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PERSPECTIVE

A kiss of death—proteasome-mediated membrane fusion and programmed cell death in plant defense against bacterial infection

Karolina Pajerowska-Mukhtar and Xinnian Dong[1]

Department of Biology, Duke University, Durham, North Carolina 27708, USA

Eukaryotes have evolved various means for controlled and organized cellular destruction, known as programmed cell death (PCD). In plants, PCD is a crucial regulatory mechanism in multiple physiological processes, including terminal differentiation, senescence, and disease resistance. In this issue of *Genes & Development*, Hatsugai and colleagues (pp. 2496–2506) demonstrate a novel plant defense strategy to trigger bacteria-induced PCD, involving proteasome-dependent tonoplast and plasma membrane fusion followed by discharge of vacuolar antimicrobial and death-inducing contents into the apoplast.

The majority of plant pathogens—including fungi, oomycetes, and bacteria—initially invade the intercellular spaces called the apoplast. This is the battleground for the host's early defense responses and pathogens' counterdefense strategies. Upon entry into the host, pathogens aim to access host nutrients and subsequently multiply by either exclusively residing in the apoplast or further breaching the host cell walls, but remaining outside of the host cytoplasm. Activation of early defense responses includes secretion of defense-related proteins and antimicrobial phytoalexins into the apoplast, and the formation of callose-rich cell wall appositions, known as papillae (Kwon et al. 2008). In order to circumvent these early defenses, pathogens have evolved specialized virulence determinants, known as effector proteins, which are delivered directly into the host cytoplasm (Jones and Dangl 2006). Collectively, these proteins alter multiple host cellular processes and suppress a variety of plant early defenses to ultimately favor pathogen growth and promote disease. In this tug-of-war, plants have evolved specific receptor molecules—known as the resistance (R) proteins—that recognize the presence or the activities of certain pathogenic effector proteins. In this scenario, the effector proteins become avirulence (Avr) signals for triggering host defense. Recognition of Avr proteins causes an increase in cellular concentration of defense signaling molecules such as salicylic acid and nitric oxide. Consequently, a form of programmed cell death (PCD), also known as hypersensitive response (HR), is triggered to arrest the pathogen growth. In plants, HR is associated with a series of dynamic cellular changes including rupturing of vacuole, lowering of the cytosolic pH, and releasing of hydrolytic enzymes into the cytoplasm. This leads to bulk degradation of the remaining cellular components and a complete cell collapse. However, the molecular mechanisms underlying the various regulatory steps of this plant PCD remain unclear. In this issue of *Genes & Development*, the team led by Hara-Nishimura (Hatsugai et al. 2009) describes the proteasome subunit-mediated fusion of the central vacuole with the plasma membrane, followed by the discharge of vacuolar contents into the extracellular compartment as the molecular basis for PCD defense against bacterial pathogens. Their paradigm-shifting study shows that the central vacuole functions as a crucial executioner of PCD, rather than just playing a supporting role as a provider of the necessary proteolytic enzymes in the dying cell.

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[1]Corresponding author.
E-MAIL xdong@duke.edu; FAX (919) 613-8177.
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Avirulent bacterial pathogen-induced PCD is associated with fusion between the central vacuole and the plasma membrane

Plant vacuoles play fundamental roles in allowing cell growth through regulation of turgor, maintaining cellular homeostasis, storage, and detoxification processes, as well as timely execution of PCD in a process known as autolysis (Marty 1999; Reisen et al. 2005). A key role for the central vacuole in plant PCD has been speculated previously (Higaki et al. 2007). An increase in vacuolar size and volume, or "vacuolation," likely resulting from water absorption and simultaneous degradation of excessive vacuolar membranes, has been observed prior to the

developmental PCD to facilitate vacuole rupture and release its contents directly into the cytosol.

Hatsugai et al. (2009) found that, during PCD induced by two avirulent bacterial strains of *Pseudomonas syringae* pv. *tomato* (*Pst*)—DC3000 AvrRpm1 and *Pst* DC3000 AvrRpt2—in *Arabidopsis* plants, the membrane of the central vacuole fuses with the plasma membrane. This fusion seemed to be specific to R protein-mediated PCD, since it was not observed in an *Arabidopsis R* gene mutant or in response to a virulent *Pst* strain, DC3000. Furthermore, Hatsugai et al. (2009) provide evidence for colocalization of vacuolar membrane and plasma membrane using transgenic plants expressing tonoplast (vacuolar membrane)-specific and cell membrane-specific fluorescent markers GFP-PIP2a and mRFP-VAM3, respectively, in the PCD-induced cells. In a complementary study by Higaki et al. (2007), vacuolar collapse was studied during PCD triggered by the fungal elicitor cryptogein, which is secreted by the oomycete pathogen *Phytophthora cryptogea*. Using the dye BCECF-AM, which specifically stains vacuolar lumen in intact cells, and the dye Evans Blue for dead cells, Higaki et al. (2007) demonstrated that at 24 h after the elicitor treatment, almost 40% of the PCD-induced cells displayed disintegrated vacuolar and plasma membranes, while a very small fraction (0.2%) had a ruptured tonoplast with intact plasma membrane. This lends further evidence that disintegration of tonoplast and plasma membrane occurs simultaneously in R protein-mediated PCD, as proposed here by Hatsugai et al. (2009).

Central vacuolar and plasma membrane fusion leads to apoplastic release of vacuolar fluid with antimicrobial and PCD-inducing activities

The vacuole is a reservoir of antimicrobial compounds that are proposed to participate in PCD (Reisen et al. 2005). In response to pathogen attack or other biotic/abiotic stresses, plant cells secrete toxic cocktails of antimicrobial compounds and proteins, such as pathogenesis-related (PR) proteins (van Loon et al. 2006). Some PR proteins contain an N-terminal signal peptide for apoplastic secretion, whereas a subset of basic forms is targeted to vacuoles. Hatsugai et al. (2009) tested whether the membrane fusion observed in their experiments is linked to the release of vacuolar antimicrobial proteins into the extracellular spaces. They generated transgenic plants expressing vacuolar-localized fluorescent protein (SP-Venus-2SC). Remarkably, they observed the fluorescence outside the cell 3 h post-inoculation, indicative of vacuolar protein discharge into the apoplast. Using Western blotting, Hatsugai et al. (2009) also found SP-Venus-2SC, as well as other vacuolar-localized endogenous proteins in the extracellular fluid of PCD-induced leaves. They further showed that the extracellular fluid of PCD-induced leaves has not only antibacterial activities, but also cell death-promoting functions. This may have an impact on the invading pathogens in two different ways: (1) containing the pathogens directly in the PCD-induced cells, and (2) restricting the growth of the pathogen by the antimicrobial fluid before it multiplies and attacks the neighboring cells. Since many PR proteins exhibit broad antibacterial and antifungal activities, an interesting follow-up experiment would involve testing the activity spectrum of the fluid. Is it specific to *P. syringae*, or can it also restrict the growth of other bacterial or nonbacterial pathogens?

The caspase-3-like activity detected during bacteria-induced PCD is attributed to PBA1, the β1 subunit of the proteasome

A PCD-induced cell undergoes several sequential changes prior to the disruption of the vacuolar membrane. These cellular events may differ in various physiological contexts. Higaki et al. (2007) demonstrated that in cryptogein-induced PCD tobacco cells, microtubule disruption, actin microfilament bundling, peripheral nucleus movement, and rearrangement of vacuolar membrane structures occur prior to vacuole rupture. This pathogen elicitor-triggered PCD is distinct from senescence-induced PCD, which involves the initial chloroplast degradation, followed by the disruption of vacuoles and the nucleus (Thomas et al. 2003). Wada et al. (2009) demonstrated recently that, during plant senescence, the entire chloroplast can be engulfed by the central vacuole through an autophagic process. Overall, a PCD-induced plant cell shares several morphological similarities with an animal apoptotic cell, including cell shrinkage, chromatin condensation, and DNA fragmentation. Moreover, a role of mitochondria in integrating cellular stress and regulating PCD in plants, as in animals, has been suggested (Lam 2004). Therefore, some regulatory mechanisms underlying PCD may be conserved between animals and plants, even though the exact molecular basis for plant PCD remains to be elucidated.

In animals, PCD is typically executed by caspases or cysteinyl aspartate-specific proteases. These enzymes are released at a crucial stage of apoptosis in order to shred all cellular proteins and dismantle the cell. Even though no orthologous caspase sequences have been discovered through plant genome sequencing, caspase activities have been detected in plant cells (Woltering et al. 2002). Typically, caspase inhibitors corresponding to a particular substrate are used to determine what caspase-like activities are required for execution of PCD under certain biological conditions. The avirulent bacteria-induced PCD observed by Hatsugai et al. (2009) in this study was effectively abolished by a caspase-3 inhibitor (Ac-DEVD-CMK). Thus, Hatsugai et al. (2009) concluded that caspase-3-like activity is crucial for proper execution of PCD upon avirulent bacterial pathogen infection. In plants, at least eight distinct caspase-like activities have been found under various stress conditions (Woltering et al. 2002; Bonneau et al. 2008). The caspase-3-like DEVDase belongs to the most-studied ones, along with the caspase-1-like YVADase (Bonneau et al. 2008). In human cells, caspase-3 is one of the executioner caspases that catalyze the specific cleavage of many key cellular proteins, resulting in membrane bulging, chromatin condensation, and DNA fragmentation, and is considered to

be the central protein in the completion of the final steps of PCD (Porter and Janicke 1999).

Besides caspases, the relationship between PCD and the proteasome is complicated. The proteasome is a highly conserved, multisubunit complex that consists of several multicatalytic proteases responsible for most of the cytosolic and nuclear protein degradation (Smalle and Vierstra 2004). In animals, protesomes affect apoptosis by controlling the stability of both pro- and anti-apoptotic molecules (Jesenberger and Jentsch 2002; Friedman and Xue 2004). To examine the role of proteasomes in the R protein-mediated PCD in plants, Hatsugai et al. (2009) used proteasome inhibitors in their study. They found that Ac-APnLD-CHO, an inhibitor of the β1 subunit, and β-lactone, a general proteasome inhibitor, could both block PCD induced by avirulent bacteria. Three of the β subunits of proteasomes have proteolytic activities: PBA (β1) has glutamyl-peptide or aspartyl-peptide hydrolase activity, PBB (β2) has trypsin-like activity, and PBE (β5) is a chymotrypsin-like protease. Since the *Arabidopsis* PBA1 has an analogous sequence to the substrate pocket of the yeast proteasome β1 subunit, Hatsugai et al. (2009) hypothesized that PBA1 may be the target for Ac-APnLD-CHO in *Arabidopsis*.

Hatsugai et al. (2009) then went a step further to make the bold assumption that the caspase-3-like (DEVDase) activity observed in the R protein-mediated PCD might have come from the proteasome β1 subunit PBA1. To test this hypothesis, Hatsugai et al. (2009) first demonstrated that the caspase-3-like DEVDase activity could be inhibited by proteasome inhibitor treatments, whereas the PBA1 activity was compromised after the caspase-3 inhibitor application. Moreover, Hatsugai et al. (2009) used a combination of genetic and biochemical approaches to show that plants with diminished expression of PBA1 were also deficient in caspase-3-like activity, and PBA1 could be pulled down using a biotinylated peptide containing the caspase-3 substrate site (biotin-DEVD-fmk). Based on these data, Hatsugai et al. (2009) propose that PBA1 is a long-sought-after caspase-3-like protein in plants.

Caspases are synthesized as inactive propeptides that are activated through a cascade of proteolytic events when an apoptotic stimulus is perceived (Han et al. 1997). PBA1, on the other hand, is a catalytically active subunit of the proteasome that is involved in constant degradation of cellular proteins in non-PCD cells (Smalle and Vierstra 2004). If PBA1 is the plant caspase-3-like protein, how is it regulated during PCD? Interestingly, it has been found that human caspase-3 and another executioner, caspase-7, can cleave a 20S proteasome activator 28 subunit 3, PA28γ, upon apoptotic induction (Araya et al. 2002). Even though the PA28 is known to stimulate multiple peptidase activities of the 20S proteasome, the biological significance of this caspase-mediated PA28γ cleavage is not known. It is possible that an analogous mechanism might be in place for PBA1 during R protein-mediated PCD, where it is activated by another protease, or even through self-cleavage, to trigger or enhance its caspase-3-like function.

Similar to PBA1, mammalian and yeast β1 proteasome subunits contain a proteolytic site that was shown to preferentially cut after acidic residues, with the highest affinity for aspartate, similar to canonical caspases (Kisselev et al. 2003). Moreover, occupancy of these caspase-like sites in proteasomes was shown to stimulate their trypsin-like activity. Taken together, the findings described by Hatsugai et al. (2009) put the well-studied proteasome in a new context as a protein complex that is directly involved in execution of HR in plant defense. As in animal systems, the plant proteasome seems to play contrasting roles in controlling PCD. There have been previous reports showing that proteasome inhibits PCD in plants. Reduced activities of two different subunits of the 26S proteasome, the α6 subunit of the 20S proteasome and RPN9 subunit of the 19S regulatory complex, resulted in the activation of PCD, accompanied by reduced proteasome activity and accumulation of polyubiquitinated proteins (Kim et al. 2003). Is it possible that these contrasting functions are performed by the different parts of the proteasome? It has been reported that the catalytic barrel (20S) of the proteasome alone has distinct activities from the whole 26S proteasome (Kurepa and Smalle 2008a). The *Arabidopsis* proteasome regulatory particle mutants, while compromised in ubiquitin (Ub)-dependent protein degradation, have increased 20S Ub-independent proteolytic activities and increased tolerance to oxidative stress (Kurepa and Smalle 2008a,b).

While the role of the Ub-independent pathway in R protein-mediated PCD remains to be explored, there is more evidence supporting the involvement of the Ub-dependent pathway. The U-box E3 ligase homolog CMPG1 is a key enzyme required for protein ubiquitination and subsequent degradation. It has been shown to be essential for signaling downstream from the tomato and tobacco resistance genes Cf-4 and Cf-9, as well as for the response to fungal elicitors and an avirulent bacterial strain (Gonzalez-Lamothe et al. 2006). Another tobacco U-box E3 ligase, ACRE276, and its *Arabidopsis* homolog, PLANT U-BOX17 (PUB17), were demonstrated to be involved in the establishment of HR conferred by tobacco Cf and *Arabidopsis* RPM1 and RPS4 resistance proteins (Yang et al. 2006). Multiple genetic screens have led to the identification of RAR1 (Required for MLA12 Resistance 1) as a key component required for several R protein functions. RAR1 and its interactor, SGT1 (Supressor of the G2 allele of SKP1), have been shown to be cochaperons for the HSP90 (Heat-Shock Protein 90) protein responsible for the stability of some R proteins (Shirasu 2009). In yeast, SGT1 interacts with SKP1 of the SCF Ub complex, linking it with HSP90. In mammals, both SGT1 and HSP90 are required for the inflammatory response induced by Nod-like receptors, which are structurally similar to plant R proteins (da Silva Correia et al. 2007; Mayor et al. 2007). Interestingly, in mammals, the receptor stability is only affected by HSP90, not SGT1. Given the new finding by Hatsugai et al. (2009) that a subunit of proteasome is involved in R protein-mediated PCD, it will be interesting to test the PBA1 activity in the *sgt1*, *rar1*, and *hsp90* mutants. Is it possible that PBA1 is activated by R proteins through their interactions with the SGT1–RAR1–HSP90 complex?

The caspase-like activities are required for the vacuolar-mediated PCD

One of the results of PBA1 activation is vacuolar collapse during PCD. Hatsugai et al. (2009) demonstrate that reduced PBA1 activity abolished the fusion of the tonoplast with the cell membrane. Identification of the PBA1 target controlling this membrane fusion will be the next logical step. In an earlier report from the same laboratory (Hatsugai et al. 2004), the reduction of caspase-1-like activity of the tobacco vacuolar processing enzyme (VPE), a protein regulating hydrolytic activity of the vacuole, also blocked the vacuolar collapse in Tobacco Mosaic Virus (TMV)-triggered PCD tobacco cells. However, this caspase-1-like activity is not required for vacuolar and plasma membrane fusion upon avirulent bacterial infection. The caspase-1-like activity of the *Arabidopsis* AtVPEγ (one of four highly similar genes in *Arabidopsis*) was also found by Rojo et al. (2004). In that report, the caspase-1-like activity was shown to be enhanced during PCD induced by multiple pathogens, including *Pst* DC3000 AvrRpm1, which was used by Hatsugai et al. (2009). In the *vpeγ* mutant, resistance against *Pst* DC3000 AvrRpm1 was moderately compromised. Since *Arabidopsis* VPEγ is developmentally regulated and functionally redundant, these potentially conflicting pieces of evidence could be due to different types of plant samples analyzed in these studies.

Multiple routes to PCD in plant immunity

The discrepancy in the above-mentioned reports may reflect the complexity of PCD during plant immune responses, where multiple pathways may be recruited to execute PCD. This is consistent with the observation that the PBA1 knockdown plants (*ipba1*) did not support bacterial growth to the levels found in the *R* gene mutant *rpm1* plants. In a recent report, RPM1-mediated PCD has also been shown to be positively affected by the release of lysosomal cathepsins, potent inducers of PCD, as well as a nonapoptotic autophagy pathway (Hofius et al. 2009). It is also tempting to search for parallels between vacuolar collapse-triggered HR and the endoplasmic reticulum (ER) stress-related unfolded protein response (UPR) pathways. It is known that multiple ER vesicles containing hydrolases and antimicrobial proteins are targeted to vacuoles. Induction of plant defense in the *Arabidopsis* mutant with heightened UPR leads to massive collapse of leaves (Wang et al. 2005). In the same study, a Vacuolar Sorting Receptor (*VSR*) gene was identified as being upregulated by the plant defense hormone salicylic acid. *VSR* expression is also induced by avirulent *P. syringae* strains expressing AvrRpm1 or AvrRpt2 (*Arabidopsis* eFP browser, http://bar.utoronto.ca). Thus, it is plausible that a common molecular mechanism is required for activation of UPR following ER stress and the early stages of HR. In this scenario, at the onset of pathogen-induced PCD, the secretory machinery would be induced to load the vacuole with antimicrobial cargo and prepare it for fusion with the plasma membrane, leading to cellular collapse.

The multiplicity of PCD pathways in plant immune responses might have resulted from the tug-of-war between plants and pathogens during evolution. R protein-mediated PCD is a predominant immune response in plants and a major hurdle for a pathogen to overcome before it can live on/off its host. Thus, rapidly coevolving effector proteins gain the ability for the pathogen to suppress PCD. It was shown recently that at least five effectors from *Pst* DC3000 were capable of suppressing the RPM1-dependent PCD when they were individually delivered through a nonpathogenic strain, *Pseudomonas fluorescens*, containing the type III secretion system (Guo et al. 2009). The ubiquitination/proteasome pathway seems to be a favorable target for bacterial effectors. These effectors manipulate host protein degradation machinery as a virulence strategy. For example, a *P. syringae* type III effector, HopM1, mediates the proteasome-dependent elimination of *Arabidopsis* ARF-GEF (ADP-ribosylation factor guanine-nucleotide exchange factors) protein AtMIN7, which is required for full resistance against bacterial infection (Nomura et al. 2006). Another *P. syringae* effector, AvrPtoB, possesses structural homology with a RING U-box type E3 Ub ligase and can possibly ubiquitinate specific host proteins (Abramovitch et al. 2006; Janjusevic et al. 2006). Indeed, AvrPtoB ubiquitinates multiple receptor kinases, including tomato Fen kinase (a member of Pto kinase family), *Arabidopsis* flagellin-sensitive 2 kinase (FLS2), bacterial EF-Tu-recognizing receptor (EFR), and chitin elicitor receptor kinase (CERK1) (Spallek et al. 2009). Several F-box-containing GALA effector proteins from the bacterial wilt pathogen *Ralstonia solanacearum* interact with members of *Arabidopsis* ASK family (equivalent of SKP, a subunit of SCF class of E3 ligases in plants) to potentially reconstitute an active SCF complex and are required for full bacterial virulence (Angot et al. 2006).

Concluding remarks and future directions

The work by Hatsugai et al. (2009) has found a missing piece of the puzzle in our understanding of plant immune system. Based on their exciting findings and other previously published data, we put forward a tentative model in Figure 1. In this model, we divided R protein-mediated PCD into three stages: (1) effector delivery and recognition; (2) signal competent state and transduction; and (3) PCD execution through the PBA1-dependent vacuolar release. Like every exciting discovery, the report by Hatsugai et al. (2009) opens a myriad of new questions, the first being: How is PBA1 activated by R proteins? It would be equally important to identify substrates of PBA1 and address the question of whether ubiquitination is important for the degradation of these substrates. A handful of similar studies have already been successfully performed in mouse and human cells using a modified yeast two-hybrid screen and led to the identification of several endogenous substrates for caspase-3 and caspase-7 (Kamada et al. 1998; Araya et al. 2002). Discovery of potential caspase substrates in plant cells will shed new light on plant PCD/HR molecular mechanisms and identify additional proteins required for cell death.

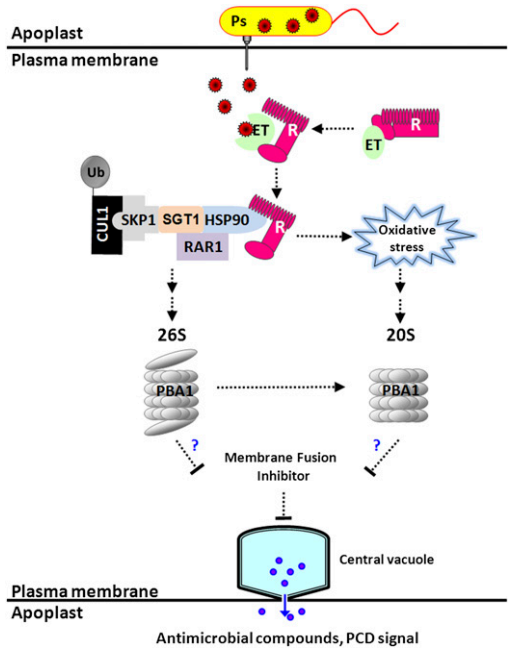

Figure 1. A model presenting the three known stages of R protein-induced PCD in plant defense. The three stages of R protein-mediated PCD include the following. (1) Effector delivery and recognition: *P. syringae* (Ps) delivers effector proteins into the host cell via the type III secretion system. Interaction of a specific type III effector with host effector target (ET) proteins is shown. R protein guarding the ET proteins indirectly recognizes the type III effector. (2) Signal-competent state and transduction: R protein forms a dynamic complex with other host proteins like HSP90, RAR1, and an F-box protein, SGT1b. SGT1b interacts with SKP1, a component of the CUL1 E3 Ub ligase, to modify R proteins and/or other regulators of the resistance response. (3) PCD execution through the PBA1-dependent vacuolar release: The caspase-3-like activity of PBA1 is triggered by the R protein through an unknown mechanism. A negative regulator of tonoplast and cytoplasmic membrane fusion is removed by PBA1 through either a Ub-dependent (26S) or a Ub-independent (20S) pathway. The release of the vacuolar content into the apoplast inhibits bacterial growth and causes host PCD.

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