation of viable *Eam* was measured 4 dpi. In leaves inoculated with TRV::*NbCathB-1* or TRV::*Nbsgt1*, approximately eight-fold more viable *Eam* cells were recovered in each case than in plants inoculated with TRV::*gfp* (Figure 2b). This demonstrates that silencing of *NbCathB* compromises non-host disease resistance to a level similar to that caused by silencing *Nbsgt1* (Figure 2b). Silencing *NbCathB* with the TRV::*NbCathB-2* construct resulted in a similar suppression of HR and increase in viable *Eam* cells (Supplementary Figure S3).Given the similar results with both *NbCathB* silencing constructs, further experiments were conducted using only the TRV::*NbCathB-1* construct.

*Pseudomonas syringae* pv. tomato (*Pst*) DC3000, when infiltrated into *N. benthamiana* at high concentrations ($10^6$ cfu $ml^{-1}$) elicits a non-host HR (Hye-Sook and Collmer, 2005). Whilst this HR was clearly visible in plants inoculated with TRV::*gfp* within 48 hpi (Figure 2c), it was suppressed in plants inoculated with either TRV::*NbCathB-1* (Figure 2c) or TRV::*Nbsgt1* (results not shown). Again, this resulted in significant (approximately five-fold in each case) increase in viable cells from TRV::*NbCathB-1* or TRV::*Nbsgt1* plants, demonstrating that disease resistance was compromised by silencing these genes (Figure 2d).

Trypan blue staining was used to visualize host cell death over time during the *Eam*-mediated HR. On TRV::*gfp* expressing plants, *Eam*-mediated cell death was clearly visible by 18 hpi (Figure 2e). However, on plants harbouring TRV::*NbCathB-1*, *Eam*-mediated cell death was no higher than background cell death in plants infiltrated with buffer alone, even at 24 hpi (Figure 2e). Significantly, these results demonstrate that non-host HR is suppressed by silencing *NbCathB*.

### *VIGS and inhibitors suppress an early* E. amylovora*-mediated increase in cathepsin B activity*

Real time RT-PCR was used to quantify *NbCathB* expression prior to and after infiltration of *Eam*, and to investigate expression of this gene following VIGS. In non-TRV-inoculated *N. benthamiana*, infiltration of *Eam* led to a modest but significant increase in expression of *NbCathB* at 6 hpi (Figure 3a). Similarly, upregulation of *NbCathB* was observed at 6 hpi with *Eam* in TRV::*gfp* control plants. These results agree with the previous observation (Avrova *et al.*, 2004) that cathepsin B is induced during the HR. In plants inoculated with TRV::*NbCathB-1* the level of expression of *NbCathB* was 10-fold lower than in TRV::*gfp* plants throughout the time course after *Eam* infiltration (Figure 3a).

A colorimetric substrate specific for mammalian cathepsin B was used to assay activity of cathepsin B during the HR. In plants inoculated with TRV::*gfp* an increase in cathepsin B activity was detected at 6 hpi (Figure 3b), mirroring the induction of gene expression witnessed using real-time RT-PCR (Figure 3a). In contrast, no such increase in activity was observed at 12 or 18 hpi (results not shown). A significant decrease in activity was observed in plants inoculated with TRV::*NbCathB-1*, and this activity showed only a negligible increase at 6 hpi with *Eam* (Figure 3b). The modest reduction in activity observed in TRV::*NbCathB-1*-inoculated plants

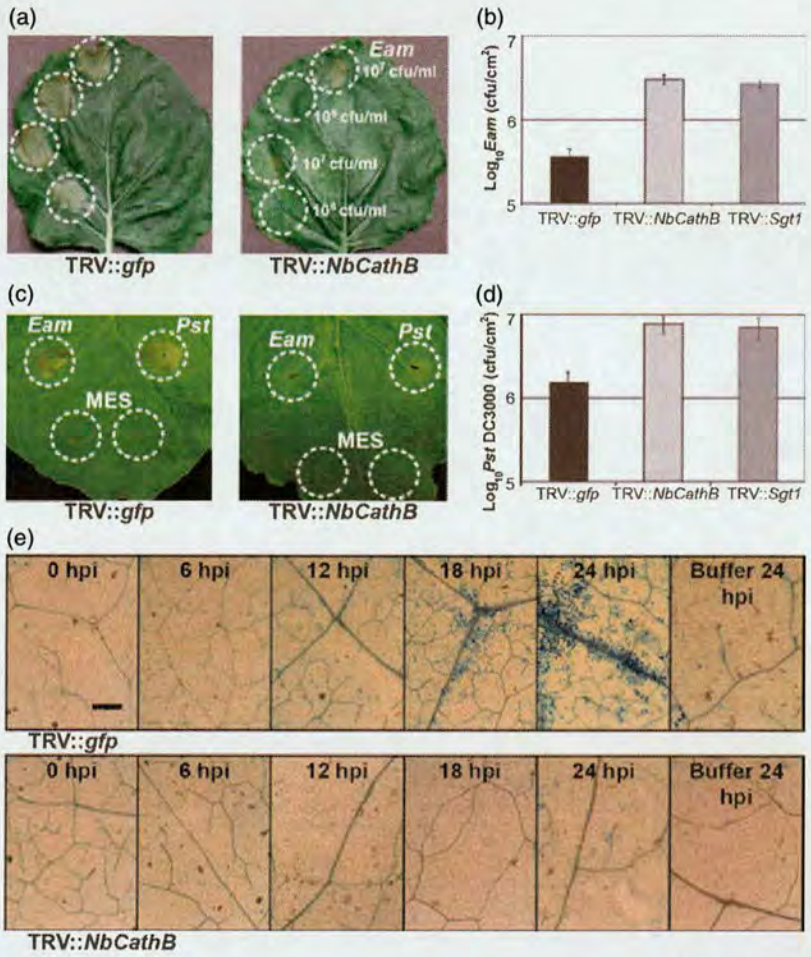



**Figure 2.** Cathepsin B plays a role in plant non-host disease resistance.

(a) The hypersensitive response (HR) caused by infiltration of $10^6$ or $10^7$ colony-forming units (cfu) $ml^{-1}$ of *Erwinia amylovora* (*Eam*) onto plants infected with TRV::*gfp* was suppressed on plants infected with TRV::*NbCathB-1*.

(b) Colony counts (cfu $cm^{-2}$) of viable *Eam* at 4 days post-inoculation in TRV::*gfp*, TRV::*NbCathB-1* or TRV::*Nbsgt1* plants.

(c) The HR caused by infiltration of $10^6$ cfu $ml^{-1}$ of *Pseudomonas syringae* pv. tomato (*Pst*) onto plants infected with TRV::*gfp* is suppressed on plants infected with TRV::*NbCathB-1*. No HR was caused by infiltration of 2-(*N*-morpholine)-ethanesulphonic acid, the salt in which the bacteria are suspended.

(d) Colony counts (cfu $cm^{-2}$) of viable *Pst* at 4 days post-inoculation in TRV::*gfp*, TRV::*NbCathB-1* or TRV::*Nbsgt1* plants. In all cases, experiments involved six replicate plants and were repeated three times with similar results. Colony counts in (b) and (d) are the mean ± SEM of three replicate experiments. Zones of infiltration are indicated by dotted circles.

(e) Detail of leaves stained with trypan blue across a time course [0–24 h post-inoculation (hpi)] after infiltration of $10^6$ cfu $ml^{-1}$ *Eam* in plants harbouring TRV::*gfp* (upper panels) or TRV::*NbCathB-1* (lower panels). Bar = 500 μm (for all images).

may be due to this assay detecting other plant protease activities in addition to cathepsin B, and further work will be required to assess the potential range of such specificity. The *Eam*-dependent increase in protease activity at 6 hpi was also observed in non-TRV-inoculated plants and this was suppressed by the inhibitors z-FA-fmk and Ac-LVK-cho (Figure 3c), which were shown to inhibit cathepsin B, amongst other papain activities, in Figure 1(c). These results demonstrate that a rapid, transient *Eam*-mediated increase in cathepsin B transcription is accompanied by an increase in protease activity at 6 hpi in *N. benthamiana*, prior to HR symptoms visualized by trypan blue staining (Figure 2e). Both transcription and activity are considerably reduced in *NbCathB*-silenced plants in which such symptoms are suppressed.

### *Induction of the HR marker gene Hsr203 is suppressed by cathepsin B silencing*

The gene *HSR203* (Pontier *et al.*, 1999) is regarded as a marker of the HR induced by a range of stimuli, including avirulent bacteria. We investigated expression of this gene after infiltration of *Eam* onto plants infected with TRV::*gfp*, TRV::*NbCathB-1* or TRV::*NbCathB-2*. *HSR203* was upregulated moderately in all cases at 12 hpi with *Eam*. By 24 hpi, this gene was upregulated approximately 20-fold in plants inoculated with TRV::*gfp*. However, regardless of the construct used for silencing *NbCathB* expression, *HSR203* expression was suppressed relative to that in TRV::*gfp* plants by approximately 70% in three cases and by approximately 30% in the fourth case (Figure 4). These results are in line with an observed reduction in *Eam*-induced cell death shown in Figure 2(e).

### *Cathepsin B is involved in a HR triggered by the R3a-Avr3a gene-for-gene interaction*

We used VIGS to investigate the role of cathepsin B in the HRs triggered by two gene-for-gene interactions. Recently, we showed that *P. infestans* AVR3a is detected by potato R3a in the host cytoplasm following *Agrobacterium tumefaciens*-mediated transient co-expression of *Avr3a* and *R3a* in

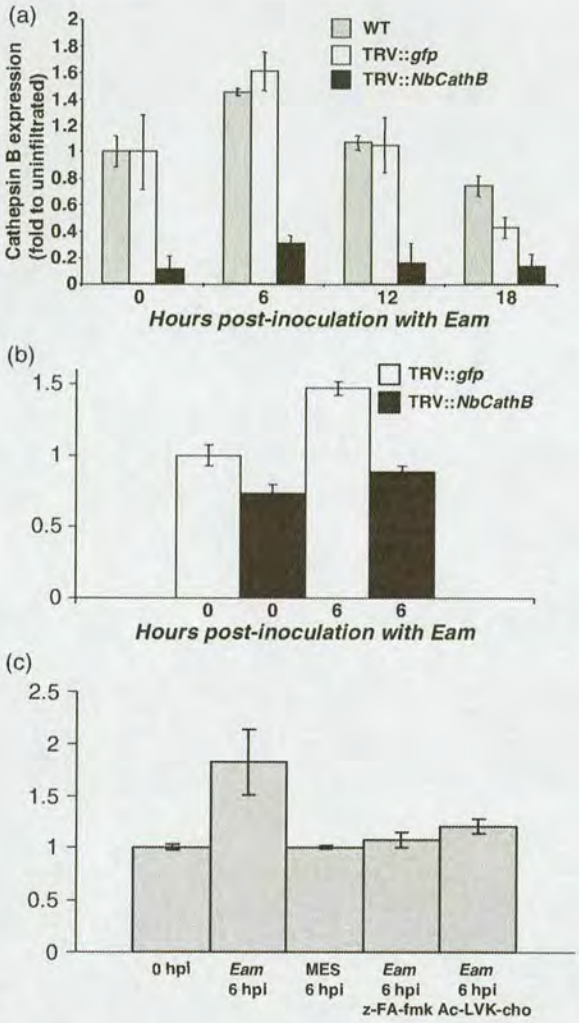



**Figure 3.** Cathepsin B transcription and activity are upregulated by *Erwinia amylovora* (*Eam*) at 6 h post-inoculation (hpi).
(a) Relative expression of *NbCathB* in uninoculated (WT) and inoculated (TRV::*gfp* or TRV::*NbCathB-1*) *Nicotiana benthamiana* leaves that were untreated (0), or at 6, 12 and 18 hpi with *Eam*. Expression in uninoculated or TRV::*gfp* plants following treatment with *Eam* was compared with the equivalent untreated plants, which was assigned a value of 1.0. Expression in TRV::*NbCathB-1* inoculated plants was relative to expression in untreated TRV::*gfp* plants.
(b) Cathepsin B activity in *N. benthamiana* leaves inoculated with TRV::*gfp* or TRV::*NbCathB-1* that were untreated (0), or at 6 hpi with *Eam*. Activity in TRV::*gfp* plants not treated with *Eam* was assigned a value of 1.
(c) Cathepsin B activity in uninfiltrated *N. benthamiana* leaves (0; assigned a value of 1), and in leaves 6 hpi with *Eam*, with only the buffer in which *Eam* was suspended [2-(*N*-morpholine)-ethanesulphonic acid, MES] or with *Eam* containing 1 mM z-FA-fmk or Ac-LVK-cho. Results in (a)–(c) are each the mean ± SEM of three independent experiments each involving six replicate plants.

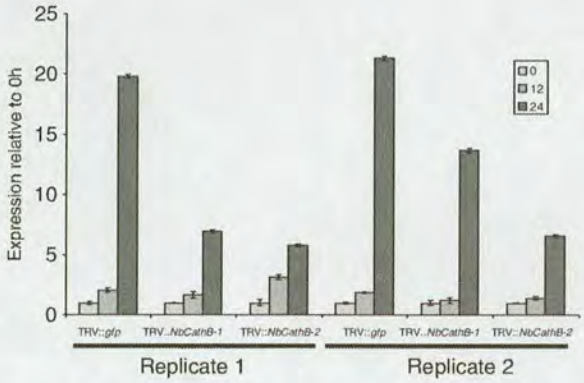



**Figure 4.** Induction of hypersensitive response marker gene *HSR203* is suppressed by virus-induced gene sampling of *NbCathB*.
*Hsr203* gene expression at 0, 12 and 24 hpi with *Erwinia amylovora* (*Eam*) in two replicates, in plants inoculated with TRV::*gfp*, TRV::*NbCathB-1* and TRV::*NbCathB-2*.

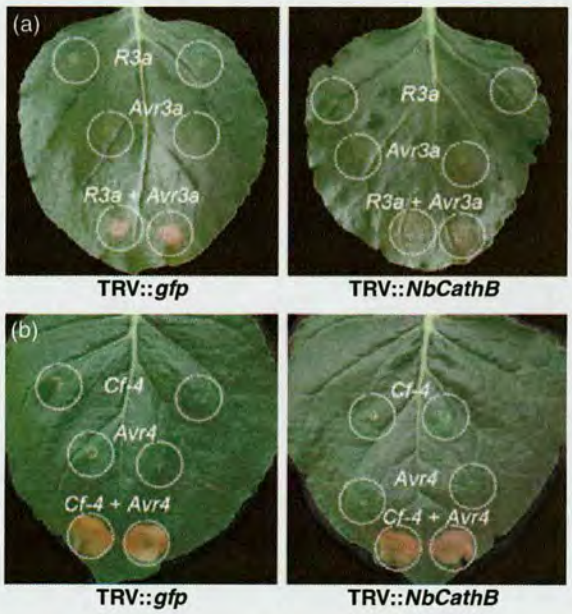



**Figure 5.** Cathepsin B plays a role in gene-for-gene disease resistance.
(a) The hypersensitive response (HR) caused by co-expression of potato *R3a* and *Phytophthora infestans Avr3a* on plants infected with TRV::*gfp* was suppressed on plants infected with TRV::*NbCathB-1*. No HR was elicited on either plant by expression of *R3a* or *Avr3a* alone.
(b) The HR caused by co-expression of tomato *Cf-4* and *Cladosporium fulvum Avr-4* on plants infected with TRV::*gfp* was not suppressed on plants infected with TRV::*NbCathB-1*. Zones of infiltration are indicated by dotted circles.

*N. benthamiana* (Armstrong *et al.*, 2005). The HR triggered by interaction of these proteins in plants harbouring TRV::*gfp* was compromised in plants inoculated with either TRV::*NbCathB-1* (Figure 5a) or TRV::*Nbsgt1* (results not shown; demonstrated recently by Bos *et al.*, 2006). These results show that the HR triggered by the cytoplasmic AVR3a-R3a interaction is dependent on both cathepsin B and SGT1.

We investigated the involvement of cathepsin B in the apoplastic gene-for-gene interaction between the products of *C. fulvum Avr4* and tomato *Cf-4*, following their

co-expression in *N. benthamiana* (Van der Hoorn *et al.*, 2000). In this case, whereas silencing of *Nbsgt1* suppressed the HR (results not shown; demonstrated recently by Gabriels *et al.*, 2006), VIGS of *NbCathB* had no effect on the Avr4-Cf-4-mediated HR (Figure 5b), indicating either that not all PCD triggered during disease resistance is dependent on cathepsin B, or that the reduction in cathepsin B levels following VIGS was insufficient to affect cell death triggered by Avr4-Cf4.

### *Cathepsin B is activated upon secretion into the apoplast*

The programme SignalP predicts that the NbCathB protein possesses a signal peptide for extracellular targeting (Supplementary Figure S1a). Using a signal sequence trap strategy, Hugot *et al.* (2004) identified a number of tobacco proteins, including cathepsin B, which appeared to be secreted at late developmental stages. These stages were also associated with increased resistance to *Phytophthora parasitica*. To obtain more evidence that cathepsin B is secreted and active in the apoplast, full-length *NbCathB* (encoding pre-proenzyme) was fused to *mRFP*, encoding a fluorescent marker that is stable in the apoplast. Expression of the NbCathB::mRFP fusion in *N. benthamiana* leaf epidermal cells resulted in largely apoplastic fluorescence (Figure 6a). This was confirmed by co-expression of the construct with a plasma membrane marker EGFP-LT16b (Kurup *et al.*, 2005), with clear mRFP fluorescence detected outside the cell membrane (Figure 6b). Furthermore, treatment with brefeldin A (BFA), an inhibitor of secretion, resulted in retention of mRFP fluorescence within the cell (Figure 6c). Similar BFA treatment of plants over-expressing mRFP fused to a signal peptide for secretion also resulted in retention of the sec::mRFP within the plant cell (Supplementary Figure S4).

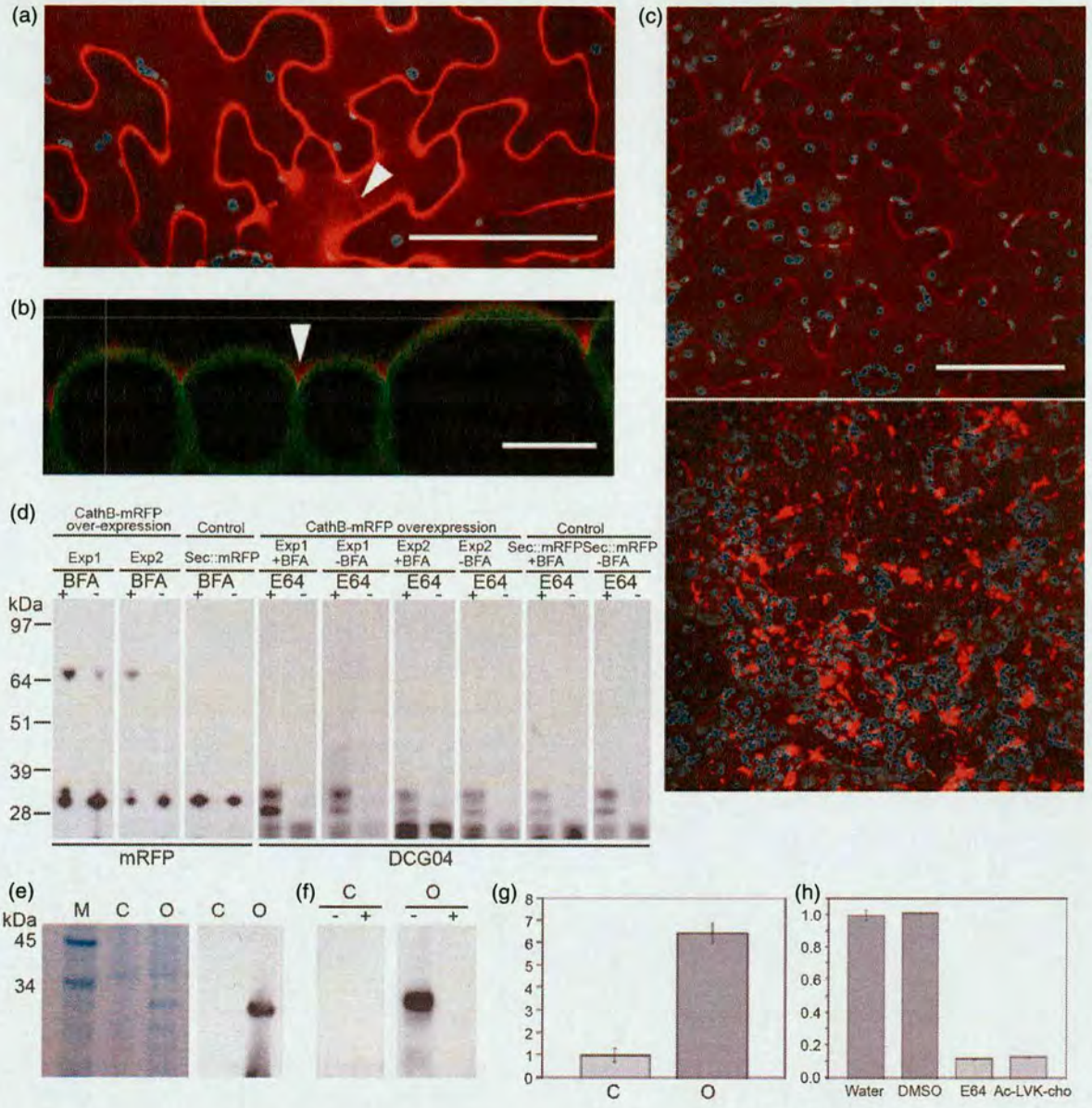
To confirm that the mRFP was fused to cathepsin B, a Western blot of total protein extracted from leaves over-expressing either NbCathB::mRFP or sec::mRFP, with or without BFA treatment, was probed with mRFP antibody. A protein of approximately 69 kDa was specifically detected in the NbCathB::mRFP over-expressing material, a size expected of the unprocessed protease, i.e. retaining the signal peptide and prodomain. In independent experiments, the concentration of this protein was considerably greater when secretion was inhibited by BFA treatment (Figure 6d). In contrast, a protein of the size of free mRFP (approximately 30 kDa) was also detected, and this was reduced in concentration upon BFA treatment (Figure 6d), suggesting that the fluorescent tag was cleaved from NbCathB upon secretion. Activity profiling of these protein samples was performed using DCG04, and biotinylated proteins were separated on a protein gel, revealing a range of papain protease activities from 25 to 38 kDa (Figure 6d). Crucially, no activity was detected from the full-length NbCathB::mRFP. These results demonstrate that the NbCathB::mRFP fusion is inactive and is cleaved upon secretion.

Apoplastic proteins were extracted from control *N. benthamiana* leaves and from leaves over-expressing the NbCathB::mRFP fusion and visualized on a polyacrylamide gel. A band of approximately 30 kDa was observed specifically in the NbCathB::mRFP over-expressing material (Figure 6e), less than half the size of the fusion protein. Western hybridization with a mRFP antibody revealed the band to contain free mRFP (Figure 6e). Nevertheless, as free mRFP and activated NbCathB (lacking the signal peptide and prodomain) are predicted to be of similar size, and would thus co-migrate on the gel, the band was excised and analysed by tandem mass spectrometry (MS/MS) and revealed the presence of mRFP and NbCathB (Supplementary Figure S5a), indicating that mRFP had been cleaved from NbCathB to generate two products of similar size.

Activity profiling of apoplastic protein from both control and over-expressing leaf material was performed using DCG-04 and biotinylated proteins were separated on a protein gel, revealing a single band of approximately 30 kDa specifically in the over-expressing sample (Figure 6f). This band was excised and analysed by MS/MS, and the identities of four peptides revealed it to be NbCathB (Supplementary Figure S5b). These peptides all correspond to the protease domain and do not contain the active site cysteine, consistent with the expectation that the prodomain is removed and the active site peptide is biotinylated. Cathepsin B activity was assayed in apoplastic protein from control and NbCathB::mRFP over-expressing leaves using the colorimetric substrate specific for mammalian cathepsin B. This revealed that whereas cathepsin B levels in control, non-pathogen-challenged samples were similar to those shown earlier (Figure 3b), cathepsin B activity more than six-fold higher was observed in the over-expressing plants (Figure 6g). This confirmed the efficacy of this assay in determining plant cathepsin B activity. Furthermore, the activity was inhibited by use of the cathepsin B inhibitor Ac-LVK-cho and the papain inhibitor E64 (Figure 6h). Taken together, the results indicate that NbCathB is secreted and is activated only upon secretion.

To confirm that secretion of active NbCathB was not an artefact of over-expression in *N. benthamiana*, we performed large-scale protease activity profiling on apoplastic proteins of the closely related tomato. Tomato was used because apoplastic papain proteases were difficult to detect in *N. benthamiana* (Figure 6g), whereas apoplastic proteins can be obtained effectively from tomato (Krüger *et al.*, 2002). Tomato leaves were vacuum-infiltrated with DCG-04, incubated, and apoplastic fluids were carefully isolated. Biotinylated proteins were separated on a protein gel and the 30 kDa region (Figure 7) was excised and analysed by MS/MS. This revealed the presence of two tomato cathepsin B-like proteases (TC175119 and TC182010) represented with seven




and six peptides, respectively (Supplementary Figure S5c). A phylogenetic tree constructed following alignment of these sequences with *NbCathB* and cathepsin B sequences from potato and tobacco revealed that *NbCathB* is likely to be the orthologue of tomato sequence TC182010 (Supplementary Figure S1b). These data demonstrate that cathepsin B is a secreted, active protease in the tomato apoplast.

## Discussion

Inhibitors have been used to implicate a diverse range of plant proteases in pathogen perception and in subsequent signalling and execution of disease resistance. They have been utilized to demonstrate roles for caspases in regulation and execution of plant PCD such as that characterizing the HR (Van der Hoorn and Jones, 2004; Woltering, 2004). In contrast, the tomato papain protease RCR3 plays a role in pathogen perception by recognition of AVR2 from *C. fulvum*, and recognition can be prevented by the general papain inhibitor E64 (Rooney *et al.*, 2005). E64 has also been shown to prevent the HR whilst not affecting caspase activities, implying the involvement of papain proteases in PCD (D'Silva *et al.*, 1998; Coffeen and Wolpert, 2004; Woltering, 2004). However, the identities of papain proteases involved in regulation or execution of the HR have proven elusive. Here, we used cathepsin B inhibitors to provide evidence of a role for this papain

**Figure 6.** Cathepsin B is activated upon secretion.
(a) Expression of the NbCathB::mRFP (monomeric red fluorescent protein) fusion in leaf epidermal cells resulted in largely apoplastic fluorescence; this image is a maximum projection of a stack of 20 confocal images covering 19 μm in depth. In highly over-expressing cells some fluorescence visible within the cell (arrowhead) appeared to be in the endoplasmic reticulum. Scale bar is 100 μm.
(b) When co-expressed with the plasma membrane marker EGFP-LT16b, NbCathB::mRFP fluorescence was observed outside the GFP-LT16b-labelled plasma membrane of the abaxial epidermal cells. This image is a cross-section through a projected stack of 72 confocal images covering 43 μm in depth. mRFP fluorescence was particularly bright close to cell junctions (arrowhead). Scale bar is 20 μm.
(c) Apoplastic localization of NbCathB::mRFP is retained following treatment with water for 6 h (upper panel) but the protein formed aggregates within the cell (lower panel) when leaves were treated with brefeldin A (BFA), which inhibits secretion. Scale bar is 50 μm.
(d) Western blot of total protein from plants over-expressing NbCathB::mRFP (from two independent experiments) or secreted mRFP (sec:mRFP), with or without BFA treatment, probed with mRFP antibody (first three panels). Protease activity profiling of total protein from plants over-expressing NbCathB::mRFP or sec::mRFP (control), with (+) or without (–) BFA treatment, using a biotinylated derivative of E-64 (DCG-04) in the presence (+) or absence (–) of 0.4 mM inhibitor E-64.
(e) Left panel shows a protein gel of apoplastic protein from control (C) and NbCathB::mRFP over-expressing (O) *Nicotiana benthamiana*, indicating a specific band of approximately 30 kDa specific to the 'O' sample. Tandem mass spectrometry (MS/MS) analysis of this band identified peptides from both NbCathB and mRFP (Supplementary Figure S5a). The right panel indicates western hybridization of anti-mRFP antibody to the protein gel shown in the left panel.
(f) Activity profiling of apoplastic protein from control (C) and NbCathB::mRFP over-expressing (O) plants using biotinylated derivative of E-64 (DCG-04) in the presence (+) or absence (–) of 0.4 mM inhibitor E-64. The MS/MS analysis of the 30 kDa band specific to the O sample identified peptides from NbCathB (Supplementary Figure S5b).
(g) Cathepsin B activity was more than six-fold higher in apoplastic protein from NbCathB::mRFP-over-expressing (O) plants than from control (C) plants (set at a value of 1 on the *y*-axis).
(h) Cathepsin B activity in apoplastic protein from NbCathB::mRFP-over-expressing plants was inhibited approximately 90% by papain inhibitor E64 and by cathepsin B inhibitor Ac-LVK-cho (compared to water or DMSO treatments). Results in (g) and (h) are each the mean ± SEM of two independent experiments.


**Figure 7.** Apoplastic localization of cathepsin B in tomato.
Biotinylated proteins isolated from tomato apoplast after DCG-04 labelling, detected with streptavidin-horseradish peroxidase (left) and colloidal Coomassie stain (right) and cathepsin B was identified by tandem mass spectroscopy analysis (Supplementary Figure S5c).

protease in *N. benthamiana* non-host disease resistance to *E. amylovora*. However, the inhibitors, whilst suppressing cathepsin B activity, also suppressed additional papain proteases and VIGS was required to demonstrate unequivocal involvement of cathepsin B not only in non-host disease resistance but also in the HR triggered by interaction of potato *R3a* and *P. infestans Avr3a* gene products.

Based on the similarities between three cathepsin B genes in *A. thaliana* and multiple EST sequence contigs in potato, cathepsin B is encoded by a family of closely related genes in plants and, in this work, VIGS involved constructs that were likely to silence the entire cathepsin B family in *N. benthamiana*. Further work will be needed to ascertain whether independent cathepsin B genes play different roles in plant development or in disease resistance. The reduction of disease resistance to *Eam* and *Pst* following silencing of *NbCathB* resulted in a five- to eight-fold increase in viable bacterial cells, similar to those seen when silencing *Nbsgt1* (Figure 2). Moreover, as VIGS of *NbCathB* suppressed the HR triggered by co-expression of *R3a* and *Avr3a*, we conclude that cathepsin B is involved in both host and non-host disease resistance. SGT1 also plays a role in both forms of disease resistance, but not in all cases (Peart *et al.*, 2002; Muskett and Parker, 2003). Similarly, as cathepsin B was not required for Cf4-AVR4-mediated HR, it also is not involved in all types of gene-for-gene disease resistance. Intriguingly, SGT1 was required for CF4-AVR4-mediated HR (Gabriels *et al.*, 2006; and this study), indicating overlapping but operationally distinct roles for these proteins in disease resistance.

In animals, cathepsin B can function as a regulator of PCD following its relocalization from the lysosome to the cytosol (Kingham and Pocock, 2001; Vancompernolle *et al.*, 1998; Guicciardi *et al.*, 2001; Zeiss, 2003). Here, VIGS of *NbCathB* suppressed PCD triggered by co-expression of AVR3a and R3a, and by infiltration of *Eam*, as visualized using trypan blue staining and suppression of the HR marker gene *Hsr203*. It is thus reasonable to speculate that cathepsin B also plays a role in regulation of PCD in plants. Nevertheless, we provided evidence that cathepsin B may not be required for all forms of PCD in plants. This is perhaps unsurprising as morphologically distinct forms of PCD suggest more than one genetically programmed route for the death of plant cells (van Doorn and Woltering, 2005). Indeed, there is more than one form of PCD in animals with varying dependence on different classes of protease (Podgorski and Sloane, 2003; Zeiss, 2003).

Apoptosis in animals is characterized by the tightly regulated activation of numerous constitutively expressed protease proenzymes, as is the case for caspases, or by the cellular relocalization of proteases such as cathepsin B (Podgorski and Sloane, 2003). The recently described plant saspases represent a class of PCD-associated proteases that are constitutively active and relocalized to the apoplast upon PCD induction (Coffeen and Wolpert, 2004). Nevertheless, *VPE*, encoding a protease with caspase-1-like activity, is

induced at the transcriptional level during the HR (Hatsugai *et al.*, 2004), revealing a difference in the induction of PCD in animals and plants. Cathepsin B represents a further protease that is constitutively expressed in animals but transcriptionally upregulated in plants during processes involving PCD in response to pathogen attack (this study and Avrova *et al.*, 2004). Indeed, cathepsin B is also induced during senescence, a process also involving PCD in plants (Gepstein *et al.*, 2003; Bhalerao *et al.*, 2003).

Many plant defence-associated proteases are secreted into the apoplast, including RCR3 (Krüger *et al.*, 2002), P69B (Tornero *et al.*, 1996), CDR1 (Xia *et al.*, 2004) and saspases (Coffeen and Wolpert, 2004). The saspases represent one of three distinct proteolytic activities responsible for victorin-mediated PCD, the others being a different caspase-like activity and a papain-like activity. Coffeen and Wolpert (2004) provided evidence that these activities were components of a signal cascade responsible for victorin- and heat-mediated PCD, and they postulated that saspase was upstream of other proteases within this signalling process, and possibly involved in their activation. It is thus interesting that cathepsin B is also secreted into the apoplast and is activated upon secretion; its potential interaction with other proteases warrants detailed investigation.

Despite cathepsin B being secreted, VIGS of cathepsin B compromised the HR triggered by intracellular recognition of AVR3a and R3a. Similar observations have been made with the extracellular cathepsin D-like protein CDR1. Antisense suppression of *CDR1* compromised the HR triggered by the intracellular recognition of *P. syringae* AvrRpm1, by mediation of an unidentified cell-to-cell peptide signalling system (Xia *et al.*, 2004). It has thus been established that, in plants, extracellular proteases can regulate the HR following intracellular recognition of pathogen effector proteins.

The presence of so many defence-associated proteases in the plant extracellular fluid highlights the potential importance of protease inhibitors secreted into the apoplast by invading pathogens in the establishment of infection (Tian *et al.*, 2004; Rooney *et al.*, 2005). The proteolytic battle for resistance or susceptibility outside the plant cell promises to be a fascinating area of study in the coming years.

## Experimental procedures

### *Bacterial inoculations, disease resistance and cell death measurement*

*Erwinia amylovora* (*Eam*) strain 1430 was cultured in King's broth supplemented with 6 mM $MgSO_4$ incubated at 30°C and 100 ***g*** over night. Bacteria were resuspended in sterile 5 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES). *Eam* suspensions ($10^6$ cfu $ml^{-1}$) in 5 mM MES were pressure infiltrated with or without 1 mM inhibitors (cathepsin B, S and L inhibitor, Z-FGNHO-Bz; cathepsin B inhibitors z-FA-fmk, Ac-LVK-cho and CA-074 Me) (Calbiochem®, Merck Biosciences; http://www.merckbiosciences.com/) into three leaves on each of six wild-type *N. benthamiana* plants. For VIGS experiments, $10^7$ or $10^6$ cfu $ml^{-1}$ *Eam* and $10^6$ cfu $ml^{-1}$ *Pst* DC3000 were infiltrated into *N. benthamiana* plants inoculated with TRV::*gfp*, TRV::*sgt1* or TRV::*NbCathB* as described previously (Cao *et al.*, 1994).

For viable *Eam* and *Pst* colony counting experiments, leaves were harvested 4 dpi. Whole leaves or leaf segments of equivalent size were ground in 1 ml of 10 mM $MgCl_2$ using micropestles. Four subsequent serial dilutions of 100 µl sample in 900 µl of 10 mM $MgCl_2$ were performed and 100 µl of the $10^{-4}$ dilution spread on King's Broth (KB) plates containing 50 mg $l^{-1}$ rifampicin and incubated at 30°C for 48 h. Trypan blue stain, used to visualize host cells dying in response to *Eam*, was performed as described (Tissier *et al.*, 1999).

### Avr3a-R3a *and* Cf-4-Avr-4 *co-expression*

*Avr3a-R3a* co-expression was performed as described (Armstrong *et al.*, 2005), except that the infiltration buffer was the same as for inhibitor infiltrations. The AGL0 strain carrying pBINplus::*R3a* was resuspended to $OD_{600}$ = 1, and mixed in a 1:1 ratio with $OD_{600}$ = 0.4 suspension of LB4404 carrying pGR106::*Avr3a* for co-inoculation. Photographs were taken after HR symptoms developed at 6 dpi. Co-expression of *C. fulvum Avr4* and tomato *Cf-4* was as described previously (Van der Hoorn *et al.*, 2000).

### *Cathepsin B activity*

Frozen leaves were finely ground, 300 µl of extraction buffer (50 mM monobasic, 50 mM dibasic potassium phosphate, pH 6.8 and 1 mM DTT) was added and the tissue homogenized. Protein samples were incubated on ice for 15 min and spun at maximum speed (16 000 ***g***) for 15 min to pellet cell debris. Supernatant was kept on ice or frozen at −70°C. Assays were performed using clear 96-well microtitre plates (Fisher Scientific, http://www.fisher.co.uk/) and a DIAS plate reader (Dynatech Laboratories; http://www.dynatechlaboratories.com).

Bradford reagent (Bio-Rad; http://www.bio-rad.com/) was diluted 1:5 with sterile distilled water (SDW), aliquoted (199 µl) to each well of a 96-well microtitre plate and 1 µl of protein extract was added and mixed. The reaction was left for 30 min at room temperature (21°C) .and optical density was measured at 595 nm. The protein concentration was calculated using a formula derived from a bovine serum albumin dilution series ranging from 0 to 6 µg.

Cathepsin B colorimetric substrate Z-Arg-Arg-*p*NA, 2HCl (Calbiochem®, Merck Biosciences) was diluted to 1 mM in SDW. Assay buffer was prepared as recommended by Calbiochem®. Assay buffer and colorimetric substrate were mixed in equal volumes, then mixed with 1.8× volume of SDW. One hundred and forty microlitres of buffer/substrate solution was aliquotted into each well of a 96-well microtitre plate and 10 µl of protein extract added, mixed and incubated at room temperature for 30 min. Optical density was read at 405 nm using a DIAS plate reader. Protein activity was divided by total protein (mg $ml^{-1}$) in crude extracts calculated using a Bradford assay, reading the optical density at 595 nm.

### *Cloning of* NbCathB

The *N. benthamiana NbCathB* gene was obtained by aligning cathepsin B DNA sequences from *S. tuberosum* (accession number AY450641) and *N. rustica* (X81995). Primers (cathB-F 5′-TTT GGGTACCTAAGCGCCTTCTTG-3′ and cathB-R 5′-TTTTCCATGGG TAAGGATCACACTCTTC-3′) designed to anneal to common sequences were used to PCR amplify the equivalent sequence from

*N. benthamiana* cDNA. The full-length coding region for *NbcathB* was obtained through 3′-rapid amplification of cDNA ends (RACE) according to specifications in the SMART RACE II kit (BD Clontech; http://www.clontech.com/). The forward primer cathB-F, for cloning into the VIGS vector, was used as the gene-specific primer in conjunction with the 3′-RACE primers from the kit. Sequencing and BLAST results indicated that the fragment had 97% homology at the nt level to the *N. rustica* cathepsin B-like cysteine protease mRNA (Not shown). Therefore, a primer to amplify the *N. benthamiana* fragment from the start codon was designed from the *N. rustica* sequence and used (CATHSTARTa: 5′-GGCACGAGGCCAAATAT G-3′) in conjunction with the 3′- RACE primer. The resulting 1400-bp amplification product was cloned into pGEM-T EASY (Promega; http://www.promega.com/) according to the manufacturer's specifications and sequenced using the Applied Biosystems Bigdye® v3.1 Terminator sequencing kit (http:http://www.appliedbiosystems.com/). The *NbCathB* accession number is DQ492287.

### *Phylogenetic analyses of cathepsin B sequences*

The 13 cathepsin B nt sequences used for the tree in Supplementary Figure S1(b) [DQ492287 from *N. benthamiana;* AF359422 from *Nicotiana tabacum*; TC137447, TC145097, TC152070 and TC142279 from potato; TC175119 and TC182010 from tomato; NM_100110 (At1 g02300); NM_100111 (At1 g02305); NM_116392 (At4 g01610) and the splice variant of NM_178950 from *A. thaliana*; and NM_001908 from *H. sapiens*] were used to obtain a back-translated alignment of their source nt sequences, using an *ad hoc* Python script that matched the protein sequence to the coding sequence fragments in the source nt sequence. This script first reduced the EST sequences to their coding sequences, and these were threaded onto the full protein sequence alignment, using t_coffee, with the protocol: t_coffee -other_pg seq_reformat -in cathB_backtrans.fas \ -in2 cathB_prot.fasta_aln -action +thread_dna_on_prot_aln -output phylip_aln. This alignment was cropped to only those regions shared by all sequences. The seqboot package was used to generate 1000 bootstrap sequences from this truncated alignment. The PHYLIP package dnaml was used to generate maximum-likelihood trees. Trees were generated based on a single alignment, and on 1000 bootstrap alignments generated from these sequences.

### *Plant material and VIGS constructs*

Virus-induced gene silencing experiments were conducted in containment glasshouses under Scottish Executive Environment and Rural Affairs Department licenses GM/203/2004 and GM/210/2004. Growth of *N. benthamiana* and use of a Tobacco rattle virus vector (TRV-2b) for VIGS was as described previously (Valentine *et al.*, 2004). Primer sequences used to clone a 364-bp portion of *NbCathB* into TRV were cathB-F, 5′-TTTGGGTACCTAAGCGCCTTCTTG-3′, and cathB-R, 5′-TTTTCCATGGGTAAGGATCACACTCTTC-3′. The *NbCathB* PCR product was subcloned into pGEM-T EASY then excised by *Ncol*–*Not*I digestion and subcloned in antisense orientation into *Ncol*–*Eag*I-digested TRV vector to generate TRV::*NbCathB-1*. The insert was also cloned into the TRV binary vector pBinTRV2b (Liu *et al.*, 2002). Silencing with this construct produced similar results to the TRV::*NbCathB-1* construct described above. An independent 247-bp fragment of *NbCathB* (see Supplementary Figure S1a) was cloned into pBinTRV2b by utilizing the primers cathB2-F, 5′-AATTGAATTCGAGAGGACTATTGGCTTC TTGC-3′, and cathB2-R, 5′-AAAAGTTAACTTGTTCCCCAGTCTTCAG AGA-3′. This *NbCathB* PCR product was subcloned into pGEM-T EASY, excised by *Eco*RI and *Hpa*I and cloned in antisense orientation into *Eco*RI- and *Hpa*I-digested binary vector pBinTRV2b to generate TRV::*NbCathB-2*. Primers for cloning a 580-bp portion of *Nbsgt1* (accession number AF494083.1) were 5′-TTTTGGTAC CTTCGCCGACCGTG-3′ and 5′-TATTCCATGGGCAGGTGTTATCTT C-3′. The PCR product was subcloned into pGEM-T EASY. Following *Avr*II digestion and blunting using T4 DNA polymerase (New England Biolabs; http://www.neb.com/), *Nbsgt1* cDNA was excised by *Not*I digestion and subcloned in antisense orientation into TRV (linearized by *Hpa*I and *Eag*I) to generate TRV::*Nbsgt1*. TRV::*gfp* (Valentine *et al.*, 2004) was used as a control of TRV infection.

### *Real time RT-PCR*

Total RNA extraction and first-strand cDNA synthesis using random hexamer primers were as described previously (Lacomme *et al.*, 2003). For SYBR green-based real-time RT-PCR (QuantiTect SYBR GreenPCR kit, Qiagen; http://www.qiagen.com/) experiments, primer pairs were designed outside the region of cDNA targeted for silencing using PRIMER EXPRESS software supplied with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) following the manufacturer's guidelines. Expression of *NbCathB* and *NbHsr203* were assessed relative to that of 26S rRNA. The primers for 26S rRNA were 5′-CACGGACCAAGGAGTCTGACAT-3′ and 5′-TCCCACCAATCAGCTTCCTTAC-3′. Primers for *NbCathB* were designed from *N. benthamiana* accession DQ492287 and were 5′-CAGCTCCGATCCACACAGTA-3′ and 5′-GAGCGAAATCCTCGTAA ACAG-3′. Additional primers (5′-CAGCTCCGATCCACACAGTA-3′ and 5′-GAGCGAAATCCTCGTAAACAG-3′) were used to assess transcription level of *NbCathB* after silencing with TRV::*NbCathB-2*. Primers for *NbHsr203* were designed from *N. tabacum* accession X77136 5′-ATGAAAAGCAAGTGATAGAGGAAGTA-3′ and 5′-GCTC GGCCATGAATTTGAC-3′. 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).

### *Western hybridization*

For immunodetection of SGT1 and mRFP, protein extraction and Western blot analyses were as previously described (Lacomme and Santa Cruz, 1999). Blots were incubated with primary antibody against SGT1 (rat polyclonal, 1:2000; Takahashi *et al.*, 2003) and for mRFP (rabbit polyclonal, 1:2000). Alkaline phosphatase-conjugated antirat IgG (Sigma; http://www.sigmaaldrich.com/) was used as a secondary antibody for SGT1 for detection in silenced and control plants, and peroxidase-conjugated antirabbit IgG (Sigma) was used for mRFP detection.

### *Activity profiling experiments.*

To investigate specificity of cathepsin B inhibitors, DCG-04 was incubated with Arabidopsis leaf extracts and competed with E-64, z-FA-fmk, Ca-074-Me and Ac-LVK-cho as described previously (Van der Hoorn *et al.*, 2004). After labelling, biotinylated proteins were purified and detected on a protein blot using streptavidin-HRP, as described previously (Van der Hoorn *et al.*, 2004).

To investigate apoplastic cathepsin B activity, 186 leaflets of tomato cultivar MoneyMaker were vacuum infiltrated with 2 mg $l^{-1}$ DCG-04 and 10 mg $l^{-1}$ L-cysteine and incubated for 5 h at room-temperature. Apoplastic fluids were subsequently isolated from ice-cooled leaflets (Krüger *et al.*, 2002) and biotinylated proteins were captured as

described previously (Van der Hoorn *et al.*, 2004). Isolated proteins were separated on a 10% SDS polyacrylamide gel and stained with colloidal Coomassie. The 30-kDa protein band was excised from the gel, treated with trypsin, and eluted peptides were analysed by MS/MS as described previously (Van der Hoorn *et al.*, 2004).

### *Confocal microscopy of cathepsin B localization*

*NbCathB* cDNA was PCR amplified and cloned into pGEM-T vector (Promega). The coding region was PCR amplified from a sequence-confirmed clone using primers designed to introduce an *AscI* site at the 5′ end and a *NotI* site at the 3′ end while removing the stop codon. This PCR fragment was cloned into a version of pENTER 1A (Invitrogen; http://www.invitrogen.com/) modified to contain *AscI* and *NotI* restriction sites in the multiple cloning region. The *NbCathB* sequence was recombined with a derivative of the Gateway vector pMDC84 (Curtis and Grossniklaus, 2003) in which the mgfp6 coding sequence had been replaced by *mRFP* (Campbell *et al.*, 2002). The cathepsin B–mRFP fusion construct was electroporated into *Agrobacterium tumefaciens* strain LBA4404 and infiltrated into leaves from 4-week-old *N. benthamiana*.

A 1:1 mixture of agrobacteria containing the *EGFP-LT16b* and *cathepsin B-mRFP* constructs was infiltrated as described for *R3a* and *Avr3a* above. Cells expressing fluorescent protein fusions were observed using a Leica TCS-SP2 AOBS confocal microscope (http://www.leica.com/) between 1 and 5 dpi. Images were obtained using an HCX APO 63×/0.9 W water-dipping lens. Monomeric red fluorescent protein was imaged using an excitation wavelength of 568 nm from a 'lime' diode laser with emissions collected between 600 and 630 nm. Green fluorescent protein was imaged using 488-nm excitation from an argon laser, with emissions collected between 500 and 530 nm. Chlorophyll-associated autofluorescence was also obtained after excitation at 488 nm and the emissions collected between 650 and 700 nm. Brefeldin A treatment was with 10 μg $ml^{-1}$ (in water), infiltrated 24 h after agroinfiltration of the cathepsin B-mRFP construct and leaves were observed under the confocal microscope 6 h later.

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## Supplementary material

The following supplementary material is available for this article online:

**Figure S1.** (a) Alignment of protein sequences of cathepsin B from *Nicotiana benthamiana*, *Nicotiana rustica*, *Solanum tuberosum*, *Arabidopsis thaliana* and *Homo sapiens*.

(b) Maximum likelihood topology generated from cathepsin B nucleotide sequences from *N. benthamiana*, *N. tabacum*, potato, tomato and from *A. thaliana*; and rooted to the outgroup cathepsin B sequence from *H. sapiens*.

**Figure S2.** (a) Relative expression of *NbCathB* in TRV::*gfp*, TRV::*NbCathB-1* and TRV::*NbCathB-2* infected plants, measured using real-time RT-PCR.

(b) Western blot analysis of SGT1 protein levels in TRV::*gfp* and TRV::*Nbsgt1b* infected plants.

**Figure S3.** Colony counts (cfu $ml^{-1}$) of viable *E. amylovora* at 4 days post-inoculation in TRV::*gfp*, TRV::*NbCathB-1* or TRV::*NbCathB-2* plants.

**Figure S4.** Use of Brefaldin A (BFA) prevents secretion of NbCathB::mRFP and sec::mRFP. Expression of NbCathB::mRFP (a) and sec::mRFP (b) in *Nicotiana benthamiana* leaves results in largely apoplastic fluorescence. However, treatment with BFA resulted in retention of the monomeric red fluorescent protein (mRFP) within the cell for both NbCathB::mRFP (c) and sec::mRFP (d).

**Figure S5.** Identification of cathepsin B peptides.

(a) Identified peptides from *Nicotiana benthamiana* cathepsin B and monomeric red fluorescent protein (mRFP) in the apoplast following over-expression of *NbCathB::mRFP*.

(b) Identified peptides from active *NbCathB* following over-expression and activity profiling with biotinylated DCG-04.

(c) Identified peptides from tomato cathepsin B-like proteases in the tomato apoplast following tandem mass spectrometry analysis.

This material is available as part of the online article from http://www.blackwell-synergy.com

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