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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 \( R^3a \) 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 *Cladosporium 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 *Solanium 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. Vacular 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 (Coffeeen 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; Coffeeen 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 cathespin D (Xia et al., 2004). We have demonstrated that a potato cathespin B-encoding gene, *StCathB*, is rapidly upregulated specifically by resistance (R) gene-mediated HRs elicited by *P. infestans* (Avrova et al., 2004). Cathespin B is structurally unrelated to cathespin D, being a member of the mammalian family of cysteine proteases. Cathespin B has been implicated in many diverse roles in animals, including PCD (Zeiss, 2003). In animals, cathespin B can activate caspases (Kingham and Pocock, 2001; Vancompemrnollee et al., 1998), and cathespin B knock-out mice fail to exhibit apoptosis (Gucciardi et al., 2001; Zeiss, 2003). Nevertheless, cathespin B can cause PCD independently of caspases (Foghsgaard et al., 2001). It was thus reasonable to regard cathespin B as a candidate cysteine protease involved in plant disease resistance.

We showed, using cathespin B inhibitors and virus-induced gene silencing (VIGS), that cathespin B plays a role in both host and non-host plant disease resistance. We determined that cathespin B transcription and enzymatic activity are induced during the HR, and showed that suppression of the HR by silencing of cathespin 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

**Cathespin 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 cathespin B in *Eam*-mediated non-host disease resistance, we used inhibitors described in the animal literature as cathespin B specific, z-FA-fmk, Ac-LVK-cho, CA-074-Me and the broad cathepsin inhibitor, E-64. Each inhibitor was infiltrated 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 cathespin B or general papain inhibitors (Figure 1a).

Two of the cathespin 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 cathespin 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, cathespin 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 cathespin 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 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 cathespin D (Xia et al., 2004). We have demonstrated that a potato cathespin B-encoding gene, *StCathB*, is rapidly upregulated specifically by resistance (R) gene-mediated HRs elicited by *P. infestans* (Avrova et al., 2004). Cathespin B is structurally unrelated to cathespin D, being a member of the mammalian family of cysteine proteases. Cathespin B has been implicated in many diverse roles in animals, including PCD (Zeiss, 2003). In animals, cathespin B can activate caspases (Kingham and Pocock, 2001; Vancompemrnollee et al., 1998), and cathespin B knock-out mice fail to exhibit apoptosis (Gucciardi et al., 2001; Zeiss, 2003). Nevertheless, cathespin B can cause PCD independently of caspases (Foghsgaard et al., 2001). It was thus reasonable to regard cathespin B as a candidate cysteine protease involved in plant disease resistance.

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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 (At1g02300); NM_100111 (At1g02305); NM_116392 (At4g01610)]. 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 unlikelyhood 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::srt1b 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 (Hyke-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...
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::Nbsgt1. 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...
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 interactions.
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 \textit{NbCathB} and cathepsin B sequences from potato and tobacco revealed that \textit{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.

\textbf{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 \textit{C. fulvum}, and recognition can be prevented by the general papain inhibitor E64 (Rooney \textit{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 \textit{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
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 relocation 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 (Podgorsk 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 saspreds 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 apoplasm, including RCR3 (Krüger et al., 2002), P69B (Tornero et al., 1996), CDR1 (Xia et al., 2004) and saspsases (Coffeen and Wolpert, 2004). The saspsases represent one of three distinct proteolytic activities responsible for victim-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 victim- 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 apoplasm 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 apoplasm by invading pathogens in the establishment of infection (Tian et al., 2003; Bhalerao et al., 2003).

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 MgSO4, incubated at 30°C and 100 g over night. Bacteria were resuspended in sterile 5 mM 2-(N-morpholine)-ethanesulfonic acid (MES). Eam suspensions (109 cfu ml−1) in 5 ml MES were pressure infiltrated with or without 1 mM inhibitors (cathepsin B, S and L inhibitor, Z-FGNNHO-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, 104 or 106 cfu ml−1 Eam and 106 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 MgCl2 using micropestles. Four subsequent serial dilutions of 100 μl sample in 900 μl of 10 mM MgCl2 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::Avr3a was resuspended to OD600 = 1, and mixed in a 1:1 ratio with OD600 = 0.4 suspension of LB4404 carrying pGR108::Avr3a for coinoculation. 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 6.5 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.dynatechalaboratories.com).

Bradyreagent (Bio-Rad; http://www.bio-rad.com/) was diluted 1:5 with sterile distilled water (SDW), aliquoted (199 l) 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-pNA, 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 aliquoted 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 (X91895). Primers (catb-F: 5’-TTTGGGTACTCATGGCCCTTCTTG-3’ and catb-R: 5’-TTTCCCATGGGTAAGGATACACTCTTC-3’) designed to anneal to common sequences were used to PCR amplify the equivalent sequence from

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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' - GCCAGGAGCCAAATAT- 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 BigDyeTM v3.1 Terminator sequencing kit (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; AF393924 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 PHYLP package dnaml was used to generate maximum-likelihood trees. Trees were generated based on 1000 bootstrap sequences from this alignment. The PHYLIP package was used to generate 1000 bootstrap sequences from this alignment. The seqboot package was used to generate 1000 bootstrap sequences from this truncated alignment. The PHYLP package dnaml was used to generate maximum-likelihood trees. Trees were generated based on 1000 bootstrap sequences from this truncated alignment. The PHYLP package was used to generate maximum-likelihood trees. Trees were generated based on 1000 bootstrap sequences from this truncated alignment. The phylib package dnaml was used to generate maximum-likelihood trees. Trees were generated based on 1000 bootstrap sequences from this truncated alignment.

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., 2003). A primers for cloning a 580-bp portion of NbCathB (see Supplementary Figure S1a) was cloned into pBINTRV2b by utilizing the primers cathB2-F, 5'-AATTGAATTCAGGACTATTGGGCTTC TTG-3', and cathB2-R, 5'-AAAGTTAATCTTGGCCAGTTT CAG AGA-3'. This NbCathB PCR product was subcloned into pGEM-T EASY, excised by EcoRI and HpaI and cloned in antisense orientation into EcoRI- and HpaI-digested binary vector pBinTRV2b to generate TRV::NbCathB-2. Primers for cloning a 580-bp portion of NbSgt1 (accession number AF490831) were 5'-TTTGGTACCTCGCCGACCCGTG-3' and 5'-TATCCCATGGCGGTGTTAATCTT C-3'. The PCR product was subcloned into pGEM-T EASY. Following AvrII digestion and blunting using T4 DNA polymerase (New England Biolabs; http://www.neb.com/), NbSgt1 cDNA was excised by NotI digestion and subcloned in antisense orientation into TRV (linearized by HpaI and EagI) 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'-CAGCGGAAACAAGGCTGACAT-3' and 5'-TCCCAACATCAGGCTTAC-3'. Primers for NbCathB were designed from N. benthamiana accession DQ492287 and were 5'-CAGCTTCGACCCCACAGTA-3' and 5'-GAGGGGAAATCTCTGTTA AAACG-3'. Additional primers (5'-CAGCTTCGATCCACACAGTA-3' and 5'-GAGCGGAAACTCCTGTTACACG-3') were used to assess transcription level of NbCathB after silencing with TRV:: NbCathB-2. Primers for NbHsr203 were designed from N. tabacum accession X77135 5'-ATGAAAACGAAGTGATAGAGGAAGTA-3' and 5'-GCCT GGCATAGAAGTTGAC-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.sigmaldrich.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 apoptosis cathepsin B activity, 186 leaflets of tomato cultivar MoneyMaker were vacuum infiltrated with 2 mg 1L-1 DCG-04 and 10 mg 1L-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
Confocal microscopy of cathepsin B localization

NbCatB 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 NbCatB sequence was recombined with a derivative of the Gateway vector pHDC84 (Curtis and Grossniklaus, 2003) in which the mgf6 coding sequence had been replaced by mRFP (Campbell et al., 2002). The NbCatB–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 fusion proteins 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 588 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 supplementary 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 NbCatB in TRV:gfp, TRV:NbCatB-1 and TRV:NbCatB-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:NbCatB-1 or TRV:NbCatB-2 plants.

Figure S4. Use of Brefeldin A (BFA) prevents secretion of NbCatB:mRFP and sec:mRFP. Expression of NbCatB: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 NbCatB: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 NbCatB:mRFP.

(b) Identified peptides from active NbCatB 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

References


