Plant proteases in the control of the hypersensitive response.

Jose Salguero-Linares and Núria S. Coll*

Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, Bellaterra, Barcelona, 08193, Spain.

***Correspondence to**: Nuria Sanchez-Coll: nuria.sanchez-coll@cragenomica.es

Highlight: This review emphasizes the crucial functions of plant proteases during the regulation of defence-related hypersensitive cell death.

Abstract

The hypersensitive response (HR) is a plant defence reaction triggered by activation of immune receptors upon pathogen recognition. It results in rapid cell death at the attempted invasion site, confining the pathogen and sending signals to distal parts of the plant that can in turn activate defences for subsequent attacks. HR cell death is a highly controlled phenomenon, requiring the concerted action of diverse plant proteases and regulatory mechanisms to keep it efficient yet confined.

Research in the last decade has significantly contributed to a better understanding of the mechanisms leading to HR, although our knowledge about the pathways that regulate this form of programmed cell death still remains scattered. In this review, we explore current knowledge on plant proteases as HR regulators. Proteases are key regulatory enzymes that not only serve degradative purposes, but also have very important signalling roles. In animals, caspases have been shown to be the major regulators and executioners of programmed cell death. Plants do not have caspases, and instead, programmed cell death is carried out by caspase-like and other protease activities owing to different protease classes. Here, we will summarise the mechanistic roles of plant proteases whose role in HR regulation is relatively well understood, which includes members of the cysteine, threonine and serine protease families.

Introduction

Due to their lack of physical mobility, plants must defend themselves against rapidly evolving pathogens. Unlike animals, plants do not possess an adaptive immune system with mobile defender cells, thus they rely on each cells´ innate immunity for effective defence responses (Jones and Dangl, 2006). In what is known as gene-for-gene interactions, plant *resistance (R)* gene products, such as surface-localised and intracellular nucleotide-binding leucine rich repeat (NB-LRR) immune receptors, perceive *avirulent (avr)* pathogen-derived gene products, also known as effector proteins, often leading to a form of confined programmed cell death, known as the hypersensitive response (HR).

The first reports on HR date back to the beginning of the 20th century when H. Marshall Ward described a variable discoloration of leaves that turned from yellow to brown/black when infected with the leaf rust *Puccina dispersa* (Ward, 1902). Additional studies at the time on the plant pathosystems *Chrysanthemum*-Uredo *(Puccinia) chrysanthemi* and wheat–*Puccinia glumarum* (leaf yellow rust), also claimed such cell death phenomenon upon pathogen infection (Gibson, 1904; Marryat, 1907). Nevertheless, it was not until 1915 that the term “hypersensitiveness” was intuitively used by Elvin C. Stackman to convey an “abnormal rapid cell death” in cereal crops when attacked by black stem rust fungus (*Puccina graminis*) (Stakman, 1915). Since the plant exhibited hypersensitiveness at the fungal entry sites, the fungus was unable to develop normally, thus the plant was deemed resistant. This phenotypic definition of HR has remained partially unchanged over the years, though in certain pathosystems we know now that HR is often uncoupled from resistance (Coll et al., 2011).

Defining HR cell death has not been an easy task due to its mixed morphological and biochemical features, partly resembling several other forms of cell death in both plants and mammals. In mammals, up to 12 types of programmed cell death (PCD, also known as regulated cell death) modalities have been described so far (Galluzzi et al., 2018). Amongst them, apoptosis is the best characterised. Apoptosis is a non-inflammatory process mainly regulated by caspases, in which the following features are observed:

cytoplasmic shrinkage, chromatin condensation, nuclear fragmentation and plasma membrane blebbing, which ultimately lead to the formation of intact vesicles (apoptotic bodies) that are engulfed and digested by phagocytes (Galluzzi *et al.*, 2018). Whilst certain features such as cytoplasmic shrinkage and chromatin condensation are also observed during HR, other events like phagocytosis of apoptotic bodies after cellular demise do not occur in plants (Table 1). As a result, resemblance of specific aspects of HR to apoptosis is not sufficient to consider HR as an apoptotic-like cell death. On the other hand, HR-presents the majority of morphological features of plant regulated necrosis cell death wherein mitochondrial swelling, shrinkage of the protoplast and early rupture of the plasma membrane is observed. These features can also be found in other types of mammalian PCD such as pyroptosis or necroptosis (Table 1) (Galluzzi et al., 2018). However, characteristics reminiscent of plant vacuolar cell death such as enlargement of the vacuole and tonoplast rupture are also exhibited during HR (van Doorn *et al.*, 2011). When considering the cytological features of HR, it is also of great importance to consider the nature of the invading pathogen. For instance, vacuolar rupture can be an effective measure to restrict viruses that proliferate in the host cytoplasm (Hatsugai *et al.*, 2004). By contrast, fusion of the tonoplast with the plasma membrane allows discharge of antimicrobial compounds to the intercellular space where bacterial pathogens tend to proliferate (Hatsugai *et al.*, 2009). In summary, HR is an atypical and confined plant cell death modality that occurs at the site of successful recognition of pathogens, and generally displays the following hallmarks: cytoplasmic shrinkage, mitochondrial swelling, chromatin condensation, chloroplast and plasma membrane disruption and vacuolisation (Table 1). Interestingly, necrotrophic pathogens such as the fungus *Cochliobolus victoriae* can hijack the HR machinery through the delivery of toxins that target the plant cell in order to kill it and feed on cell remnants (Lorang *et al.*, 2012). In the course of this cell death, expected features that resemble necrosis (protoplast shrinkage and a mitochondrial permeability transition) are displayed, though membrane and tonoplast integrity is maintained (Curtis and Wolpert, 2004).

Despite its early discovery more than a century ago, a thorough understanding of the mechanisms regulating HR is lacking. In the last few decades, a growing

body of evidence indicates that plant proteases are involved in pathogen perception and induction of effective local and systemic defence responses, that are often accompanied by an HR-related cell death confined to the attempted pathogen ingress site (Bozkurt *et al.*, 2011; Coll *et al.*, 2010; Rooney *et al.*, 2005)

Proteases are ubiquitous enzymes required for the correct functioning of living cells. Operating at the post-translational level, proteases catalyse irreversible hydrolytic reactions wherein peptide bonds of target substrates are cleaved, giving rise to new protein products. (van der Hoorn, 2008). Whilst traditionally believed to act solely as destructing enzymes, we currently know that proteases can also influence the activity of other proteins, regulate protein fate and localisation, modulate protein-protein interactions, and contribute to processing of cellular information through signal transduction (Turk, 2006).

Based on the MEROPs database, an integrated information resource of proteases (http://merops.sanger.ac.uk), there are five mechanistic classes of proteases found on living organisms depending on the catalytic residue involved in the cleavage of the substrate peptide bond, namely cysteine, aspartate, threonine, serine and metalloproteases (Rawlings *et al.*, 2018). In the case of cysteine, threonine and serine proteases, the orchestrated action of a catalytic triad comprised of a nucleophile (Cys, Thr or Ser), a base (usually His) and in certain cases an acid (Asp), allows cleavage of the peptide bond (Lopez-Otin and Bond, 2008). A second classification in the MEROPS database discriminates between clan types (usually denoted by a letter) where proteases fall into distinct categories depending on their protein tertiary structure. Within each clan, a third and final subdivision is made into distinct families of proteases based on their evolutionary relationships (Rawlings *et al.*, 2018).

As occurs in animals, plant proteases are directly implicated in the regulation of host responses to pathogen infection, including PCD. This review is intended to underscore the crucial functions of the distinct classes of plant proteases in the regulation of HR. Due to space limitations, we will only emphasise proteases whose mechanistic roles in HR regulation are well understood, which involve members of the cysteine, threonine and serine protease class.

Cysteine proteases: PLCPs, Metacaspases and VPEs

In mammals, apoptosis requires an evolutionarily conserved group of cysteine proteases termed caspases. Since particular characteristics are shared between animal apoptosis and the defence-related hypersensitive cell death in plants, it was reasoned in the past that a certain level of conservation on the molecular components involved in PCD should be present across kingdoms (del Pozo and Lam, 1998). Nevertheless, although certain structurally unrelated plant proteases have been shown to exhibit caspase-like activities in the course of defence-related HR (Chichkova *et al.*, 2004; del Pozo and Lam, 1998; Hatsugai *et al.*, 2004), no caspase homologues are found within plant genomes. Plant genomes encode approximately 140 cysteine proteases which fall into 5 distinct clans. In the context of plant-pathogen interactions, the CA clan, comprising proteases with a papain-like fold named papain-like cysteine proteases (PLCPs), and the CD clan, comprising proteases with a caspase-like fold, have been well documented (Misas-Villamil *et al.*, 2016). Biochemical tools such as specific protease inhibitors as well as activity-based probes have been pivotal in the discovery of many cysteine proteases implicated in plant defence by monitoring their protease activity (van der Hoorn and Kaiser, 2012). Here, we will discuss the role in HR of 3 PLCPs (Cathepsin B, Rcr3, Pip1), 3 Metacaspases (AtMC1, AtMC2 and AtMC4) and the Vacuolar Processing Enzymes (VPEs).

PLCPs

PLCPs are released as a preproteases bearing a signal peptide at the N-terminal end, an autoinhibitory domain or prodomain and the catalytic domain (bearing the catalytic triad: Cys, His and Asn). A granulin domain with unknown function is usually present at the C-terminus. PLCPs are predominately secreted into the apoplast, a major battleground wherein the fate of either a successful pathogen infection or an effective plant defence response is dictated (Figure 1) (Du *et al.*, 2016).

A first evidence for a role of Cathepsin B (CathB) in PCD came from studies in animals where CathB was shown to activate caspases (Kingham and Pocock, 2001) and cathepsin B knock-out mice exhibited impaired apoptosis (Guicciardi *et al.*, 2001). Thereafter plant CathB was proved to be involved in the regulation defence-related HR and basal disease resistance (Gilroy *et al.*, 2007; McLellan *et al.*, 2009). In plants, CathB is activated in the apoplast upon secretion. Through the use of specific animal CathB inhibitors and virus-induced gene silencing (VIGS) of potato cathepsin B (*StCathB*), Gilroy and co-workers demonstrated that the HR elicited by two bacterial pathogens, *Erwinia amylovora* and *Pseudomonas syringae* pv. tomato (*Pst*) DC3000, was remarkably impaired in the absence of CathB, resulting in enhanced disease susceptibility in *N. benthamiana* (Figure 1) (Gilroy *et al.*, 2007). Likewise, transient co-expression of the pathogen-derived effector Avr3a from *Phytophthora infestans* and the potato NB-LRR R3a resulted in compromised HR when CathB transcript levels were reduced (Table 2) (Armstrong *et al.*, 2005) Conversely, VIGS of cathepsin B in *N. benthamiana* did not attenuate HR following perception of *Cladosporium fulvum* effector Avr4 by the plant receptor-like protein Cf-4 (Gilroy *et al.*, 2007). In Arabidopsis, although required for basal resistance to *Pst, AtCathB*1-3 genes are dispensable for avirulent *R*-gene mediated resistance to strains carrying the effectors AvrB and AvrRps4. Interestingly, *AtCathB*1-3 genes act redundantly to positively regulate HR development triggered by *Pst* strains expressing AvrB, owing to the fact that *atcathb* triple mutants, but not double or single mutants *atcathb* lines, exhibit abrogated HR (Figure 1 and Table 2) (McLellan *et al.*, 2009). Taken together, these observations indicate that CathB is not a universal HR regulator and its role in defence-related HR seems to be pathogen-specific.

The tomato cysteine proteases, Rcr3 and PHYTOPTHORA INHIBITED PROTEASE 1 (Pip1), are two interesting examples of secreted PLCPs that mediate pathogen perception in the apoplast. These pathogenesis-related proteases are targeted by phylogenetically unrelated pathogens and appear to be under strong diversifying selection (Shabab *et al.*, 2008). The fungal pathogen *C. fulvum* secretes the effector Avr2 into the apoplast. Avr2 binds and inhibits Rcr3 and Pip1, forming Avr2-Rcr3 and Avr2-Pip1 complexes,

respectively (Figure 1) (Rooney *et al.*, 2005; Shabab *et al.*, 2008). Avr2-mediated perturbations on Rcr3 are perceived by the LRR-containing receptor-like protein (RLP) Cf-2, triggering HR which, in this case, results in full resistance to *C. fulvum* (Figure 1 and Table 2) (Rooney *et al.*, 2005). Interestingly, *rcr3* mutant lines do not exhibit higher susceptibility compared with tomato lines missing the *Cf-2* gene cluster, implying that Rcr3 inhibition does not contribute to virulence (Dixon *et al.*, 2000). Moreover, Pip1 accumulates to higher levels compared to Rcr3 in the apoplast upon SA treatment or diverse pathogen infections. Hence, in agreement with the decoy model, it can be hypothesised that Rcr3 evolved as a decoy to perceive effector-mediated perturbations and that the original operational target of Avr2 is Pip1 (Shabab *et al.*, 2008; van der Hoorn and Kamoun, 2008). In addition to *C. fulvum,* the oomycete, *Phytophthora infestans*, and the root parasitic nematode *Globodera rostochiensis* are also able to inhibit Rcr3 via secretion of apoplastic effectors EPIC1 and EPIC2B, and Gr-VAP1, respectively (Lozano-Torres *et al.*, 2012; Song *et al.*, 2009). However, whilst the weak interaction between EPICs and Rcr3 is not sufficient to trigger HR, complex formation between the allergen-like effector Gr-VAP1 and Rcr3 is sensed by the guardian Cf-2 which ultimately induce HR at the attempted site of infection (Table 2) (Lozano-Torres *et al.*, 2012; Rooney *et al.*, 2005; Song *et al.*, 2009). Consequently, Rcr3 exemplifies a striking example of antagonistic evolutionary arms race wherein a plant PLCP evolved as a decoy to trap diverse pathogen effectors into a recognition event.

VPEs

VACUOLAR PROCESSING ENZYMEs (VPEs) are cysteine proteases of the CD clan C13 family which cleave their substrate after asparagine or aspartate residues (Thomas and van der Hoorn, 2018). Despite their low sequence similarities, VPEs are evolutionarily related and share structural homology to caspases (Misas-Villamil *et al.*, 2013). Moreover, they exhibit caspase 1-like activity. By means of VPE inhibitors and VIGS experiments of VPEs, it was revealed that HR triggered by tobacco mosaic virus (TMV) in *N. benthamiana*

carrying an *N* resistance gene, requires active VPEs (Figure 1 and Table 2) (Hatsugai *et al.*, 2004). A prerequisite of HR is the rupture of the tonoplast through vacuolar collapse and the subsequent release of hydrolytic enzymes to the cytoplasm. Notably, vacuoles of TMV-infected plants deficient in VPEs were similar to wild type plants, suggesting that VPEs are necessary for tonoplast disruption (Hatsugai *et al.*, 2004). Besides their role in TMV-triggered cell death, VPEs are also required for an HR-like cell death triggered by the bacterial elicitor harpin and by fumonisin B1 (FB1), a toxin naturally produced by the maize necrotrophic fungal pathogen *Fusarium moniliforme* (Figure 1 and Table 2) (Kuroyanagi *et al.*, 2005; Zhang *et al.*, 2010). Remarkably, a necrotrophic pathogenic strategy to induce cell death and an HR-like cell death response mediated by the host as a plant defence strategy are both mediated by VPEs. Nonetheless, VPEs are not universal HR regulators. HR-like cell death induced by elicitors such as fungal nep1 and oomycete boehmerin do not require VPE activity (Zhang *et al.*, 2010). Moreover, in the course of compatible interactions between the oomycete obligate biotroph *Hyaloperosnospora arabidopsis (Hpa)* and Arabidopsis, a host VPE (γVPE) activity is increased upon infection, leading to enhanced disease susceptibility. Since sporulation of *Hpa* on *vpe* mutant plants is remarkably reduced, it can be hypothesised that VPEs play a role during compatible interactions that is independent of cell death (Misas-Villamil *et al.*, 2013). Collectively, it can be concluded that the role of VPEs in host pathogen-triggered PCD also appears to be dependent on the pathosystem.

Metacaspases

Along with VPEs in the CD clan and belonging to the C14 family, are metacaspases (Rawlings *et al.*, 2018). Metacaspases are an ancient group of cysteine proteases found in protozoa, fungi, plants and bacteria, predominantly known for their pivotal roles in PCD of non-metazoan organisms (Minina *et al.*, 2017). From an evolutionary standpoint, metacaspases are distantly related to animal caspases, though bioinformatic analysis predict close structural homology to animal caspases at the catalytic domain which harbours a

caspase-like His-Cys catalytic dyad and a caspase-hemoglobinase fold (Tsiatsiani *et al.*, 2011). With regards to their biochemical features, metacaspases are quite distinct compared to caspases, owing to their lack of aspartate specificity and their preference for substrate cleavage after Arg or Lys residues (Gonzalez *et al.*, 2007; Vercammen *et al.*, 2004; Watanabe and Lam, 2005). Metacaspases are classified into type I and type II based on their domain architecture. In plants, type I metacaspases bear an N-terminal prodomain extension that is absent in type II metacaspases. Type II metacaspases, however, possess an extended linker region between catalytic subunits and the C-terminus (Tsiatsiani *et al.*, 2011). Evidence for a direct role of metacaspases in HR came mainly from studies in Arabidopsis wherein an upregulation of type I metacaspase 1 (AtMC1) was initially reported upon pathogen infection (Zimmermann *et al.*, 2004). Thereafter genetic analysis of AtMC1 function through knockout mutants revealed that AtMC1 is required for the HR-like runaway cell death phenotype of the lesion mimic mutant *lesion stimulating disease 1 (lsd1)* (Coll *et al.*, 2010). In parallel, *atmc1* plants exhibit suppression of HR triggered by infection with an avirulent strain of *Pst* DC3000 (*AvrRpm1*) (see Figure 1) or an avirulent strain of the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) (Table 2). Of note, pathogen growth is unaffected in *atmc1* plants, providing another example of HR uncoupled from disease resistance. Interestingly, AtMC2, a closely related type I metacaspase in Arabidopsis, genetically serves the opposite function of AtMC1 by negatively regulating HR as *AtMC2* overexpression phenocopies the abrogated HR phenotype of *atmc1* mutant plants, whereas *atmc2* mutants show exacerbated HR (Figure 1 and Table 2). Remarkably, whilst AtMC1 function is dependent on its catalytic activity, AtMC2 exerts its negative HR regulation in spite of the presence or absence of its cysteine catalytic residue (Coll *et al.*, 2010).

Being such a potent HR mediator, plant cells must ensure appropriate AtMC1 activation under distinct stress settings. Consequently, besides the negative regulation of AtMC1 mediated by AtMC2, plants have evolved alternative means to keep AtMC1 at bay under basal conditions. LSD1 negatively regulates AtMC1 by directly interacting with the LSD1-like zinc finger region of the N-terminal prodomain of AtMC1 (Coll *et al.*, 2010). Presumably, such interaction

with the prodomain impedes autoprocessing of AtMC1, thus preventing its activation. Furthermore, AtSERPIN1, functions as a suicide inhibitor by covalently and irreversibly inhibiting AtMC1 (Asqui *et al.*, 2018).

In parallel to type I metacaspases, the constitutively expressed Arabidopsis type II metacaspase, AtMC4, has been found to contribute to the HR-like cell death response triggered by fungal mycotoxin FB1 and avirulent *Pseudomonas syringae* pv. maculicola ES4326 carrying AvrRpt2 (*Pma AvrRpt2*) (Figure 1 and Table 2) (Watanabe and Lam, 2005). Two independent knock out mutant lines of AtMC4 display attenuated and delayed HR-like cell death upon mycotoxin-treatment and *Pma* (*AvrRpt2*) infection, respectively. Conversely, AtMC4 overexpressor lines treated with mycotoxin FB1 induce a more pronounced HR-like cell daeth when compared to wild-type plants. Notably, during mycotoxin FB1-induced HR-like cell death, catalytic activity and self-processing of AtMC4 in the cytosol is of critical importance to exert a wild-type-like cell death response (Watanabe and Lam, 2011).

Although solid evidence for the implication of AtMC1, AtMC2 and AtMC4 in HR regulation exists, the molecular mechanism by which metacaspases exerts its pro-death function during mycotoxin FB1 treatment and downstream of NB-LRR activation is far from clear. Future elucidation of metacaspase physiological substrates in the course of pathogen infection by means of protein degradomics studies will be of critical importance to complement our fragmented knowledge of HR.

Threonine proteases: PBA1 subunit of the proteasome

The ubiquitin-proteasome system (UPS) is a protein degradation system that has long been known for its role in many fundamental cellular processes, including plant immunity (Ustun *et al.*, 2016). Ubiquitinated proteins destined for degradation are recognised and degraded by the 26S proteasome, an ATP-dependent protease complex comprised of 31 subunits that are further subdivided into two subcomplexes, the 20S core protease (CP) and the 19S regulatory particles (RPs). Owing to its caspase 3-like activity, one of the

subunits of the CP subcomplex, PBA1/β1, has been deeply scrutinised in the context of HR (Hatsugai *et al.*, 2009). PBA1/β1 is a threonine protease which belongs to the PB clan and T1 family of cysteine proteases in Arabidopsis (Thomas and van der Hoorn, 2018). In the course of an avirulent bacterial infection, the central vacuole of plant cells fuses with the plasma membrane. By doing so, anti-microbial hydrolytic enzymes can be released to the apoplast where bacteria proliferate (Hatsugai *et al.*, 2009). Upon Arabidopsis infection with avirulent *Pst* DC3000 carrying AvrRpt2 or AvrRpm1, inhibition of the PBA1/β1 subunit of the proteasome through caspase 3 and proteasome specific inhibitors, impede vacuolar membrane fusion with the plasma membrane, which supposedly prevents discharge of anti-microbial enzymes into the apoplast (Figure 1 and Table 2). In the same manner, Arabidopsis *PBA1/β1*-silenced plants exhibit the exact same phenotype. Consequently, HR is remarkably reduced in Arabidopsis PBA1/β1 defective plants compared to wild-type controls, and such impairment is dependent on the caspase 3-like activity of PBA1/β1 (Figure 1 and Table 2). Of note, concomitant with the reduction of HR is an increase in plant susceptibility to the above-mentioned avirulent bacterial strains (Hatsugai *et al.*, 2009). Other catalytic subunits of the proteasome such as PBB and PBE do not exhibit caspase-3 like activity, though silencing of *PBB* and *PBE* replicates the HR suppression observed in PBA1/β1 deficient plants (Hatsugai *et al.*, 2009). Finally, a subunit of the RP subcomplex, RPN1a is required for effective resistance to powdery mildew and mildew-induced cell death. Perturbation of other subunits of the proteasome such as RPT2a and RPN8a also impair powdery mildew resistance and mildew-induced cell death (Yao *et al.*, 2012). Nevertheless, *rpn1a* mutant Arabidopsis plants infected with avirulent *Pst (AvrRpt2)* and *Pst (AvrRPS4)* display normal HR induction compared to wild-type plants, suggesting a powdery mildew-specific function of RPN1a subunit during mildew-induced cell death (Yao *et al.*, 2012).

Subtilisin-like proteases: saspases and phytaspases

Given the importance of caspases in animal PCD processes, over the past few decades the hunt for caspase-like proteases in plants has been unceasingly

intense. A thorough scrutiny of caspase-like activities in plants led to the conclusion that, although sharing minor structural resemblance to animal caspases, the majority of caspase-like activities displayed in plants can be attributed to subtilisin-like proteins or subtilases (Vartapetian *et al.*, 2011). Subtilases are serine proteases, belonging to the SB clan and S8A family, which rely on the catalytic triad: aspartate, histidine and serine for execution of their catalytic activity (Rawlings *et al.*, 2018). In the context of HR, saspases and phytaspases represent two examples of serine proteases that might play indirect and direct roles, respectively, in the regulation of cell death upon biotic insults (Chichkova *et al.*, 2010; Coffeen and Wolpert, 2004).

Saspases

The necrotrophic fungus *Cochliobolus victoriae,* the causative agent of Victoria blight of oats (*Avena sativa*), produces the host selective toxin victorin. In a gene-specific manner, victorin triggers a form of cell death reminiscent of HR. Proteolysis of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), an *in vitro* substrate of victorin, was demonstrated to be inhibited by caspase-specific and general inhibitors of cysteine and serine proteases (Navarre and Wolpert, 1999). In parallel, purification of two specific caspase-like activities of victorin-treated *A. sativa* protein extracts followed by substrate cleavage assays of caspase-like synthetic tretrapeptides, suggested the existence of a proteolytic signalling cascade upstream of Rubisco cleavage (Coffeen and Wolpert, 2004). Purification of active proteolytic enzymes followed by N-terminal sequencing revealed two peptidases that shared extensive homology to diverse plant subtilases, particularly, rice subtilisin-like serine proteases. As a result, the term “saspases” was intuitively coined owing to its serine catalytic residue and their “aspase” activity (Coffeen and Wolpert, 2004). Alike animal caspases, saspases appear to serve a processing enzymatic function rather than a degradative one, owing to their low activity towards general protease substrates, including Rubisco. Collectively, it appears likely that, upon victorin sensitivity, saspases may be constituents of a proteolytic cascade that leads to Rubisco cleavage and PCD. Interestingly, saspases localise to the extracellular fluid at the early

stages of victorin-induced PCD, in what appears to be a tightly regulated secretion event rather than a consequence of PCD (Figure 1). As a result, the subcellular localisation of saspases and rubisco makes it unlikely that saspases cleave rubisco directly (Coffeen and Wolpert, 2004; Vartapetian *et al.*, 2011). Unfortunately, besides the intriguing biochemical data on saspases, no direct genetic evidence for their involvement in HR has been identified to date.

Phytaspases

An alternative approach to scout around for caspase-like proteases was based on the previous knowledge that the *Agrobacterium tumefaciens*-encoded protein VirD2 is cleaved by human caspase 3 at D^{400} within a TATD motif (Chichkova *et al.*, 2004). VirD2 from *A. tumefaciens* harbours a nuclear-localisation signal (NLS) and assists in the transfer of single-stranded DNA fragments (T-DNA) into the genome of the plant (Tinland *et al.*, 1995). Since the NLS of Vir2D is essential for successful nuclear uptake of foreign DNA, Chichkova and colleagues hypothesised the existence of a plant protease capable of cleaving VirD2 in a caspase 3-like manner (Chichkova *et al.*, 2004). In order to test such hypothesis, Vir2D was utilised as a substrate to detect a "plant caspase" that operates in the course of a TMV infection in *Nicotiana tabacum* plants carrying an *N* resistance gene. In this manner, they elucidated a caspase 3-like activity that was exclusively present in plants undergoing TMV-induced PCD (Chichkova *et al.*, 2004). Purification of the protein responsible for the activity in tobacco and rice followed by mass spectrophotometer analysis suggested that the protein was a subtilisin-like protease of the S8 family, which was thereafter named, phytaspase (Chichkova *et al.*, 2010). This enzyme is comprised of a signal peptide, a prodomain and a protease-associated domain within its peptidase domain (Vartapetian *et al.*, 2011). *In vitro* cleavage assays further demonstrated the aspartate specificity of phytaspases. Moreover, mutational analysis on the catalytic Ser^{537} of recombinant protein corroborated a Ser^{537} dependence for substrate cleavage and maturation of the protease, thus demonstrating autocatalytic processing of the proenzyme (Chichkova *et al.*, 2010).

With regards to their role in HR and defence, several lines of evidence suggest that phytaspases are required for TMV-triggered HR in tobacco plants harbouring an *N* resistance gene (Chichkova *et al.*, 2010). Transgenic tobacco plants overproducing phytaspases exhibit enhanced HR upon TMV infection. By contrast, impairment in phytaspase production, in phytaspase-silenced plants, results in attenuated HR triggered by TMV (Figure 1 and Table 2). Notably, this latter phenotype can be restored by heterologous expression of rice wild-type phytaspase but not by its catalytically inactive mutant (Chichkova *et al.*, 2010). In this pathosystem, HR triggered by phytaspases appear to serve a protective function, owing to the fact that as opposed to phytaspase-silenced plants, which tend to accumulate high levels of TMV, phytaspase-overproducing plants reduce TMV accumulation compared to wild-type control plants (Chichkova *et al.*, 2010).

As opposed to animal caspases that retain an intracellular localisation, phytaspases seem to be constitutively synthesised as zymogens and processed into prodomain-less mature forms which are secreted to the apoplast (Figure 1). Intriguingly, upon viral infection, phytaspases shuttle their subcellular localisation into the cytoplasm where they may cleave intracellular substrates required to induce HR, pointing towards a spatial regulation of their activity (Chichkova *et al.*, 2010). This re-entry into the cytoplasm would be needed to explain the observed VirD2 cleavage by *N. tabacum* phytaspase. Such protease redistribution is exclusive to phythaspases since other apoplastic proteases that exhibit caspase-like activities, such as CathB, are confined in the apoplast throughout the entire course of a viral infection (Gilroy *et al.*, 2007; Vartapetian *et al.*, 2011).

Concluding remarks

Now that more than a century since the HR discovery went by, we are still far from understanding how this type of PCD is mechanistically carried out. However, research in the last decade has significantly contributed to a better understanding of the proteases involved in this phenomenon, largely due to the successful efforts of the expanding plant protease community, which has

developed many tools and methods to efficiently dissect their function, mode of action and substrates.

Now we know that plants do not have caspases. Their structural relatives in plants are important both for HR and for other types of PCD, but have a different mode of action. Besides, plants have evolved several different caspase-like activities catalysed by other families or even classes of proteases. These caspase-like activities in plants are not only involved in HR, but also in processes not related to cell death.. In this review we report only the function of a few proteases in HR belonging to the cysteine, threonine and serine protease families, which are the best characterized. Still, for most of them we do not know the substrates nor their upstream regulators. In addition, several other plant proteases that have been directly or indirectly linked to HR await characterisation. Our current knowledge of HR execution consists of scattered protease activities in different cell compartments of different plant species infected by different pathogens. These separated pieces of the HR puzzle will hopefully come into place during the next years, thanks to the coalescing efforts that the plant protease community is putting together.

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	Animal PCD			Plant PCD		
Characteristics	**Apoptosis**	**Necroptosis**	**Pyroptosis**	**HR**	**Regulated necrosis**	**Vacuolar cell death**
Cytoplasmic shrinkage	✓	✗	✗	✓	✗	✓
Cytoplasmic swelling	✗	✓	✓	✗	✓	✗
Chromatin condensation	✓	✗	✓	✓	✗	✓
Mitochondrial swelling	✗	✓	✓	✓	✓	✗
Vacuolization	✗	✗	✗	✓	✗	✓
Chloroplast rupture	NA*	NA*	NA*	✓	✓	✗
Plasma membrane blebbing	✓	✗	✗	✗	✗	✗
Plasma membrane rupture	✗	✓	✓	✓	✓	✗
Tonoplast rupture	✗	✗	✗	✓	✗	✓
Nuclear fragmentation	✓	✗	✓	✗	✗	✓
Apoptotic bodies	✓	✗	✗	✗	✗	✗

Table 1: Hallmarks of animal (apoptosis, necroptosis and pyroptosis) and plant (HR, regulated necrosis and vacuolar) PCD. Although additional cell death modalities exist in animals, we considered these three cell death types

were the most representative for conveying comparisons with HR in plants. *NA = Not applicable

Table 2: Plant proteases involved in HR and HR-like cell death.

Class	Protease	Protein ID (UNIPROT)	Clan and family	Plant species	HR or HR-like cell death trigger	Subcellular localisation	Regulatory role in HR	References
CYSTEINE	**Cathepsin B**	Q40413, F4HVZ1, Q93VC9, Q94K85	CA/PLCP,C1	*N.bethamiana and A.thaliana*	• *Pst (AvrB),* • *E. amylovora* • *Pst DC3000*	Apoplast	Positive	Gilroy *et al*., 2007 McLellan *et al*., 2009
	Rcr3	Q8S333	CA/PLCP,C1	*Solanum Lycopersicum*	• *C. fulvum* • *G. rostochiensis*	Apoplast	Positive	Rooney *et al*., 2005 Lozano-Torres *et al*., 2012
	Pip1	Q156I2	CA/PLCP,C1	*Solanum Lycopersicum*	• *C. fulvum*	Apoplast	Positive	Shabab *et al*., 2008
	VPE	Q39119, Q60G64, Q60G63	CD/ Legumain,C13	*N.bethamiana and A.thaliana*	• TMV • Mycotoxin FB1 • Bacterial harpin	Vacuole	Positive	Hatsugai *et al*., 2004, Kuroyanagi *et al*., 2005, Zhang *et al*., 2010
	AtMC1	Q7XJE6	CD,C14	*A. thaliana*	• *Pst (AvrRPM1)* • *H.arabidopsis*	Cytoplasm	Positive	Coll *et al*., 2010
	AtMC2	Q7XJE5	CD,C14	*A. thaliana*	• *Pst (AvrRPM1)* • *H.arabidopsis*	Cystoplasm	Negative	Coll *et al*., 2010
	AtMC4	O64517	CD,C14	*A. thaliana*	• *P.m.a(AvrRpt2)* • Mycotoxin FB1	Cystoplasm	Positive	Watanabe and Lam, 2005
THREONINE	**PBA1**	F4JRY2	PB,T1	*A. thaliana*	• *Pst(AvrRPM1)* • *Pst (AvrRpt22)*	Cystoplasm	Positive	Hatsugai *et al*., 2009
SERINE	**Saspase**	-	SB,S8A	*A. sativa*	• Victorin	Apoplast	Positive	Coffeen and Wolpert, 2004
SERINE	**Phytaspase**	C7E4J6	SB,S8A	*Nicotiana tabacum and Oryza Sativa*	• TMV	Apoplast/ cytoplasm	Positive	Chichkova *et al*., 2010

Figure Legend

Figure 1. Mechanistic role of cysteine, serine and theronine proteases in the regulation of HR cell death in plants. Cysteine (orange), serine (green) and threonine (blue) protease activities highlighted in this review have been represented separately in three schematic cells. **Cysteine proteases** (bottom cell in orange) – PLCPs: Avr2 effector from *C. fulvum* binds to its virulence host target Pip1 and the host decoy cysteine protease Rcr3. Avr2-Rcr3 complex formation is sensed by the immune receptor Cf-2, leading to HR. CathB is necessary for HR cell death induced by *P. syringae* carrying the effector *AvrB*. Metacaspases: *P. syringae* carrying the *AvrRpm1* effector is perceived by intracellular immune receptors which trigger AtMC1 activation and HR. Such AtMC1-mediated cell death event is genetically inhibited by AtMC2. AtMC4 is required for HR triggered by *P. syringae* carrying the *AvrRpt2* effector and *F. moniliforme* mycotoxin FB1. VPEs are involved in TMV-induced HR and, fungal mycotoxin FM1 and bacterial harpin-triggered HR. **Serine proteases** (upper left cell in green) - Saspases are thought to be constituents of a proteolytic cascade upstream of Rubisco cleavage and victorin-induced cell death upon treatment of *A. sativa* leaves with victorin. Phytaspases are imported from the apoplast to the cytosol upon TMV-induced cell death and are required for HR. **Threonine protease** (upper right cell in blue). The PBA1/β1 subunit of the proteasome is required for vacuolar membrane fusion with the plasma membrane upon infection with avirulent *P. syringae* carrying the AvrRpm1 or AvrRpt2 effectors. Such membrane fusion facilitates discharge of anti-microbial hydrolytic enzymes, ultimately leading to HR. In the figure, bacteria are represented in light pink, viruses in light grey and fungi in white.

Figure 1