REVIEW PAPER

The front line of defence: a meta-analysis of apoplastic proteases in plant immunity

Alice Godson and Renier A.L. van der Hoorn*

The Plant Chemetics Laboratory, Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, UK

* Correspondence: renier.vanderhoorn@plants.ox.ac.uk

Received 5 October 2020; Editorial decision 16 December 2020; Accepted 23 December 2020

Editor: Marina Klemenčič, University of Ljubljana, Slovenia

Abstract

Secreted proteases act at the front line of defence and play pivotal roles in disease resistance. However, the criteria for apoplastic immune proteases are not always defined and followed. Here, we critically reviewed 46 apoplastic proteases that function in plant defence. We found that most apoplastic immune proteases are induced upon infection, and 17 proteases are genetically required for the immune response. Proteolytic activity has been confirmed for most of the proteases but is rarely shown to be required for biological function, and the apoplastic location of proteases can be subjective and dynamic. Pathogen-derived inhibitors have only been described for cysteine and serine proteases, and the selection pressure acting on immune proteases is rarely investigated. We discuss six different mechanisms by which these proteases mediate plant immunity and summarize the challenges for future research.

Keywords: Apoplast, defence, hypersensitive response, immunity, pathogen, plant, protease, recognition, signalling.

Introduction

Proteases are present throughout the tree of life, determining the fate of proteins by irreversibly cleaving peptide bonds. This cleavage serves not only to degrade proteins, thereby rendering them non-functional and facilitating protein turnover, but also to activate proteins through the removal of inhibitory or regulatory domains and changing their subcellular location (Van der Hoorn, 2008).

Plant proteases perform critical functions during the interaction between plants and pathogens. Upon pathogen entry, pathogen-associated molecular patterns (PAMPs) such as chitin and flagellin are recognized by pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Adapted pathogens use effector proteins to perturb PTI. Resistant plants can recognize some of these effectors through nucleotide-binding leucine-rich repeat (NB-LRR) proteins, resulting in effector-triggered immunity (ETI) (Zipfel, 2014). ETI often culminates in a form of programmed cell death (PCD) known as the hypersensitive response (HR) at the site of infection, limiting the spread of the pathogen (Morel and Dangl, 1997). Both PTI and ETI trigger similar downstream responses, including the release of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), and the production of pathogenesis-related (PR) proteins such as chitinases, glucanases, and proteases (Dodds and Rathjen, 2010). Local defence responses often trigger systemic acquired resistance (SAR), underpinned in part by the signalling hormone salicylic acid (SA).
Whilst much of the plant immune response comprises intracellular events such as transcriptional reprogramming and MAPK signalling, most plant–pathogen interactions occur in the extracellular space known as the apoplast. This is the first and often final destination for pathogens that have entered the plant through wounds, stomata, and hydathodes, and is an important site for pathogen proliferation (Jones and Dangl, 2006). The apoplast can be thought of as an ancient ‘battlefield’ that must be fiercely defended by the host (Jones and Dangl, 2006; Doehlemann and Hemetsberger, 2013; Jashni et al., 2015). Plants are armed with critical weaponry in the form of apoplastic proteases, which they secrete from their cells both constitutively and inducibly (Fig. 1). These proteases are highly stable and active at acidic pH, crucial for their function in the proteolytically challenging environment of the apoplast (Simões and Faro, 2004; Richau et al., 2012). The MEROPS database (Rawlings et al., 2008) provides a classification of proteases into four main groups depending on their catalytic mechanism: aspartic proteases, cysteine proteases, metalloproteases, and serine proteases (Van der Hoorn, 2008).

There are several reviews that discuss the role of proteases in plant immunity (Figueiredo et al., 2014; Misas-Villamil et al., 2016; Balakireva and Zamyatnin, 2018; Thomas and Van der Hoorn, 2018), but a recent comprehensive overview on the role of apoplastic proteases is missing. In this review we will outline the catalytic classes of plant proteases, and then undertake a meta-analysis of the current literature on apoplastic immune proteases. We will finally discuss the various functions these proteases perform to illustrate the critical role apoplastic immune proteases play at multiple stages of the defence response.

**Overview of the catalytic classes of plant proteases**

Plants produce 500–1000 proteases, which belong to four main catalytic classes and a few additional catalytic classes (Van der Hoorn, 2008).

Aspartic (Asp) proteases are characterized by the presence of two Asp residues which support a molecule of water that acts as a nucleophile during proteolysis (Van der Hoorn, 2008). Asp proteases are grouped into 14 different families in the MEROPS database (Rawlings et al., 2008), of which five contain plant proteases. The majority of plant Asp proteases belong to the A1 family of pepsin-like proteases (pepsins) (Simões and Faro, 2004). These pepsins may be typical, atypical, or nucellin-like, depending on their active site motif and other sequence features such as the presence of a plant-specific insert called a swaposin (Simões and Faro, 2004). The majority of plant pepsins are intracellular, usually with a vacuolar location, but there are several plant pepsins accumulating in the apoplast (Simões and Faro, 2004).

Cysteine (Cys) proteases utilize a catalytic Cys residue as a nucleophile during proteolysis (Van der Hoorn, 2008). Plant Cys proteases are represented in five MEROPS clans (Rawlings et al., 2008). Papain-like cysteine proteases (papains) are the only family containing extracellular Cys proteases and have long been implicated in plant immunity (Misas-Villamil et al., 2016). Papains belong to MEROPS clan CA, family C1 and are grouped into nine subfamilies (Richau et al., 2012). The general papain structure consists of a papain-like fold of an α-helix and β-sheet domain, and two papain subfamilies also carry a C-terminal granulin domain. Being very stable enzymes with multiple disulfide bridges, papains are often found in proteolytically harsh environments, such as the apoplast and vacuole (Richau et al., 2012). Their apoplastic location puts papains in direct contact with pathogens and their associated proteins, making them prime candidates for involvement in the plant immune response.

Metalloproteases utilize a catalytic metal cofactor to activate water that acts as a nucleophile during proteolysis (Van der Hoorn, 2008). There are >50 families of metalloproteases in the MEROPS database (Rawlings et al., 2008). Plants have representatives that belong to 19 families, which are grouped into 11 evolutionarily unrelated clans (Van der Hoorn, 2008). The

---

**Fig. 1.** Apoplastic immune proteases at the extracellular battlefield. Proteases of the four major catalytic classes are secreted from the cell into the apoplast, an important site for pathogen colonization. Here, they coordinate the plant immune response through six main strategies: direct antimicrobial activity; immune hydrolase activation; damage-associated molecular pattern (DAMP) release; effector perception; initiation of the hypersensitive response (HR); and the regulation of both systemic acquired resistance and priming.
Table 1. Criteria for apoplastic immune proteases

| Name       | Criteria: | MEROPS Family | Organism | Apoplastic? | A | B1 Genetically required? | B2 Induced? | B3 Inhibited? | B4 Diversifying selection? | B5 Secretion prevented? | C1 Protease? | C2 Activity required? | Reference                                      |
|------------|-----------|----------------|----------|-------------|---|--------------------------|-------------|----------------|--------------------------------|------------------------|-------------|----------------------|-----------------------------------------------|
| AED1       | A01       | At             | Y        | –           | Y | –                        | –           | –             | –                              | –                      | –           | –                    | Breitenbach et al. (2014)                     |
| GmAP1      | A01       | Gm             | Y        | Y           | – | –                        | –           | –             | Y                              | Y                     | Y           | –                    | Guo et al. (2019)                           |
| StAP1/3    | A01       | St             | Y        | –           | Y | –                        | –           | –             | –                              | Y                     | –           | –                    | Guevara et al. (1999, 2001, 2002, 2004); Mendieta et al. (2006); Muñoz et al. (2010) |
| OsAP7      | A01       | Os             | –        | Y           | Y | Y                        | –           | –             | –                              | –                      | –           | –                    | Alam et al. (2014)                          |
| LeAspP     | A01       | Sl             | –        | –           | Y | –                        | –           | –             | –                              | –                      | –           | –                    | Schaller and Ryan (1996)                    |
| CDR1       | A01       | At             | Y        | Y           | Y | –                        | –           | –             | –                              | Y                     | Y           | –                    | Xia et al. (2004); Simões et al. (2007)       |
| SAP1/2     | A01       | At             | Y        | Y           | Y | –                        | –           | –             | –                              | Y                     | Y           | –                    | Wang et al. (2019)                          |
| C14        | C1A       | Sl             | Y        | –           | Y | Y                        | N           | Y             | Y                              | –                      | –           | –                    | Harrak et al. (2001); Kaschani et al. (2010); Bozkurt et al. (2011); Kovács et al. (2016); Shindo et al. (2016) Avrova et al. (1999); Kaschani et al. (2010); Kaschani and Van der Hoorn (2011) |
| CathB      | C1A       | At             | Y*       | Y           | Y | –                        | –           | –             | Y                              | –                      | –           | –                    | McLellan et al. (2009); Ge et al. (2016); Porodko et al. (2018) |
| CP1/2      | C1A       | Zm             | Y        | –           | Y | Y                        | –           | Y             | –                              | –                      | –           | –                    | Mueller et al. (2013); Ziemann et al. (2018); Misses Villami et al. (2019) Kaschani et al. (2010); Bozkurt et al. (2011); Paireder et al. (2016) Pechan et al. (2000, 2002); Mohan et al. (2008); Lopez et al. (2007); Fescemyer et al. (2013); Louis et al. (2015); Varsani et al. (2016) |
| CP14       | C1A       | Nb             | Y*       | Y           | – | –                        | –           | –             | Y                              | –                      | –           | –                    | Gilroy et al. (2007)                        |
| Mir1       | C1A       | Zm             | Y        | –           | Y | Y                        | –           | Y             | –                              | –                      | –           | –                    | Avrova et al. (2004)                        |
| MRP1/2     | C1A       | Mj             | Y        | –           | – | –                        | Y           | –             | –                              | Y                     | –           | –                    | Dong et al. (2014)                          |
| Name       | Criteria: | Organism | Apoplastic? | Genetically required? | Induced? | Inhibited? | Diversifying selection? | Secretion prevented? | Protease? | Activity required? | Reference                                      |
|------------|-----------|----------|-------------|-----------------------|----------|------------|---------------------------|----------------------|-----------|---------------------|-----------------------------------------------|
| Papain     | C1A       | Cp       | Y           | –                     | Y        | Y          | –                         | –                    | Y         | –                   | El Moussaoui et al. (2001); Konno et al. (2004); Azarkan et al. (2006); Gumtow et al. (2018) |
| Pip1       | C1A       | Sl       | Y           | Y                     | Y        | Y          | –                         | –                    | Y         | –                   | Tian et al. (2007); Shabab et al. (2008); Ilyas et al. (2015); Shindo et al. (2016); Planas-Márquez et al. (2018) |
| CsRD21a    | C1A       | Cs       | Y           | –                     | –        | Y          | –                         | –                    | Y         | –                   | Clark et al. (2018) |
| Rcr3       | C1A       | Sl       | Y           | Y                     | Y        | Y          | –                         | –                    | Y         | –                   | Dixon et al. (2000); Krüger et al. (2002); Rooney et al. (2005); Shabab et al. (2008); Song et al. (2009); Lozano-Torres et al. (2012); Dong et al. (2014); Ilyas et al. (2015); Shindo et al. (2016); Paulus et al. (2020) |
| CsSAG12-1  | C1A       | Cs       | –           | –                     | –        | –          | –                         | –                    | Y         | –                   | Clark et al. (2018) |
| XCP2       | C1A       | Os       | –           | –                     | Y        | Y          | –                         | –                    | –         | –                   | Niño et al. (2020) |
|            |           | Zm       | Y           | –                     | Y        | Y          | –                         | –                    | –         | –                   | van der Linde et al. (2012); Mueller et al. (2013) |
| At-MMPs    | M10       | At       | Y           | Y                     | Y        | –          | –                         | –                    | –         | –                   | Maidment et al. (1999); Marino et al. (2014); Zhao et al. (2017) |
| GmMMP2     | M10       | Gm       | –           | –                     | Y        | –          | –                         | Y                    | –         | –                   | Liu et al. (2001) |
| M1MMP1     | M10       | Nt       | Y           | –                     | Y        | –          | –                         | Y                    | –         | –                   | Schiemeyer et al. (2009); Mandal et al. (2010) |
| Sl-MMPs    | M10       | Sl       | Y           | Y                     | Y        | –          | –                         | Y                    | –         | –                   | Li et al. (2015); Zimmermann et al. (2016) |
| HvPR-17a/-17b | M10?    | Hr       | Y           | –                     | Y        | –          | –                         | –                    | –         | –                   | Christensen et al. (2002) |
| NbPP27     | M10?      | Nb       | Y           | –                     | Y        | –          | –                         | –                    | –         | –                   | Xie and Goodwin (2009) |
| NtPP27     | M10?      | Nt       | Y           | –                     | Y        | –          | –                         | –                    | –         | –                   | Okushima et al. (2000) |
| WCI-5      | M10?      | Ta       | Y           | –                     | –        | –          | –                         | –                    | –         | –                   | Görlich et al. (1996); Schweizer et al. (1999) |
| OsBISCPL1  | S10       | Os       | –           | –                     | Y        | –          | –                         | –                    | –         | –                   | Liu et al. (2008) |
### Table 1. Continued

| Name       | Criteria: MEROPS Family | Organism | Apoplastic? | B1 Genetically required? | B2 Induced? | B3 Inhibited? | B4 Diversifying selection? | B5 Secretion prevented? | C1 Protease? | C2 Activity required? | Reference                                                                 |
|------------|------------------------|----------|-------------|--------------------------|-------------|---------------|-----------------------------|--------------------------|--------------|----------------------|---------------------------------------------------------------------------|
| P69B       | S8                     | Sl       | Y           | –                        | Y           | Y             | –                           | –                        | Y            | Y                    | Granell et al. (1987); Tomero et al. (1997); Jordà et al. (1999); Zhao et al. (2003); Tian et al. (2004, 2005); Zimmermann et al. (2016); Planas-Marquès et al. (2018); Wang et al. (2020); Paulus et al. (2020) |
| P69C       | S8                     | Sl       | Y           | –                        | Y           | –             | –                           | –                        | Y            | Y                    | Tomero et al. (1996); Jordà et al. (1999); Planas-Marquès et al. (2018) |
| S/Phyt-1/2 | S8                     | Sl       | Y           | –                        | –           | –             | –                           | –                        | Y            | –                    | Beloshistov et al. (2018); Reichardt et al. (2018) |
| Phytaspase | S8                     | Nt       | Y           | Y                       | Y           | –             | –                           | –                        | –            | Y                    | Chichkova et al. (2004, 2010) |
| SAS-1/-2   | S8                     | As       | Y           | –                        | N           | –             | –                           | –                        | –            | Y                    | Chichkova et al. (2010) |
| Gb/SBT1    | S8                     | Gb       | Y           | Y                       | Y           | –             | –                           | –                        | –            | –                    | Duan et al. (2016) |
| SBT3       | S8                     | Sl       | Y           | Y                       | Y           | –             | –                           | –                        | Y            | Y                    | Meyer et al. (2016a, b) |
| SBT3.3     | S8                     | At       | Y           | Y                       | Y           | –             | –                           | –                        | Y            | Y                    | Ramírez et al. (2013) |
| SBT3.5     | S8                     | At       | Y           | –                        | –           | –             | –                           | –                        | –            | –                    | Bethke et al. (2014); Sénchal et al. (2014) |
| Nb/SBT5.2  | S8                     | Nb       | Y           | –                        | –           | –             | –                           | –                        | –            | –                    | Wang et al. (2020); Paulus et al. (2020) |
| S/SBTc-3   | S8                     | St       | Y           | –                        | Y           | –             | –                           | –                        | Y            | Y                    | Fernández et al. (2012, 2015) |
| Hb/SPA     | S8                     | Hb       | Y           | –                        | Y           | Y             | –                           | –                        | –            | –                    | Ekchaweng et al. (2017) |

A total of 46 proteases are evaluated against the ABC criteria for apoplastic immune proteases: (A) apoplastic location; (B) biological function in immunity; and (C) catalytic activity as a protease. As, Avena sativa; At, Arabidopsis thaliana; Cp, Carica papaya; Cs, Citrus sinensis; Gb, Gossypium barbadense; Gm, Glycine max; Hb, Hevea brasiliensis; Hv, Hordeum vulgare; Mj, Mirabilis jalapa; Nb, Nicotiana benthamiana; Nt, Nicotiana tabacum; Os, Oryza sativa; Sl, Solanum lycopersicum; St, Solanum tuberosum; Ta, Triticum aestivum; Zm, Zea mays; Y (Yes), evidence that protein meets criteria; N (No), evidence that protein does not meet criteria; – protein not tested against criteria; *not shown in planta.
matrix metalloproteases (MMPs) of family M10 are the only plant metalloprotease family with apoplastic proteases. MMPs are zinc-dependent endopeptidases that share a conserved catalytic domain containing a zinc-binding sequence and a conserved Met residue that forms a ‘Met-turn’ (Marino and Funk, 2012). All plant MMPs contain a signal peptide and are either secreted into the plant apoplast or anchored to the plasma membrane (Flinn, 2008; Marino and Funk, 2012).

Serine (Ser) proteases are the largest class of proteases in plants and are unified by the use of Ser in the active site as a nucleophile (Van der Hoorn, 2008). Plants contain 14 Ser protease families which are grouped into nine evolutionarily unrelated clans. Subtilisin-like proteases (subtilases) of subfamily S8A are often secreted and are the most extensively studied (Schaller et al., 2018). Serine carboxypeptidase-like proteins (SCPLs) are found in MEROPS subfamily S10 and comprise another abundant serine hydrolase subfamily (Van der Hoorn et al., 2011). Whilst many SCPLs have carboxypeptidase activity (Li et al., 2001; Casamitjana-Martínez et al., 2003), some catalyse transacylation reactions rather than cleaving C-terminal peptide bonds (Mugford and Milkowski, 2012). All Arabidopsis SCPLs contain signal peptides targeting them for secretion (Fraser et al., 2005) and many SCPLs are detected in the apoplast (Sueldo et al., 2014; Grosse-Holz et al., 2018).

The MEROPS database divides proteases into a further three smaller classes, in addition to the four major classes discussed above. These are Asn peptide lyases, Glu proteases, and Thr proteases. Asn peptide lyases and Glu proteases have not been studied extensively and plant members are yet to be identified (Rawlings et al., 2011). Thr proteases such as Arabidopsis PBA1 form subunits of the 26S proteasome that mediates protein degradation (Tanaka, 2009). PBA1 has caspase-3-like activity and is involved in the induction of PCD upon infection with avirulent bacteria (Hatsugai et al., 2009), but is not an apoplastic immune protease. These three protease classes are not further discussed in this review because they do not contain apoplastic immune proteases.

**Meta-analysis of apoplastic immune proteases**

Scientific advances have identified and characterized an increasing number of apoplastic immune proteases. We undertook a meta-analysis of proteases that are both apoplastic and involved in immunity. We identified 46 putative apoplastic immune proteases, summarized in Table 1. For the purpose of the analysis, we counted orthologues of a gene in different species separately. Closely related paralogues from the same species that show indistinguishable immune phenotypes or act redundantly, CathB1, -B2, and -B3 in Arabidopsis for instance (McLellan et al., 2009), are counted only once.

To qualify as an apoplastic immune protease, experimental evidence is required for each of these three defining words: apoplastic, immune, and protease. This can be summarized as the ABC criteria: (A) apoplastic location; (B) biological function in immunity; and (C) catalytic activity as a protease. We critically evaluated each of the 46 putative apoplastic immune proteases for fulfilling these criteria, summarized in Table 1 and discussed below.

**Criterion A (apoplast): apoplastic location can be subjective and dynamic**

In total, 35 of the 46 identified proteins (76%) have been shown to be secreted, although whether a protein is considered apoplastic is dependent on definition. We consider a protease to be apoplastic if its catalytic domain is located outside of the plasma membrane. This encompasses the extracellular space and the xylem, but also the cell wall and latex. The picture is complicated when considering that proteins are often found in multiple locations within and outside the cell. The papain C14, for example, is found in the vacuoles, vesicles, endoplasmic reticulum, and apoplast of tomato (Bozkurt et al., 2011).

The literature is abounding with examples of proteases whose apoplastic location is predicted on the basis of the extracellular location of orthologous proteases, or the use of prediction software such as ApoplastP, which utilizes machine learning to predict the location based on amino acid enrichment and depletion patterns (Sperschneider et al., 2018), and SignalP, which predicts signal peptides and their cleavage sites (Almagro Armenteros et al., 2019). These methods of prediction are not infallible, and experimental evidence is usually obtained to confirm the location of the protease. This might include detection of the protease from apoplastic fluid or confocal microscopy with fluorescent fusion proteins.

Protein location is also dynamic, and both import to and export from the apoplast are inducible upon both abiotic and biotic stress. Proteases are released from the vacuole into the apoplast upon PCD induced by avirulent bacteria (Hatsugai et al., 2009). Conversely, phytaspase is constitutively secreted into the apoplast of tobacco and rice before being reimported into the cell upon PCD (Chichkova et al., 2010). Similarly, cotton subtilase GbSBT1 relocates from the apoplast to the cytoplasm during defence against the fungus *Verticillium dahliae* (Duan et al., 2016). Extracellular GbSBT1 interacts with the secreted *V. dahliae* effector prohibitin, which may trigger the movement of GbSBT1 into the cell (Duan et al., 2016).

**Criterion B (biological role)**

*B1: 17 proteases are genetically required for immunity*

Genetic requirement for pathogen resistance has been demonstrated for 17 of the 46 identified proteases (37%). For example, antisense *Pip1* tomato plants are highly susceptible to *Cladosporium fulvum*, *Pseudomonas syringae*, and *Phytophthora*...
infection (Planas-Marquès et al., 2018). However, not all immune proteases are induced. The oat subtilases SAS-1 and SAS-2 are constitutively transcribed and present in the cell, but apoplastic activity is induced by relocation into the apoplast at the onset of PCD induced by the fungal toxin victorin (Coffeen and Wolpert, 2004).

B3: identification of pathogen-derived inhibitors has been restricted to Cys and Ser proteases

Inhibition by pathogen-derived inhibitors has been described for 11 of the 46 immune proteases (24%). Notably, nine of these 11 proteases are Cys proteases. In fact, the Cys proteases Rcr3, Pip1, and C14 are each targeted by multiple inhibitors (Tian et al., 2007; Shabab et al., 2008; Kaschani et al., 2010; Lozano-Torres et al., 2012; Shindo et al., 2016), and the same inhibitor often targets multiple Cys proteases (Clark et al., 2018). The concept of adaptation of a pathogen and its inhibitor repertoire to its host is neatly demonstrated by the case of Phytophthora minabilis, an oomycete closely related to P. infestans, which infects the four-o’clock flower (Mirabilis jalapa). Whilst EPIC1 of P. infestans inhibits Solanum Rcr3, PipEPIC1 of P. minabilis has specialized to inhibit the Rcr3-related proteases MRPI and MRP2 of M. jalapa. The specialization of each effector to its corresponding protease was underpinned by a single amino acid polymorphism in the host protease along with a reciprocal single amino acid change in the pathogen effector (Dong et al., 2014).

Despite the bias in the literature towards the identification of Cys protease inhibitors, there are also some known examples of Ser protease inhibitors. Tomato P69B is inhibited by two Kazal-like Ser protease inhibitors of P. infestans, EP11 and EP110 (Tian et al., 2004, 2005). These inhibitors are distinct, differing in the number and sequence of the Kazal-like domains they possess. Inhibition of P69B by two divergent inhibitors suggests that this is an important infection strategy for the pathogen. The Phytophthora palmivora homologue of EP110 is secreted into the apoplast during infection of the rubber tree (Hevea brasiliensis) (Chinnapun et al., 2009) and inhibits the apoplastic subtilase HsSPA, which may otherwise mediate resistance to the pathogen (Ekchweng et al., 2017). Whilst inhibitors of apoplastic Asp and metalloproteases have not yet been reported, there is no inherent reason why they could not be targeted by pathogen-derived inhibitors.

B4: selection pressure acting on immune proteases is rarely investigated

Only two of the 46 immune proteases (4%) have been shown to be under diversifying selection: Rcr3 and StC14 (Shabab et al., 2008; Kaschani et al., 2010; Kaschani and Van der Hoorn, 2011). Their variant residues locate around the substrate-binding site as a footprint of an arms race with pathogen-derived inhibitors. The positions of the variant residues in Pip1 and P69B are also consistent with the presence of diversifying selection caused by pathogen-derived inhibitors (Shabab et al., 2008; Hörger and Van der Hoorn, 2013). This low number is not because of

In total, 38 of the 46 proteases (83%) are induced at the transcript level and/or protein level. This suggests that nearly all immune proteases are PR proteins, or alternatively that proteases that are not induced, but do function in immunity, are being overlooked. For instance, mRNA corresponding to the tomato Asp protease LeAspP is induced in leaves upon wounding (Schaller and Ryan, 1996), and there is a rapid accumulation of soybean GmMMP2 transcripts upon infection with Phytophthora sojae and P. syringae pv. glycinea (Liu et al., 2001). Likewise, experiments with coffee cultivars showed an increase in apoplastic Ser protease activity upon Hemileia vastatrix infection, particularly in resistant cultivars (Guerra-Guimarães et al., 2015), and activity-based protein profiling (ABPP; explained in section C1) uncovered an increased activity of Cys and Ser proteases Pip1, P69B, and P69C in tomato upon Ralstonia solanacearum infection (Planas-Marquès et al., 2018). However, not all immune proteases are induced. The oat subtilases SAS-1 and SAS-2 are constitutively transcribed and present in the cell, but apoplastic activity is induced by relocation into the apoplast at the onset of PCD induced by the fungal toxin victorin (Coffeen and Wolpert, 2004).

B3: identification of pathogen-derived inhibitors has been restricted to Cys and Ser proteases

Inhibition by pathogen-derived inhibitors has been described for 11 of the 46 immune proteases (24%). Notably, nine of these 11 proteases are Cys proteases. In fact, the Cys proteases Rcr3, Pip1, and C14 are each targeted by multiple inhibitors (Tian et al., 2007; Shabab et al., 2008; Kaschani et al., 2010; Lozano-Torres et al., 2012; Shindo et al., 2016), and the same inhibitor often targets multiple Cys proteases (Clark et al., 2018). The concept of adaptation of a pathogen and its inhibitor repertoire to its host is neatly demonstrated by the case of Phytophthora minabilis, an oomycete closely related to P. infestans, which infects the four-o’clock flower (Mirabilis jalapa). Whilst EPIC1 of P. infestans inhibits Solanum Rcr3, PipEPIC1 of P. minabilis has specialized to inhibit the Rcr3-related proteases MRPI and MRP2 of M. jalapa. The specialization of each effector to its corresponding protease was underpinned by a single amino acid polymorphism in the host protease along with a reciprocal single amino acid change in the pathogen effector (Dong et al., 2014).
negative results for the remaining proteases, but rather because this aspect has not been investigated for most immune proteases. Only SLIC14 is confirmed to be under stabilizing, rather than diversifying, selection (Shabab et al., 2008). The difference in selection pressure between tomato and potato C14 probably reflects the specialization of P. infestans to wild potato, its natural host (Kaschani et al., 2010).

**B5: two proteases are prevented from being secreted into the apoplast**

The prevention of protease secretion into the apoplast has been identified for two of the 46 proteases (4%). Secretion of the tomato Cys protease C14 is prevented by the P. infestans RxLR effector Atr12B, which accumulates around haustoria. An Atr12B mutant impaired in haustorial localization allowed apoplastic C14 accumulation and reduced the growth of P. infestans (Bozkurt et al., 2011). Likewise P. sojae, the cause of soybean stem and root rot, secretes the plasma membrane-localized RxLR-type effector PaAtt240 that prevents the secretion of the soybean Asp protease GmAPI into the apoplast (Guo et al., 2019). GmAPII positively contributes to resistance against Phytophthora species, and so, by preventing its secretion, GmAPII-mediated defence is compromised. In both instances, the underlying molecular mechanism is unknown.

**Criterion C (catalytic activity)**

**C1: protease activity is shown for most candidates**

Protease activity has been confirmed for 34 of the 46 identified proteases (74%). In particular, catalytic activity has been confirmed for the majority of the identified Cys proteases (81%). This probably reflects the relatively thorough characterization of papains and the tools available to monitor their activity.

Catalytic activity is demonstrated by the ability of a protease to degrade a biological or commercial substrate. For instance, gelatin and casein are general protease substrates that were used to confirm the protease activity of tomato Rcr3 and potato StSBTc-3, respectively (Krüger et al., 2002; Fernández et al., 2015). Similarly, myelin basic protein and Z-Leu-Arg-MCA have confirmed the catalytic activity of soybean metalloprotease GmMMP2 (Liu et al., 2001) and Nicotiana benthamiana Cys protease CP14 (Paired et al., 2016), respectively. Protease activity can also be inferred by ABPP. This technique uses chemical probes that mimic substrates but covalently bind to the active site of proteases as a readout for their activity (Morimoto and van der Hoorn, 2016). For example, ABPP labelling was used to infer the catalytic activity of Citrus sinensis RD21a (Clark et al., 2018), tomato P69C (Planas-Marquês et al., 2018), and NbSBTS2.5 (Paulus et al., 2020). Finally, catalytic activity is implicit if criterion C2 is satisfied, even if the activity has not been shown directly—for instance, an active site mutant of soybean Asp protease GmAPI no longer confers resistance to Phytophthora capsici, indicating that the wild-type protease must display catalytic activity (Guo et al., 2019).

**C2: the requirement of catalytic activity for biological function is rarely shown**

The active site of proteases has been shown to be required for the biological function of only nine of the 46 immune proteins (20%). The requirement for catalytic activity can primarily be shown by a catalytically inactive mutant no longer being able to perform the described immune function. For instance, catalytic mutants of Asp protease SAP1 are no longer able to suppress P. syringae growth (Wang et al., 2019). Similarly, tobacco silenced for PCD-promoting phytaspase show an abolished Tobacco mosaic virus (TMV)-induced HR that can be restored by complementation with wild-type rice phytaspase, but not its catalytic mutant (Chichkova et al., 2010).

Alternatively, the requirement for catalytic activity can be shown through the use of an inhibitor preventing the biological function of the protease. However, this can only be taken as evidence if only the protease in question is being inhibited. Since most commercial inhibitors are not specific to a single protease, the protease must be purified and inhibition studied in vitro. For instance, papain itself is released into the latex of papaya upon wounding, where it provides a strong toxicity and growth inhibition against a range of lepidopteran pests (Konno et al., 2004). Papaya leaves painted with the papain inhibitor E-64 support significantly greater larval growth, but this is not proof of the catalytic requirement of papain itself since E-64 may also inhibit other papain-like Cys proteases present in latex (Konno et al., 2004).

Likewise, the antimicrobial activity of extracellular Asp proteases StAPI and StAP3 was demonstrated by in vitro experiments showing the inhibition of P. infestans cyst and Fusarium solani conidia germination upon incubation with the purified proteases (Guevara et al., 2002, 2004). This antimicrobial activity can be abolished by the addition of the Asp protease inhibitor pepstatin A to the purified proteases (Guevara et al., 2002, 2004). Addition of pepstatin A to apoplastic fluid also increases susceptibility to F. solani, but this is not enough evidence that these proteases have antimicrobial activity in vivo, and that catalytic activity is required for this.

**Six functions of secreted immune proteases**

The molecular mechanism by which apoplastic proteases confer immunity appears resolved in eight cases: StAPI/3, SAP1/2, Mir1, papain, Rcr3, P69B, SPhyt-1/-2, and SBT3.5, although genetic requirement for the immune response has not yet been demonstrated for StAPI/3, papain, and SPhyt-1/-2. We divide these mechanisms into four groups: antimicrobial activity, immune hydrolase activation, damage-associated molecular pattern (DAMP) release, and effector perception. A single protease may act on multiple substrates and hence perform multiple functions, as is the case for tomato P69B (Paulus et al., 2020; Wang et al., 2020).
Apoplastic proteases display direct antimicrobial activity

Apoplastic proteases are in a perfect place to directly damage the pathogen, impeding growth and proliferation (Konno et al., 2004; Mendieta et al., 2006). One of the first papains to be characterized, MIR1 from maize has direct antimicrobial activity against insect pests. This was first indicated by growth inhibition of fall armyworm and tobacco budworm larvae feeding on callus expressing MIR1 (Pechan et al., 2000). Direct antimicrobial activity was later demonstrated by a sharp increase in the permeability of Spodoptera frugiperda peritrophic matrix to Blue Dextran upon the addition of purified MIR1 (Mohan et al., 2006). MIR1 accumulates at the wound site within 1 h of larval feeding, assisted by the protease’s movement through the plant vascular tissues (Lopez et al., 2007). The protease inhibits larval growth by degrading insect intestinal mucin (IIM), which cross-links the chitin fibrils in the peritrophic matrix (Fescemyer et al., 2013). This induces IIM permeabilization, which impairs digestion and nutrient absorption (Pechan et al., 2002; Mohan et al., 2006). Larvae compensate for this damage by altering their midgut transcriptome to up-regulate IIM replacement components (Fescemyer et al., 2013) and produce Cys protease inhibitors in the midgut (Li et al., 2009). MIR1 also provides enhanced resistance to corn leaf aphids above-ground and to western corn rootworm below-ground (Louis et al., 2015; Varsani et al., 2016).

Direct antimicrobial activity has also been shown for secreted aspartic protease 1 and 2 (SAP1 and SAP2) in Arabidopsis. Purified SAP1 and SAP2 are capable of suppressing P. syringae growth in vitro, demonstrating their antimicrobial activity (Wang et al., 2019). SAP1 and SAP2 contribute redundantly to defense against P. syringae through the cleavage of the bacterial protein MucD, which is required for bacterial growth (Wang et al., 2019). Cleavage of MucD by SAP1 and SAP2 suppresses bacterial growth without causing bacterial death. SAP homologues are found throughout the Brassicaceae family as well as in tomato and rice, suggesting that this role in immunity could be evolutionarily conserved (Wang et al., 2019).

Apoplastic proteases activating immune hydrolases

Apoplastic proteases are able to activate other immature hydrolases by removing inhibitory prodomains. For instance, tomato subtilase Pe69B cleaves after Asp residues to remove the prodomain of Rcr3, thereby activating this immune protease (Paulus et al., 2020). Nicotiana benthamiana subtilase SBT5.2 can also process proRcr3 into mature Rcr3, suggesting that activation of immune proteases by subtilases is common in Solanaceous plants (Paulus et al., 2020).

Likewise, Arabidopsis subtilase SBT3.5 activates pectin methylesterases (PMEs) such as PME17 (Sénéchal et al., 2014). PMEs are found in the apoplast and are responsible for the demethylation of homogalacturonan, the major constituent of pectin in the cell wall. Altering the properties of the cell wall can have dramatic consequences for the resistance of plants to pathogens, and there is evidence of both positive and negative roles for PMEs in immunity (Lionetti et al., 2007; Körner et al., 2009). PME17 is required for immunity because its transcripts are up-regulated in response to P. syringae and Alternaria brassicicola infection, and pme17 mutants are more susceptible to P. syringae (Bethke et al., 2014). This implicates SBT3.5 in immunity through the activation of a cell wall-modifying enzyme.

Apoplastic proteases mediating DAMP release

Another function of apoplastic proteases is mediating the release of peptides that act as DAMPs (Hou et al., 2019). Tomato phytasps SAP1 and SAP2 can process the defence peptide systemin from its precursor, prosystemin (Beloshistov et al., 2018). Systemin release is required for wound signalling, which is critical in the response to herbivory (Savatin et al., 2014). Prosystemin is found in the cell cytoplasm whilst phytasps are apoplastic (Chichkova et al., 2010). Therefore, the processing of prosystemin can occur only upon wounding, when cellular integrity is disrupted and the proteins can interact (Beloshistov et al., 2018). The relocation of phytasps into the cell during PCD (Chichkova et al., 2010) could also trigger systemin activation, providing a link between PCD and the systemic wound response in tomato (Ryan, 2000).

Maize papains are required for the processing of the propeptide Prozip1 to release Zip1, a small peptide DAMP that activates SA signalling (Ziemann et al., 2018). Zip1 release induces papain activation, thus establishing a positive feedback loop, and promotes SA-mediated defence responses including the up-regulation of defence-related genes such as those encoding chitinases and β-1,3-glucanases, and the mitigation of infection by biotrophic fungi (Ziemann et al., 2018). However, the individual protease responsible for Prozip1 processing has not yet been identified.

Apoplastic proteases perceive pathogen effectors

Two apoplastic immune proteases act in the perception of pathogen effectors. The tomato papain-like Rcr3 is critical for recognition of the pathogen effector Avr2 produced by the fungus C. fulvum. The complex formed when Avr2 inhibits Rcr3 is perceived by the LRR receptor-like protein Cf2, triggering the HR (Rooney et al., 2005). Rcr3 is essential for Avr2 perception and, in the absence of Cf2, Rcr3 does not contribute to immunity to C. fulvum, suggesting that it acts as a decoy mimicking the more abundant Rcr3 paralogue Pip1 (Ilyas et al., 2015). However, Rcr3 contributes to P. infestans resistance in the absence of Cf2, indicating also a direct role in immunity (Ilyas et al., 2015). Like C. fulvum, P. infestans secretes effectors to inhibit Rcr3 and other papains. Yet unlike Avr2, EPIC1 and EPIC2B are ‘stealthy’ effectors that inhibit host proteases without being detected by Cf2 (Song et al., 2009).
The second protease involved in effector perception utilizes a very different mechanism. The Cys-rich secreted protein PC2 produced by $P.\text{infestans}$ is cleaved by apoplastic subtilases including P69B, triggering an immune response including cell death, the accumulation of ROS, and the up-regulation of defence-related genes (Wang et al., 2020). This cleavage is essential for the PC2-triggered immune response and presumably produces a small peptide that is recognized at the cell surface. Interestingly, $P.\text{infestans}$ is able to inhibit PC2 cleavage and therefore cell death by producing Ser protease inhibitors such as EPI1 (Wang et al., 2020).

### Apoplastic proteases required for the HR

Several apoplastic proteases contribute to HR, although relatively little is understood about the mechanisms by which they regulate and initiate this process. For instance, overexpression and silencing of tobacco phytaspase results in TMV-induced lesions that are larger or smaller, respectively, than those of wild-type plants, providing evidence of a clear involvement of phytaspase in the HR. How this is achieved at the mechanistic level has not yet been elucidated (Chichkova et al., 2010).

The first subtilases associated with PCD, SAS-1 and SAS-2, were identified in 2004 and have caspase-6-like activity. These saspsases are thought to be involved in a signalling cascade leading to the cleavage of Rubisco (Coffeen and Wolpert, 2004). Similarly, increased caspase-3-like activity is implicated in the production of necrotic lesions in potato leaves that restrict $P.\text{infestans}$ growth (Fernández et al., 2012). This activity was later attributed to potato subtilase SSBTc-3 which was isolated from $P.\text{infestans}$-infected leaves (Fernández et al., 2015). A role in PCD for SSBTc-3 is further suggested by the ability of the purified protease to induce cytoplasmic shrinkage and decrease the viability of tomato cell cultures in vitro (Fernández et al., 2015). Likewise, the tomato phytaspases FPht-2, -3, -4, -5, and -6 were able to trigger cell death, observed by trypan blue staining, when overexpressed in tomato leaves (Reichardt et al., 2018).

Cathepsin B (CathB) is a papain implicated in PCD in both plants and animals. Silencing of CathB in N. benthamiana restricts HR triggered by both Envinia amylovora and P. syringae, compromising resistance (Gilroy et al., 2007). In Arabidopsis, three CathB genes have caspase-3-like activity and contribute redundantly to HR (McLellan et al., 2009; Ge et al., 2016). However, the importance of CathB in HR appears to be context dependent. For instance, NbCathB induces HR triggered by the Avr/R combination Avr3a/R3a, but is not required for HR triggered by Avr4/Ci4 (Gilroy et al., 2007). Likewise, AtCathB mutants are more susceptible to virulent $P.\text{syringae}$, yet, despite a reduction in AvrB/RPM1-mediated HR, AtCathB mutant plants are not compromised in reducing bacterial growth (McLellan et al., 2009). In spite of the evidence that CathB participates in HR regulation, the location of CathB action—aoplastic or intracellular—remains unclear. Studying the role of proteases during the HR is challenged by the fact that protease location cannot be resolved whilst the cell is undergoing PCD.

### Apoplastic proteases mediate SAR and priming

Three apoplastic proteases (AED1, CDR1, and SBT3.3) are involved in signalling leading to local and systemic defence responses, as well as priming the plant for future pathogen invasion. In each case, the substrates of the immune proteases and their position within the signalling cascade are not known.

Constitutive Disease Resistance 1 (CDR1) is an ‘atypical’ Arabidopsis pepsin-like Asp protease identified by T-DNA activation tagging (Xia et al., 2004). CDR1 overexpression results in increased resistance to virulent $P.\text{syringae}$ as well as a phenotype mimicking constitutive SAR activation, whilst antisense CDR1 lines show enhanced bacterial susceptibility (Xia et al., 2004). Upon pathogen invasion, CDR1 accumulates in the apoplast, where it induces both local and systemic defence responses in an SA-dependent manner. Activation of the systemic defence response relies on a mobile elicitor present in extracellular fluids and thought to be generated by CDR1, but its identity is still unknown (Xia et al., 2004). Likewise, rice OsCDR1 induces the expression of defence-related genes and enhances disease resistance to multiple pathogens when overexpressed in both rice and Arabidopsis (Prasad et al., 2009). Local OsCDR1 expression in Arabidopsis induces a systemic defence response, suggesting that CDR1 has a conserved function in SA-mediated disease resistance (Prasad et al., 2009). The contrasting roles for CDR1 and AED1 (Criterion B1) raise fascinating questions regarding their evolution and substrate selectivity.

Arabidopsis subtilase SBT3.3 is involved in the regulation of immune priming (Ramírez et al., 2013). Priming is the process by which plants mount a stronger and faster immune response. The expression of SBT3.3 is induced upon pathogen invasion, and sbt3.3 mutants are more susceptible to $P.\text{syringae}$ and Hyaloperonospora arabidopsidis. SBT3.3 primes plants for the transcriptional activation of defence-related genes following pathogen invasion by inducing chromatin remodelling in the form of activating histone marks at the promoters of SA-regulated defence-related genes including PR1, and at its own promoter, initiating a positive feedback loop (Ramírez et al., 2013). However, the substrate and the mechanism of SBT3.3 activity remain enigmatic.

### Outlook

There is no doubt that proteases play important roles in plant immunity, and the emerging picture is that these roles are very diverse. Unravelling these different roles holds several major challenges.

The first challenge lies in unravelling a robust proteolytic network with intrinsic redundancies. Genetic redundancy improves the robustness of defence when multiple proteins, each
with different sensitivities to, for example, pathogen-derived inhibitors, act on one or several substrates (Whitacre, 2012; Fares, 2015). A similar robust network has been described for diverse ‘helper’ and ‘sensor’ NLRs that confer immunity to a broad range of plant pathogens (Wu et al., 2017). The expansion of the P69 family in tomato (Jordà et al., 2000; Reichardt et al., 2018) as well as the clustering of papains in several plant families (Richau et al., 2012) support the concept of selection for redundant protease networks. Compensation between different protease classes, such as Asp and Cys proteases, is also increasingly likely because redundant proteases may cleave at different sites within the same region of a substrate to produce the same outcome.

A second challenge is to understand how apoplastic proteases are regulated. Activation of apoplastic proteases may be dependent on endogenous regulators (Zimmermann et al., 2016), proteolytic cascades (Paulus et al., 2020), pH (Meyer et al., 2016b), or redox status (Balakireva and Zamyatnin, 2019). Understanding how these elements coordinate protease function during pathogen invasion remains a challenge for future research.

The third, obvious challenge is to determine if the prevalence of Cys and Ser proteases, particularly papains and subtilases, is due to their relative importance in immunity, or the result of a research bias, sparked by leading examples and supported by robust detection assays. Future efforts should also include unbiased approaches to identify secreted immune proteases and consider less well-characterized proteases.

In addition, we are left with several intriguing questions. For example, which apoplastic proteases are responsible for DAMP and PAMP release in vivo? For instance, proteases responsible for releasing the bacterial PAMPs from flagellin and EF-Tu remain to be identified. Furthermore, how do secreted proteases act collectively and consecutively on substrates? In addition, how do plant proteases distinguish between plant and pathogen substrates in order to prevent self-degradation? Finally, are extracellular protease repertoires different between plant species and is this important for co-evolution with other secreted host proteins? Or for being a non-host? These are just a few of the fascinating remaining questions waiting to be answered.

Acknowledgements

We would like to thank other members of the Van der Hoorn and Preston labs and the reviewers for useful suggestions. This work was financially supported by the Biotechnology and Biological Sciences Research Council (BBSRC) Interdisciplinary Biosciences DTP (AG) and the European Research Council (ERC) consolidator grant 616449 ‘GreenProteases’ and BBSRC 18RM1 grant BB/S003193/1 ‘Pip1S’ (RvdH).

References

Alam MM, Nakamura H, Ichikawa H, Miyao A, Hirochika H, Kobayashi K, Yamaoka N, Nishiguchi M. 2014. Response of an aspartic protease gene OSAP77 to fungal, bacterial and viral infections in rice. Rice 7, 9.

Almagro Armenteros JJ, Tsirigkos GD, Sanderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nature Biotechnology 37, 420–423.

Avrova AO, Stewart HE, De Jong WD, Heilbronn J, Lyon GD, Birch PR. 1999. A cysteine protease gene is expressed early in resistant potato interactions with Phytophthora infestans. Molecular Plant-Microbe Interactions 12, 1114–1119.

Avrova AO, Taleb N, Rokka VM, et al. 2004. Potato oxysterol binding protein and cathepsin B are rapidly up-regulated in independent defence pathways that distinguish R gene-mediated and field resistances to Phytophthora infestans. Molecular Plant Pathology 5, 45–56.

Azarkan M, Dibiani R, Baulard C, Baeyens-Volant D. 2006. Effects of mechanical wounding on Carica papaya cysteine endopeptidase accumulations and activity. International Journal of Biological Macromolecules 38, 216–224.

Balakireva AV, Zamyatnin AA Jr. 2019. Cutting out the gaps between proteases and programmed cell death. Frontiers in Plant Science 10, 704.

Balakireva AV, Zamyatnin AA. 2018. Indispensable role of proteases in plant innate immunity. International Journal of Molecular Sciences 19, 629.

Beloshistov RE, Dreizler K, Galullina RA, et al. 2018. Phytopase-mediated precursor processing and maturation of the wound hormone systemin. New Phytologist 218, 1167–1178.

Bethke G, Grundman RE, Sreekanta S, Truman W, Katagiri F, Glazebrook J. 2014. Arabidopsis PECTIN METHYLESTERASEs contribute to immunity against Pseudomonas syringae. Plant Physiology 164, 1090–1107.

Bozkurt TO, Schomack S, Win J, et al. 2011. Phytophthora infestans effector AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. Proceedings of the National Academy of Sciences, USA 108, 20832–20837.

Breitenbach HH, Wenig M, Wittek F, et al. 2014. Contrasting roles of the apoplastic aspartyl protease APOPLASTIC, ENHANCED DISEASE SUSCEPTIBILITY1-DEPENDENT1 and LEGUME LECTIN-LIKE PROTEIN1 in Arabidopsis systemic acquired resistance. Plant Physiology 165, 791–809.

Casamitjana-Martínez E, Hofhuis HF, Xu J, Liu CM, Heidstra R, Scheres B. 2003. Root-specific CLE19 overexpression and the solf1/Tu suppressors implicate a CLV-like pathway in the control of Arabidopsis root meristem maintenance. Current Biology 13, 1435–1441.

Chichkova NV, Kim SH, Titova ES, Kalkum M, Morozov VS, Rubtsov YP, Kalinina NO, Taliansky ME, Vartapetian AB. 2004. A plant caspase-like protease activated during the hypersensitive response. The Plant Cell 16, 157–171.

Chichkova NV, Shaw J, Galullina RA, et al. 2010. Phytaspease, a relocasalised cell death promoting plant protease with caspase specificity. The EMBO Journal 29, 1149–1161.

Chinnapun D, Tian M, Day B, Churngchow N. 2009. Inhibition of a Hevea brasiliensis protease by a Kazal-like serine protease inhibitor from Hevea brasiliensis. Biotechnology 37, 420–423.

Christensen AB, Cho BH, Nasby M, Gregersen PL, Brandt J, Madriz-Ordeñana K, Collinge DB, Thordal-Christensen H. 2002. The molecular characterization of two barley proteases establishes the novel PR-17 family of pathogenesis-related proteins. Molecular Plant Pathology 3, 135–144.

Clark K, Franco JY, Schwizer S, et al. 2018. An effector from the Huanglongbing-associated pathogen targets citrus proteasomes. Nature Communications 9, 1718.

Coffeen WC, Wolpert TJ. 2004. Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in Avena sativa. The Plant Cell 16, 857–873.
Dixon MS, Golstein C, Thomas CM, Van der Biezen EA, Jones JDG. 2000. Genetic complexity of pathogen perception by plants: the example of Rcr3, a tomato gene required specifically by Cf-2. Proceeding of the National Academy of Sciences, USA 97, 8807–8814.

Dodds PN, Rathjen JP. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. Nature Reviews Genetics 11, 539–548.

Doehlemann G, Hemetsberger C. 2013. Apoplastic immunity and its suppression by filamentous plant pathogens. New Physiologist 198, 1001–1016.

Dong S, Stam R, Cano LM, et al. 2014. Effector specialization in a lineage of the Irish potato famine pathogen. Science 343, 552–555.

Duan X, Zhang Z, Wang J, Zuo K. 2016. Characterization of a novel cotton subtilisin-like gene GbSBT1 in response to extracellular stimulations and its role in verticillium resistance. PLoS One 11, e0153988.

Ekchaweng K, Evangelisti E, Schornack S, Tian M, Churngwch N. 2017. The plant defense and pathogen counterdefense mediated by Hevea brasiliensis serine protease HbSPA and Phytophthora palmivora extracellular protease inhibitor PpEP110. PLoS One 12, e0175796.

El Moussaoui A, Nijs M, Paul C, Wintjens R, Vincentelli J, Azarkan M, Looze Y. 2001. Revisiting the enzymes stored in the laticifers of Carica papaya in the context of their possible participation in the plant defence mechanism. Cellular and Molecular Life Sciences 58, 556–570.

Fares MA. 2015. The origins of mutational robustness. Trends in Genetics 31, 373–381.

Fernández MB, Daleo GR, Guevara MG. 2012. DEVDase activity is induced in potato leaves during Phytophthora infestans infection. Plant Physiology and Biochemistry 61, 197–203.

Fernández MB, Daleo GR, Guevara MG. 2015. Isolation and characterization of a Solanum tuberosum subtilisin-like protein with caspase-3 activity (StSBTc-3). Plant Physiology and Biochemistry 86, 137–146.

Fescemyer HW, Sandoya GV, Gill TA, Ozkan S, Marden JH, Duthe L. 2013. Maize toxin degrades peritrophic matrix proteins and stimulates compensatory transcriptome responses in fall armyworm midgut. Insect Biochemistry and Molecular Biology 43, 280–291.

Figueiredo A, Monteiro F, Sebastiana M. 2014. Subtilisin-like proteases in plant–pathogen recognition and immune priming: a perspective. Frontiers in Plant Science 5, 739.

Flinn BS. 2008. Plant extracellular matrix metalloproteinases. Functional Plant Biology 35, 1183.

Fraser CM, Rider LW, Chappie C. 2005. An expression and bioinformatics analysis of the Arabidopsis serine carboxypeptidase-like gene family. Plant Physiology 138, 1136–1148.

Ge Y, Cai YM, Bonnaud L, Rotari V, Danon A, McKenzie EA, McLellan H, Mach L, Gallois P. 2016. Inhibition of cathepsin B by caspase-3 inhibitors blocks programmed cell death in Arabidopsis. Cell Death and Differentiation 23, 1493–1501.

Gilroy EM, Hein I, van der Hoorn R, et al. 2007. Involvement of cathepsin B in the plant disease resistance hypersensitive response. The Plant Journal 52, 1–13.

Görlich J, Volrath S, Knauf-Beiter G, et al. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. The Plant Cell 8, 629–643.

Granell A, Bellés JM, Conejero V. 1987. Induction of pathogenesis-related proteins in tomato by citrus exocorticis viroid, silver ion and ethephon. Physiological and Molecular Plant Pathology 31, 83–90.

Grosse-Holz F, Kelly S, Blaskowski S, Kaschani F, Kaiser M, Van der Hoorn R. 2018. The transcriptome, extracellular proteome and active secretome of agroinfiltrated Nicotiana benthamiana cover a large, diverse proteome repertoire. Plant Biotechnology Journal 16, 1068–1084.

Guerra-Guimarães LL, Tenente RER, Pinheiro CC, Chaves IL, Silva MDC, Cardoso FF, Planchon SS, De Barros DRD, Renault JJ, Ricardo CPC. 2015. Proteomic analysis of apoplastic fluid of Coffea arabica leaves highlights novel biomarkers for resistance against Hemileia vastatrix. Frontiers in Plant Science 6, 478.

Guevara MG, Daleo GR, Oliva CR. 2001. Purification and characterization of an aspartic protease from potato leaves. Physiologia Plantarum 112, 321–326.

Guevara MG, Oliva CR, Huarte M, Daleo GR. 2002. An aspartic protease with antimicrobial activity is induced after infection and wounding in intercellular fluids of potato tubers. European Journal of Plant Pathology 108, 131–137.

Guevara MG, Oliva CR, Machinandiarena M, Daleo GR. 1999. Purification and properties of an aspartic protease from potato tuber that is inhibited by a basic chitinase. Physiologia Plantarum 106, 164–169.

Guevara MG, Veríssimo P, Pires E, Faro C, Daleo GR. 2004. Potato aspartic proteases: induction, antimicrobial activity and substrate specificity. Journal of Plant Pathology 86, 233–238.

Gumtow R, Wu D, Uchida J, Tian M. 2018. A Phytophthora palmivora extracellular cystatin-like protease inhibitor targets papain to contribute to virulence on papaya. Molecular Plant-Microbe Interactions 31, 363–373.

Guo B, Wang H, Yang B, et al. 2019. Phytophthora sojae effector PsAvh240 inhibits host aspartic protease secretion to promote infection. Molecular Plant 12, 552–564.

Harrak H, Azelmat S, Baker EN, Tabaeizadeh Z. 2001. Isolation and characterization of a gene encoding a drought-induced cysteine protease in tomato (Lycopersicon esculentum). Genome 44, 369–374.

Hatsugai N, Iwasaki S, Tamura K, Kondo M, Fuji K, Ogasawara K, Nishimura M, Hara-Nishimura I. 2009. A novel membrane fusion-mediated plant immunity against bacterial pathogens. Genes & Development 23, 2496–2506.

Höger AC, Van der Hoorn RAL. 2013. The structural basis of specific protease–inhibitor interactions at the plant–pathogen interface. Current Opinion in Structural Biology 23, 842–850.

Hou S, Liu Z, Shen H, Wu D. 2019. Damage-associated molecular pattern-triggered immunity in plants. Frontiers in Plant Science 10, 646.

Ilyas M, Höger AC, Bozkurt TO, et al. 2015. Functional divergence of two secreted immune proteases of tomato. Current Biology 25, 2300–2306.

Jashni MKH, Mehrabi R, Collemare J, Mesarich CH, de Wit PJ. 2015. The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant–pathogen interactions. Frontiers in Plant Science 6, 584.

Jones JD, Dangl JL. 2006. The plant immune system. Nature 444, 323–329.

Jordá L, Conejero V, Vera P. 2000. Characterization of P69E and P69F, two differentially regulated genes encoding new members of the subtilisin-like protease family from tomato plants. Plant Physiology 122, 67–74.

Jordá L, Coego A, Conejero V, Vera P. 1999. A genomic cluster containing four differentially regulated subtilisin-like processing protease genes is in tomato plants. Journal of Biological Chemistry 274, 2360–2365.

Kaschani F, Shabab M, Bozkurt T, Shindo T, Schornack S, Gu C, Ilyas M, Win J, Kamoun S, van der Hoorn RA. 2010. An effector-targeted protease contributes to defense against Phytophthora infestans and is under diversifying selection in natural hosts. Plant Physiology 154, 1794–1804.

Kaschani F, Van der Hoorn RA. 2011. A model of the C14–EPIC complex indicates hotspots for a protease–inhibitor arms race in the oomycete–plant interaction. Plant Signaling & Behavior 6, 100–112.

Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, Kohno K. 2004. Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. The Plant Journal 34, 552–555.

Kovács J, Godson and van der Hoorn 2016. Characterization of a novel membrane fusion–mediated protease in potato leaves during Phytophthora infestans infection. Plant Physiology 1794–1804.

Körner E, von Dahl CC, Bonaventure G, Baldwin IT. 2009. Pectin methylesterase NaPME1 contributes to the emission of methanol during insect herbivory and to the elicitation of defense responses in Nicotiana attenuata. Journal of Experimental Botany 60, 2631–2640.

Kovács J, Poór P, Szepesi Á, Tari I. 2016. Salicylic acid induced cysteine protease activity during programmed cell death in tomato plants. Biologia Futura 67, 148–158.

Krüger J, Thomas CM, Golstein C, Dixon MS, Smoker M, Tang S, Mulder L, Jones JDG. 2002. A tomato cysteine protease required for
Cf-2-dependent disease resistance and suppression of autonecrosis. Science 296, 744–747.

Li C, Song X, Li G, Wang P. 2009. Midgut cysteine protease-inhibiting activity in Trichoplusia ni protects the peritrophic membrane from degradation by plant cysteine proteases. Insect Biochemistry and Molecular Biology 39, 726–734.

Li D, Zhang H, Song Q, Wang L, Liu S, Hong Y, Huang L, Song F. 2015. Tomato SIS-MMP, a member of the matrix metalloproteinase family, is required for disease resistance against Botrytis cinerea and Pseudomonas syringae pv. tomato DC3000. BMC Plant Biology 15, 143.

Li J, Lease KA, Tax FE, Walker JC. 2001. BRS1, a serine carboxypeptidase, regulates BR1 signaling in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA 98, 5916–5921.

Lionetti V, Raiola A, Camardella L, Giovane A, Obel N, Pauly M, Favaron F, Cervone F, Bellincampi D. 2007. Overexpression of peptidyl methylesterase inhibitors in Arabidopsis restricts fungal infection by Botrytis cinerea. Plant Physiology 143, 1871–1880.

Liu H, Wang X, Zhang H, Yang Y, Ge X, Song F. 2008. A rice serine carboxypeptidase-like gene OsSISCPL1 is involved in regulation of defense responses against biotic and oxidative stress. Gene 420, 57–65.

Liu Y, Dammann C, Bhattacharya MK. 2001. The matrix metalloproteinase gene GmMMP2 is activated in response to pathogenic infections in soybean. Plant Physiology 127, 1788–1797.

Lopez L, Camas A, Shivaji R, Ankala A, Williams P, Luthe D. 2007. Mr-1-CFP, a novel defense cysteine protease accumulates in maize vascular tissues in response to herbivory. Planta 226, 517–527.

Louis J, Basu S, Varsani S, Castano-Duque L, Jiang V, Williams WP, Felton GW, Luthe DS. 2015. Ethylene contributes to maize insect resistance-1-mediated defense against the phloem sap-sucking corn leaf aphid. Plant Physiology 169, 313–324.

Lozano-Torres JL, Wilbers RH, Gawronska P, et al. 2012. Dual disease resistance mediated by the immure receptor Ct-2 in tomato requires a common virulence target of a fungus and a nematode. Proceedings of the National Academy of Sciences, USA 109, 10119–10124.

Maidment JM, Moore D, Murphy GP, Murphy G, Clark IM. 1999. Matrix metalloproteinase homologues from Arabidopsis thaliana—expression and activity. Journal of Biological Chemistry 274, 34706–34710.

Mandal MK, Fischer R, Schillingberg S, Schiermeyer A. 2010. Biochemical properties of the matrix metalloproteinase NIMP1 from Nicotiana tabacum cv. BY-2 suspension cells. Planta 232, 899–910.

Marino G, Funk C. 2012. Matrix metalloproteinases in plants: a brief overview. Physiologia Plantarum 145, 196–202.

Marino G, Huesgen PF, Eckhard U, Overall CM, Schröder WP, Funk C. 2014. Family-wide characterization of matrix metalloproteinases from Arabidopsis thaliana reveals their distinct proteolytic activity and cleavage site specificity. The Biochemical Journal 457, 335–346.

McLellan H, Gilroy EM, Yun BW, Birch PR, Loake GJ. 2009. Functional redundancy in the Arabidopsis cathespin B gene family contributes to basal defence, the hypersensitive response and senescence. New Physiologist 183, 408–418.

Mendieta JR, Pagano MR, Muñoz FF, Daleo GR, Guevara MG. 2006. Antimicrobial activity of potato aspartic proteases (ISAPs) involves membrane permeabilization. Microbiology 152, 2039–2047.

Meyer M, Huttenlocher F, Cezdich A, Procopio S, Stroeder J, Pauwels SJ, Bernardo-Faura ML, Paulus J, Kaschani F, Mehofer U, Tholen S, et al. 2013. An extracellular proteolytic cascade in tomato activates immune protease Rcr3. Proceedings of the National Academy of Sciences, USA 110, 17409–17417.

Mohan S, Ma PW, Pechan T, Bassford ER, Williams WP, Luthe DS. 2006. Degradation of the S. frugiperda peritrophic matrix by an inducible maize cysteine protease. Journal of Insect Physiology 52, 21–28.

Morel JB, Dangl JL. 1997. The hypersensitive response and the induction of cell death in plants. Cell Death and Differentiation 4, 671–683.

Morimoto K, van der Hoorn RA. 2016. The increasing impact of activity-based protein profiling in plant science. Plant & Cell Physiology 57, 446–461.

Mueller AN, Ziemann S, Treitschke S, Altmann D, Doehlemann G. 2013. Compatibility in the Ustilago maydis–maize interaction requires inhibition of host cysteine proteases by the fungal effector Pi2. PLoS Pathogens 9, e1003177.

Mugford ST, Milkowski C. 2012. Serine carboxypeptidase-like acyltransferases from plants. Methods in Enzymology 516, 279–297.

Muñoz FF, Mendieta JR, Pagano MR, Paggi RA, Daleo GR, Guevara MG. 2010. The swapasin-like domain of potato aspartic protease (StAsp-FPS1) exerts antimicrobial activity on plant and human pathogens. Peptides 31, 777–785.

Niño MC, Kang KK, Cho YG. 2020. Genome-wide transcriptional response of papain-like cysteine protease-mediated resistance against Xanthomonas oryzae pv. oryzae in rice. Plant Cell Reports 39, 457–472.

Okushima Y, Koizumi N, Kusano T, Sano H. 2000. Secreted proteins of tobacco cultured BY-2 cells: identification of a new member of pathogenesis-related proteins. Plant Molecular Biology 42, 479–488.

Paireder M, Mehofer U, Tholen S, et al. 2016. The death enzyme CP14 is a unique 33-kD cysteine-like protease with a pronounced S2 subsite selectivity. Archives of Biochemistry and Biophysics 603, 110–117.

Paulus JK, Kourielis J, Ramasubramanian S, et al. 2020. Extracellular proteolytic cascade in tomato activates immune protease Rcr3. Proceedings of the National Academy of Sciences, USA 117, 17409–17417.

Pechan T, Cohen A, Williams WP, Luthe DS. 2002. Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proceedings of the National Academy of Sciences, USA 99, 13319–13323.

Pechan T, Ye L, Chang Y, Mitra A, Lin L, Davis FM, Williams WP, Luthe DS. 2000. A unique 33-kD cysteine protease accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other Lepidoptera. The Plant Cell 12, 1031–1040.

Planas-Marqués M, Bernardo-Faura M, Paulus J, Kaschani F, Kaiser M, Valls M, van der Hoorn RAL, Coll NS. 2018. Protease activities triggered by Ralstonia solanacearum infection in susceptible and tolerant tomato lines. Molecular & Cellular Proteomics 17, 1112–1125.

Porokod A, Cirkniski A, Petrov D, et al. 2018. The two cathepsin B-like proteases of Arabidopsis thaliana are closely related enzymes with discrete endopeptidase and carboxydipeptidase activities. Biological Chemistry 399, 1223–1235.

Prasad BD, Creissen G, Lamb C, Chattoo BB. 2009. Overexpression of the peptidase domain of potato aspartic protease (StAsp-PSI) exerts antinastrophic activity on plant and human pathogens. Proc. Natl. Acad. Sci. USA 106, 18417–18422.

Reichardt S, Repper D, Tuzhikov AI, Galiluina RA, Planas-Marqués M, Chichkova NV, Vartapetian AB, Stintzi A, Schaller A. 2018. The tomato subtilase family includes several cell death-related proteases with caspase specificity. Scientific Reports 8, 10531.

Ramirez V, Lopez A, Mauch-Mani B, Gil MJ, Vera P. 2013. An extracellular subtilase switch for immune priming in Arabidopsis. PLoS Pathogens 9, e1003445.

Rawlings ND, Barrett AJ, Bateman A. 2011. Asparagine peptide lyases. Journal of Biological Chemistry 286, 38321–38328.

Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. 2008. MEROPS: the peptidase database. Nucleic Acids Research 36, D320–D325.

Reichardt S, Repper D, Tuzhikov AI, Galiluina RA, Planas-Marqués M, Chichkova NV, Vartapetian AB, Stintzi A, Schaller A. 2018. The tomato subtilase family includes several cell death-related proteases with caspase specificity. Scientific Reports 8, 10531.

Richau KH, Kaschani F, Verdoes M, Pansuriya TC, Niessen S, Stüber K, Colby T, Overkleeft HS, Bogoy M, Van der Hoorn RA. 2012. Subclassification and biochemical analysis of plant papain-like cysteine proteases displays subfamily-specific characteristics. Plant Physiology 158, 1583–1599.

Roeauty HCE, Van’t Klooster JW, Van der Hoorn RAL, Joosten MHAJ, Jones JDG, De Wit PJGM. 2005. Cladosporium Avr2 inhibits tomato Rcr3
A Kazal-like extracellular serine protease inhibitor from Phytophthora infestans targets the tomato pathogenesis-related protease P69B. Journal of Biological Chemistry 279, 26370–26377.

Tian M, Win J, Song J, van der Hoorn R, van der Knaap E, Kamoun S. 2007. A Phytophthora infestans cystatin-like protein targets a novel tomato papain-like apoplastic protease. Plant Physiology 143, 364–377.

Tornero P, Conejero V, Vera P. 1997. Identification of a new pathogen-induced member of the subtilisin-like processing protease family from plants. Journal of Biological Chemistry 272, 14412–14419.

Tornero P, Maya E, Gómez MD, Cañias L, Conejero V, Vera P. 1996. Characterization of LRP, a leucine-rich repeat (LRR) protein from tomato plants that is processed during pathogenesis. The Plant Journal 10, 315–330.

Van der Hoorn RAL. 2008. Plant proteases: from phenotypes to molecular mechanisms. Annual Review of Plant Biology 59, 191–223.

Van der Hoorn RA, Colby T, Nickel S, Richau KH, Schmidt J, Kaiser M. 2011. Mining the active proteome of Arabidopsis thaliana. Frontiers in Plant Science 2, 89.

van der Linde K, Hemesberger C, Kastner B, Kaschani F, van der Hoorn RA, Kumlehn J, Doehlemann G. 2012. A maize cystatin suppresses host immunity by inhibiting apoplastic cysteine proteases. The Plant Cell 24, 1285–1300.

Varsani S, Basu S, Williams WP, Felton GW, Luthe DS, Louis J. 2016. Intraplantar communication in maize contributes to defense against insects. Plant Signaling & Behavior 11, e1212800.

Wang S, Xing R, Wang Y, et al. 2020. Cleavage of a pathogen apoplastic protein by plant subtilases activates immunity. New Phytologist doi: 10.1111/nph.17120.

Wang Y, Garrido-Oter R, Wu J, Winkelmüller TM, Agler M, Colby T, Nobori T, Kemen E, Tsuda K. 2019. Site-specific cleavage of bacterial MucD by secreted proteases mediates antibacterial resistance in Arabidopsis. Nature Communications 10, 1–12.

Whitacre JM. 2012. Biological robustness: paradigms, mechanisms, and systems principles. Frontiers in Genetics 3, 67.

 Wu CH, Abd-El-Halim A, Bozkurt TO, Belhaj K, Terauchi R, Vossen JH, Kamoun S. 2017. NLR network mediates immunity to diverse plant pathogens. Proceedings of the National Academy of Sciences, USA 114, 8113–8118.

Xia Y, Suzuki H, Borevitz J, Blount J, Guo Z, Patel K, Dixon RA, Lamb C. 2004. An extracellular aspartic protease function in Arabidopsis disease resistance signaling. The EMBO Journal 23, 980–988.

Xie W, Goodwin PH. 2009. A PRR27 gene of Nicotiana benthamiana contributes to resistance to Pseudomonas syringae pv. tabaci but not to Colletotrichum destructivum or Colletotrichum orbiculare. Functional Plant Biology 36, 351–361.

Zhang P, Zhang F, Liu D, Imani J, Langen G, Kogel KH. 2017. Matrix metalloproteinases operate redundantly in Arabidopsis immunity against necrotrophic and biotrophic fungal pathogens. PLoS One 12, e0183577.

Zhao Y, Thilmony R, Bender CL, Schaller A, He SY, Howe GA. 2003. Virulence systems of Pseudomonas syringae pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. The Plant Journal 36, 485–499.

Zimmermann D, Gomez-Barrera JA, Pasule C, Brack-Frick UB, Siefker E, Nicholson TM, Pfannstiel J, Stintzi A, Schaller A. 2016. Cell death control by matrix metalloproteinases. Plant Physiology 171, 1456–1469.

Zipfel C. 2014. Plant pattern-recognition receptors. Trends in Immunology 35, 345–351.