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REVIEW PAPER

Cutting the line: manipulation of plant immunity by bacterial type III effector proteases

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Abstract

Pathogens and their hosts are engaged in an evolutionary arms race. Pathogen-derived effectors promote virulence by targeting components of a host's innate immune system, while hosts have evolved proteins that sense effectors and trigger a pathogen-specific immune response. Many bacterial effectors are translocated into host cells using type III secretion systems. Type III effector proteases irreversibly modify host proteins by cleavage of peptide bonds and are prevalent among both plant and animal bacterial pathogens. In plants, the study of model effector proteases has yielded important insights into the virulence mechanisms employed by pathogens to overcome their host's immune response, as well as into the mechanisms deployed by their hosts to detect these effector proteases and counteract their effects. In recent years, the study of a larger number of effector proteases, across a wider range of pathogens, has yielded novel insights into their functions and recognition. One key limitation that remains is the lack of methods to detect protease cleavage at the proteome-wide level. We review known substrates and mechanisms of plant pathogen type III effector proteases and compare their functions with those of known type III effector proteases of mammalian pathogens. Finally, we discuss approaches to uncover their function on a system-wide level.

Keywords: Degradomics, effector proteases, host–pathogen interactions, hypersensitive response, *Pseudomonas syringae*, regulated cell death, type III secretion system.

Introduction

Plants have evolved multifaceted innate immune responses that are sufficient to overcome most pathogen challenges. This sophisticated and robust innate immune system comprises two interconnected tiers (Jones *et al.*, 2016). The first tier, known as pattern-triggered immunity (PTI), relies on the detection of highly conserved pathogen molecules or 'PAMPs' (pathogen-associated molecular patterns; e.g. bacterial flagellin or its 22 amino acid peptide flg22) at the cell surface by pattern

recognition receptors (PRRs) that subsequently activate the immune response. Alternatively, some PRRs recognize 'DAMPs' (damage-associated molecular patterns), a variety of host-derived factors that commonly arise following pathogen attack, such as extracellular ATP and protein or cell wall fragments (Yamaguchi and Huffaker, 2011; Hou *et al.*, 2019). PTI signals originating at the plasma membrane (PM) are transduced downstream by intracellular kinases and secondary messengers to activate the hallmark features of PTI (Dodds and Rathjen, 2010). These include transcriptional reprogramming to activate defence-related genes, stomatal closure to limit pathogen entry, the generation of reactive oxygen species (ROS) toxic to microbes, and callose deposition to reinforce the cell wall (Bigeard *et al.*, 2015; Li *et al.*, 2016). Thus, PTI provides protection against a broad spectrum of pathogens.

To counteract these defences, pathogens secrete repertoires of proteins known as 'effectors' to interfere with PTI and promote infection. Notably, bacterial pathogens may utilize the type III secretion system (T3SS) to deliver effectors directly into the cytosol of host cells where they can suppress key immune regulators by a variety of mechanisms (Toruño *et al.*, 2016; Khan *et al.*, 2018; Langin *et al.*, 2020). However, while pathogen-derived effectors target specific components of a host's PTI response to promote pathogenicity, adapted hosts have evolved proteins—typically members of the polymorphic nucleotide-binding/leucine-rich repeat (NLR) family—that sense effectors and trigger a pathogen-specific immune response, termed effector-triggered immunity (ETI) (Cui *et al.*, 2015; Toruno *et al.*, 2016). ETI is often, but not necessarily, associated with a localized form of regulated cell death termed the hypersensitive response (HR) (Laflamme *et al.*, 2020; Pitsili *et al.*, 2020). Several mechanisms of effector detection by NLRs have been described, including direct binding interactions as well as 'indirect' surveillance of effector activities (Cui *et al.*, 2015; Kourelis and van der Hoorn, 2018). The outcome of host–pathogen interactions thus depends on the set of effectors expressed by a given pathogen and the presence or absence of cognate NLRs in the host, resulting in an evolutionary arms race between plant pathogens and their hosts.

Over the past four decades, the model plant pathogen *Pseudomonas syringae* has played key roles in the discovery of effector function and ETI regulation (Xin *et al.*, 2018). Over 14 600 putative T3S effectors (T3SEs) have been identified in strains of *P. syringae* (Dillon *et al.*, 2019), several of which function as proteases that target components of PTI to enhance virulence (Hou *et al.*, 2018; Figaj *et al.*, 2019). An outstanding feature of proteases among other effectors is the ability to interfere with host processes using proteolysis as a site-specific, irreversible post-translational protein modification (Marshall *et al.*, 2017). As is the case with other proteases, T3SE proteases belong to several mechanistic classes that are classified into different clans and families depending on the structure and sequence similarity of their peptidase domain (Rawlings *et al.*, 2018), with cysteine and threonine proteases found in the effector protease repertoire of *P. syringae* (Table 1). Once inside the host cell, T3SE proteases cleave peptide bonds within proteins to inactivate immune functions, activate latent functions, or expose recognition sites for rapid degradation by the host ubiquitin–proteasome system (UPS) (Dissmeyer *et al.*, 2018; Ravalin *et al.*, 2019). Notably, several protease families are conserved among bacterial pathogens that infect animals and plants (Shao *et al.*, 2002; Nimchuk *et al.*, 2007; Dowen *et al.*, 2009), highlighting their effectiveness in targeting eukaryotic innate immune responses. Remarkably, T3SE repertoires also include proteolytic enzymes that interfere with UPS-mediated proteolytic signalling in the host by cleaving isopeptide bonds within chains of polyubiquitin or ubiquitin-like proteins (e.g. SUMO) (Pruneda *et al.*, 2016; Xiang *et al.*, 2020). Here we focus on T3SE proteases, but for a detailed discussion of effector-mediated manipulation of the host proteolytic machinery we refer the readers to an excellent recent review (Langin *et al.*, 2020).

In this review, we summarize the current knowledge on T3SE proteases in phytopathogenic bacteria with a focus on (i) their mode of action as virulence factors and the co-evolution with cognate plant NLRs; (ii) their role in the regulation of regulated cell death in both plants and animals; and (iii) their evolutionary conservation and diversity across plant and animal pathogens. Finally, considering the state of the field and the urgent need to identify proteome-wide targets of T3SE proteases, we also briefly discuss mass spectrometry (MS)-based methods that may overcome some of the current limitations (Box 1).

Suppression of PTI by *P. syringae* T3SE proteases

As indicated above, effector proteases act primarily as virulence factors that dampen innate immune responses in plants. Plants recognize flagellin fragments such as a 22 amino acid residue peptide flg22 via the PM-bound receptor-like kinase (RLK) FLAGELLIN-SENSITIVE2 (FLS2). In the absence of a pathogen threat, FLS2 constitutively associates with the PBS1-like (PBL) family VII receptor-like cytoplasmic kinase (RLCK) *BOTRYTIS*-INDUCED KINASE1 (BIK1) at the PM (Lu *et al.*, 2010). Upon flagellin detection, FLS2 forms a co-receptor complex with fellow RLK BRI1 ASSOCIATED RECEPTOR KINASE1 (BAK1), triggering a series of phosphorylation events that initiate PTI signalling (Bigeard *et al.*, 2015). Phosphorylated BIK1 dissociates from the receptor complex and activates downstream immune responses including influx of Ca^{2+} (Tian *et al.*, 2019) and ROS production (Kadota *et al.*, 2014). Both BAK1 and BIK1 are targets of effector proteases secreted by *P. syringae* to impede early PTI signals, as well as downstream signalling pathways (Fig. 1).

BAK1 can be cleaved by *P. syringae* HopB1 (Fig. 1; Table 1) (Li *et al.*, 2016; Figaj *et al.*, 2019). When expressed directly in protoplasts, HopB1 constitutively interacts with FLS2 (Li

Table 1. Overview of the bacterial T3SE proteases discussed in this review

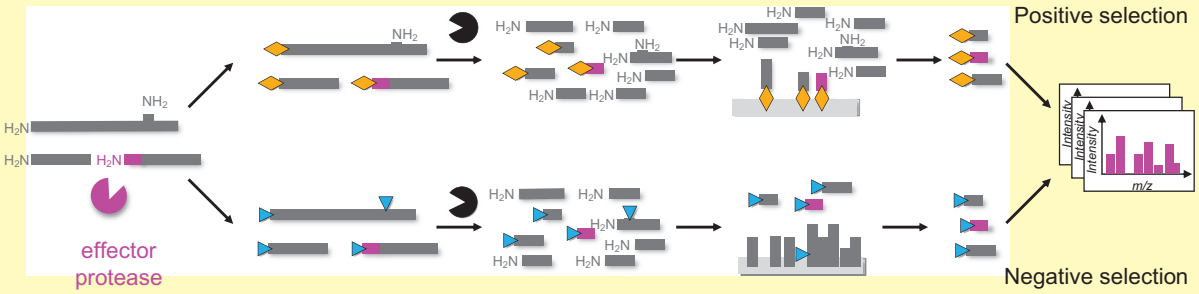
Pathogen	Effector	Type	Clan	Family	Substrate/target	Function	Source
Pseudomonas syringae	AvrPphB	CP	CA	C58B	PBS1, RIPK, PBS1-like kinases (PBL), BIK1	Supresses PTI	Ade *et al.* (2007); Zhang *et al.* (2010)
	AvrRpt2	CP	CA	C70	RIN4, NOIs	Block RPM1 activation	Eschen-Lippold *et al.* (2016); H.S. Kim *et al.* (2005)
					?	Prevents MKK4 activation, modifies auxin signalling	Cui *et al.* (2013); Eschen-Lippold *et al.* (2016)
	HopB1	TP		T8	Activated BAK1	Supresses PTI	Li *et al.* (2016)
	HopC1	n.a.	n.a.	n.a.			
	HopZ1a	CP	CE	C55			
	HopZ2	CP	CE	C55			
	HopZ3	CP	CE	C55			
	HopZ1	CP	CE	C55			
	HopX1 (AvrPphE)	CP	CA	C103	JAZ repressors		Gimenez-Ibanez *et al.* (2014)
	HopN1	CP	CA	C58B	PsbQ	Supresses HR, ROS, and callose production	Rodríguez-Herva *et al.* (2012)
Sinorhizobium fredii	NopT	CP	CA	C58		Regulating symbiosis	Dai *et al.* (2008)
Ralstonia solanacearum	RipE1	CP	CA	103	JAZ repressors		Nakano and Mukaihara (2019)
Erwinia amylovora	Eop1	CP	CE	C55			Nissinen *et al.* (2007)
	AvrRpt2EA	CP	CA	C70	RIN4		Vogt *et al.* (2013)
Shigella flexneri	OspD3	CP	na	C118	RIPK1, RIPK3	Blocks necroptosis	Ashida *et al.*, (2020)
Enteropathogenic *Escherichia coli* (*EPEC*)	EspL	CP	na	C118	RIPK1, RIPK3, TRIF, and ZBP1/DAI	Blocks necroptosis	Pearson *et al.* (2011)
	NleC	MP	MA	M85	NF-κB, p65	Blocks inflammation	Baruch *et al.* (2011)
	NleD	MP	MA	M91	JNK, p38	Blocks apoptosis/inflammation	Baruch *et al.* (2011)
Yersinia pestis	YopT	CP	CA	C58A	Rho GTPases	Disruption of actin cytoskeleton	Shao *et al.* (2002)

Selected bacterial effector proteases secreted by type III secretion systems. Green, phytopathogens; red, mammalian pathogens. Type: CP, cysteine protease; TP, threonine protease; MP, metalloprotease. Clan and family membership according to MEROPS nomenclature. Families combine homologous proteases with significant sequence similarity, while clans combine families arising from a common ancestor based on a similar protein structure and/or order of catalytic amino acids in the primary sequence (Rawlings *et al.*, 2014).

Box 1. Degradomics for unbiased effector protease substrate discovery

Shotgun proteomics, where proteomes are digested into peptides for mass spectrometric analysis, enables large-scale quantitative proteome comparisons even at near-complete coverage (Mergner *et al.*, 2020). By determining changes in protein abundance, such approaches allow identification of candidate substrates, particularly for degradative proteases (Demir *et al.*, 2018). In contrast, site-specific proteolytic cleavages are defined by the new protease-generated neo-N- and neo-C-termini, but their identification in the complex background of a proteome digest is challenging and therefore requires selective enrichment (Niedermaier and Huesgen, 2019). This can be achieved by (i) selective tagging of protein termini before digest, followed by enrichment (termed 'positive selection'); (ii) by complete modification of protein termini with a labelling reagent, followed by proteome digest and depletion of the peptides generated by the digest (termed 'negative selection'); or (iii) based on the peptide charge (Bogaert and Gevaert, 2020; Perrar *et al.*, 2019). Due to the compatibility with amine-reactive isotope labelling reagents, comparative ease of use, and superior sensitivity, enrichment of N-termini by negative selection is currently most frequently applied.

All methods allow for identification of candidate substrates by comparison of proteomes with differential exposure with the protease of interest, ideally using a catalytically inactive version carrying a point mutation in the (presumed) active site as a control. *In vitro* incubation of the candidate substrate protein, or of a cell extract with recombinant protease constructs, provides the most direct proof of protease/substrate relationships. However, this 'reverse' degradomics approach (Julien and Wells, 2017) is prone to 'false-positive' cleavage events, for example in proteins destabilized by the incubation conditions or in proteins with distinct subcellular localization(s) *in vivo*. Alternatively, substrates can be identified in a 'forward' approach based on differential activity *in vivo*, for example by constitutive or inducible expression of effector proteases *in planta*. This overcomes the issues of 'non-native' substrate cleavage (although strong expression may still result in improper subcellular localization) and provides for host factors and post-translational modifications that may be required for protease activation. More complex scenarios such as delivery by an otherwise effector-depleted pathogen strain or comparison in wild-type infection experiments are needed if effector substrate recognition depends on modifications induced by pathogen perception or the presence of other effectors. While cleavages observed in these systems are more likely to be relevant, they can also be masked by subsequent processing or degradation, or arise from a plethora of indirect effects. Therefore, a combination of these approaches, including targeted genetic or biochemical validation, is needed to establish direct, physiologically relevant protease–substrate relationships (Demir *et al.*, 2018).



et al., 2016). After flg22-induced formation of the FLS2–BAK1 co-receptor complex, BAK1 is phosphorylated at Thr455, prompting its cleavage by HopB1 between Arg297 and Gly298 (Li *et al.*, 2016). HopB1 cleavage of BAK1 impairs flg22-triggered immune responses (Wu *et al.*, 2020) and disrupts downstream signals including a reduction in the levels of phosphorylated BIK1 leading to increased *P. syringae* growth (Li *et al.*, 2016).

BIK1 is itself targeted by AvrPphB (also known as HopAR1) (Zhang *et al.*, 2010) (Fig. 1; Table 1). AvrPphB cleaves several PBL kinases including BIK1, PBS1, PBL1, PBL2, and PBL3 (Shao *et al.*, 2003; Nimchuk *et al.*, 2007; Zhang *et al.*, 2010). To access BIK1, AvrPphB must be targeted to the PM. Following its delivery in the host cell, AvrPphB first undergoes autoproteolytic cleavage *in planta* to expose embedded residues Gly63 and Cys64 at the N-terminus of the larger (C-terminal)

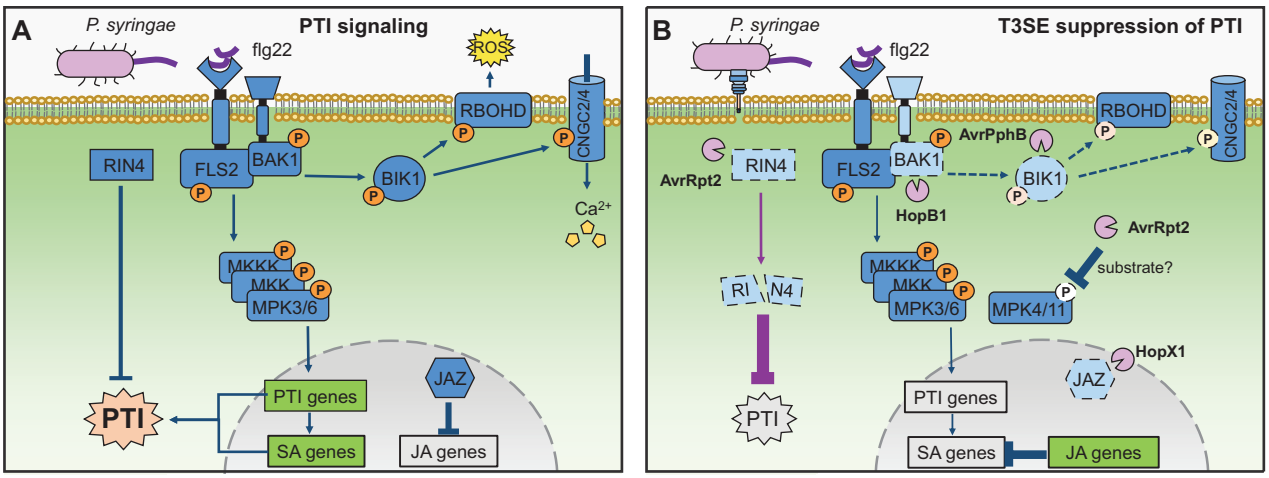


Fig. 1. T3SE proteases interfere with plant innate immune signalling. (A) PTI signalling pathway. The FLS2–BAK1 co-receptor complex initiates PTI signalling upon perception of flg22. Phosphorylated BIK1 dissociates from the receptor complex and promotes ROS production and Ca^{2+} influx by phosphorylating RBOHD and the CNGC2/4 calcium channel (Tian *et al.*, 2019). MAPK cascades transduce PTI signals intracellularly, resulting in the up-regulation of defence genes including SA-responsive genes. RIN4 generally functions as an inhibitor of PTI. (B) T3SE protease suppression of PTI. HopB1 cleaves phosphorylated BAK, inhibiting downstream signalling and BIK1 phosphorylation. BIK1 is itself cleaved by AvrPphB, thus reducing RBOHD phosphorylation and ROS production. In the nucleus, HopX1 cleaves JAZ transcriptional repressors, activating JA-responsive genes and, as a consequence of JA signalling activation, suppressing SA genes. Additionally, AvrRpt2 cleavage of RIN4 yields three fragments, two of which hyperactively suppress PTI. Pink pac-man, T3SE proteases; blue, host proteins, with light blue colour and dashed lines indicating T3SE protease targets; dashed lines indicate processes that are disrupted as a consequence of T3SE protease activity.

AvrPphB fragment (Puri *et al.*, 1997; Nimchuk *et al.*, 2000). Processed AvrPphB is myristoylated and palmitoylated *in vivo* at these N-terminal sites, prompting its translocation to the PM (Dowen *et al.*, 2009). Expression of transgenic AvrPphB in Arabidopsis inhibits PTI responses triggered by multiple PAMPs including flg22, elf18 (derived from bacterial Elongation Factor-Tu), and fungal chitin (Zhang *et al.*, 2010). Abolition of AvrPphB protease activity by a Cys98Ser substitution significantly reduces its suppression of the flg22-inducible marker gene *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*), indicating that protease activity is required for its immunosuppressive function (Shao *et al.*, 2003; Zhang *et al.*, 2010).

Besides direct regulation by kinases or secondary messengers such as ROS and Ca^{2+}, phytohormones are major regulators of transcriptional reprogramming during PTI. The principal immune hormones ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) accumulate in response to flg22 (Berens *et al.*, 2017). Each hormone controls an extensive network of response genes. For example, >3600 Arabidopsis genes are responsive to JA (Hickman *et al.*, 2017). In general, the SA network is particularly effective against biotrophic or hemibiotrophic pathogens (such as *P. syringae*), while JA and ET are associated with the response to necrotrophs (Glazebrook, 2005). The contrasting roles played by these hormones can lead to complex signalling interactions, typified by a mutual antagonism between the SA and JA pathways (Berens *et al.*, 2017). These interactions are subject to manipulation by effectors to favour pathogen virulence.

HopX1 from *P. syringae* pv. *tabaci* cleaves JASMONATE-ZIM DOMAIN (JAZ) proteins (Gimenez-Ibanez *et al.*, 2014), which function as major repressors of JA-responsive transcription factors (Pauwels *et al.*, 2010; Pauwels and Goossens, 2011) (Fig. 1; Table 1). *In planta*, HopX1 accumulates in the cytoplasm and nucleus, and interacts with the conserved zinc-finger inflorescence meristem (ZIM) domain of JAZ repressors, leading to their elimination, with no detectable fragments remaining (Gimenez-Ibanez *et al.*, 2014). The HopX1 catalytic residue Cys179 is required for degradation of JAZ5 *in vitro* (Gimenez-Ibanez *et al.*, 2014), indicating that JAZ proteins are targeted directly for proteolysis. Ectopic expression of HopX1 in Arabidopsis alleviates repression of JA response genes while reducing the expression of SA-inducible marker genes important for combatting *P. syringae* infection (Gimenez-Ibanez *et al.*, 2014). The recently characterized homologue RipE1 from *Ralstonia solanacearum* also promotes the degradation of JAZ repressors, with similar outcomes (Nakano and Mukaihara, 2019). The activity of HopX1 during infection is comparable with the effect of coronatine, a structural mimic of JA-Ile secreted by *P. syringae* to activate the JA pathway (Zheng *et al.*, 2012; Gimenez-Ibanez *et al.*, 2014), highlighting the diverse strategies employed by pathogens to overcome hormonal regulation of the host immune response.

The *P. syringae* T3S papain-like cysteine protease AvrRpt2 cleaves nitrate-induced (NOI) domain-containing proteins, including RPM1-INTERACTING PROTEIN4 (RIN4) (Axtell *et al.*, 2003; Chisholm *et al.*, 2005; H.S. Kim *et al.*, 2005; Eschen-Lippold *et al.*, 2016; Goslin *et al.*, 2019) (Fig. 1; Table

1). RIN4 is a PM-localized central immune regulator that generally inhibits PTI and is targeted by multiple effectors (M.G. Kim *et al.*, 2005; Toruño *et al.*, 2016; Ray *et al.*, 2019). Less is known about the function of other NOI domain-containing proteins that are also targeted by AvrRpt2 (Eschen-Lippold *et al.*, 2016). An important aspect of AvrRpt2 function is its activation by the cyclophilin/peptidyl-prolyl isomerase ROC1 in Arabidopsis (Coaker *et al.*, 2005, 2006; Figaj *et al.*, 2019). Activated AvrRpt2 then undergoes autoproteolytic processing and is probably myristoylated at Gly72 to facilitate co-localization with RIN4 at the PM (Coaker *et al.*, 2005, 2006; H.S. Kim *et al.*, 2005).

AvrRpt2 cleavage of RIN4 yields two fragments termed ACP2 (AvrRpt2-cleavage product 2) and ACP3 containing the majority of the N- and C-terminal NOI domains, respectively (Toruno *et al.*, 2016). Although the elimination of a negative immune regulator by pathogen proteases appears counter-productive, the ACP2 and ACP3 fragments were found to hyperactively suppress PTI in comparison with the full-length protein (Toruño *et al.*, 2016; Ray *et al.*, 2019) (Fig. 1). Both ACP2 and ACP3 appear to be short lived *in planta*, but the exact mechanism of their removal is unclear (Axtell *et al.*, 2003; Axtell and Staskawicz, 2003; Goslin *et al.*, 2019). Fragments generated by AvrRpt2 cleavage of several other NOI proteins (NOI1, NOI6, and NOI11) are substrates for the N-degron pathway (Goslin *et al.*, 2019), a ubiquitin-dependent protein degradation pathway that targets substrate proteins for degradation based on the identity of their N-terminal residue (Dissmeyer *et al.*, 2018; Holdsworth *et al.*, 2020). It remains unclear whether these NOI proteins or their cleavage products exert any functional influence on PTI or are merely inadvertent targets of AvrRpt2, with RIN4 as the operative target. However, AvrRpt2 also appears to promote virulence of *P. syringae* independently of RIN4 (Lim and Kunkel, 2004), suggesting the existence of other targets that participate in the immune response. Notably, it has been reported that AvrRpt2 also stimulates turnover of Aux/IAA negative regulators to enhance auxin signalling during infection, although direct cleavage by AvrRpt2 was not detected in this case (Cui *et al.*, 2013). Similarly, AvrRpt2 has been shown to disrupt mitogen-activated protein kinase (MAPK) signalling by suppressing the flg22-induced phosphorylation of MPK4 and MPK11 in Arabidopsis. However, the identity of the AvrRpt2 substrate(s) responsible for this down-regulation remains unknown (Eschen-Lippold *et al.*, 2016).

Detection of effector protease activity in plants

Recognition of AvrPphB protease activity

It was first reported by Simonich and Innes (1995) that Arabidopsis plants carrying the gene *RESISTANCE TO P. SYRINGAE5* (*RPS5*) were resistant to *P. syringae* pv. tomato DC3000 (*Pto*) strains carrying AvrPphB (then known as AvrPph3). Subsequent investigations revealed that RPS5-mediated resistance requires AvrPphB cleavage of PBS1 (Shao *et al.*, 2003; Zhang *et al.*, 2010). Although more recent studies have revealed that AvrPphB also cleaves other PBS1-like proteins such as BIK1 (Zhang *et al.*, 2010), only cleavage of PBS1 is sufficient to trigger ETI (Ade *et al.*, 2007) (Fig. 2). Considering that BIK1 plays a major role in PTI signalling while PBS1 makes a relatively minor contribution (Zhang *et al.*, 2010), PBS1 has been described as a 'decoy' target guarded by RPS5, while BIK1 (and possibly other PBL kinases) are the 'operative' targets of AvrPphB (Sun *et al.*, 2017; Pottinger and Innes, 2020). According to the current model of RPS5 activation, PBS1 interacts with the N-terminal coiled-coil (CC) domain of RPS5 in pathogen-free conditions, maintaining RPS5 in an inactive, ADP-bound state (Ade *et al.*, 2007; Qi *et al.*, 2014). Upon infection, cleavage of PBS1 by AvrPphB induces a structural change in RPS5, permitting

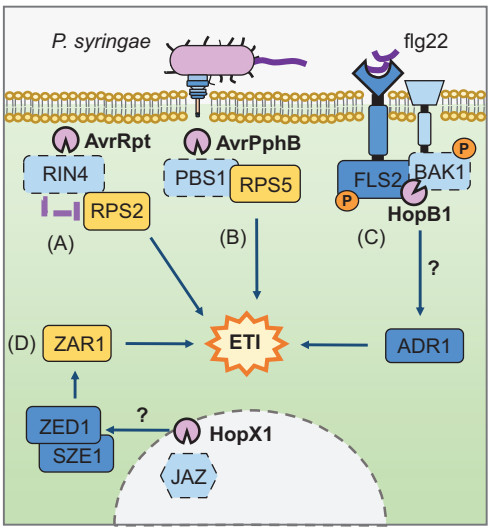


Fig. 2. Detection of effector protease activity by cytosolic plant immune receptors. Plant NLRs induce ETI in response to *P. syringae* T3SE proteases. (A) RIN4 interacts with and inhibits RPS2. Cleavage of RIN4 by AvrRpt2 relieves RPS2 from repression, triggering the activation of ETI. (B) PBS1 interacts with RPS5. AvrPphB cleavage of PBS1 induces a conformational change in RPS5, triggering the onset of ETI. (C) HopB1 interacts with the FLS2 receptor to access phosphorylated (active) BAK1 for cleavage. ETI activated in response to HopB1 requires the 'helper' NLR ADR1 and probably involves other unknown receptors, such as 'sensor' NLRs. (D) HopX1 appears to promote an interaction between RLCKs ZED1 and SZE1, leading to the activation of ZAR1-mediated ETI. To date, no link has been established between ZAR1 activation and the protease activity of HopX1. Pink pac-man, T3SE proteases; blue, host proteins, with light blue colour and dashed lines indicating T3SE protease targets; yellow, NLRs involved in the detection of T3SE proteases and onset of ETI; question marks indicate unknown mechanisms and components; dashed lines indicate processes that are disrupted as a consequence of T3SE protease activity.

the exchange of ADP for ATP and thereby activating ETI signalling and HR (Ade *et al.*, 2007).

Recent studies have revealed that AvrPphB protease activity is also recognized by other plant species including barley and wheat (Sun *et al.*, 2017; Carter *et al.*, 2019). Barley contains two *PBS1* orthologues that can be cleaved by AvrPphB, leading to the activation of defence responses by the NLR *AvrPphB Response1* (*PBR1*) (Carter *et al.*, 2019). The conservation of PBS1 can be exploited to expand the scope of RPS5-mediated ETI across different plant species and their specific pathogen interactors (Kim *et al.*, 2016). For example, expressing a modified soybean PBS1 orthologue containing a motif recognizable by the NIa protease of the *Soybean mosaic virus* (SMV) in place of the standard AvrPphB cleavage site confers immunity to the virus (Helm *et al.*, 2019; Pottinger and Innes, 2020).

Recognition of AvrRpt2 cleavage of RIN4

Another T3SE protease for which NLR-mediated recognition has been dissected in detail is AvrRpt2. The relationship between AvrRpt2 and the cognate Arabidopsis CC-NLR RPS2 was first discovered in the mid 1990s (Innes *et al.*, 1993; Bent *et al.*, 1994; Mindrinos *et al.*, 1994). The activation of RPS2-mediated defences by AvrRpt2 was later correlated with the elimination of RIN4 (Axtell and Staskawicz, 2003). In the absence of pathogen challenge, RIN4 physically associates with RPS2, maintaining it in an inactive state to preclude ETI signalling. After AvrRpt2 cleavage, RIN4 fragments are unable to maintain an interaction with RPS2 and can no longer abrogate RPS2-dependent HR (Coaker *et al.*, 2005; Day *et al.*, 2005, 2006) (Fig. 2). However, elimination of RIN4 alone is not sufficient for AvrRpt2-induced activation of RPS2 (Toruño *et al.*, 2016). NONSPECIFIC DISEASE RESISTANCE1 (NDR1) is a PM-anchored immune regulator required for the full activation of multiple NLRs including RPS5 and RPS2 (Coppinger *et al.*, 2004). A physical interaction between NDR1 and RIN4 is required for RPS2 activation by AvrRpt2 (Day *et al.*, 2006). Unlike RPS2, NDR1 can also interact with the ACP3 fragment of RIN4 after cleavage by AvrRpt2 (Day *et al.*, 2006). Although the exact mechanism underlying the role of NDR1 remains unknown, it has been proposed that its interaction with RIN4 may protect RPS2 from negative regulation during infection (Day *et al.*, 2006).

AvrRpt2-induced defence responses have been described in other plant–pathogen species contexts. The MR5 CC-NLR from wild apple recognizes an AvrRpt2 homologue from the fireblight pathogen *Erwinia amylovora* based on its cleavage of apple *Md*RIN4 (Vogt *et al.*, 2013; Broggini *et al.*, 2014; Prokchorchik *et al.*, 2020). Unlike the *At*RIN4–RPS2 complex, *Md*RIN4 does not appear to inhibit MR5 autoactivation (Prokchorchik *et al.*, 2020). Rather, the *Md*RIN4 ACP3 cleavage fragment generated by AvrRpt2 activates MR5 (Prokchorchik *et al.*, 2020). Ptr1 is a CC-NLR identified in the tomato-like nightshade *Solanum lycopersicoides* that also confers resistance to *P. syringae* expressing AvrRpt2 (Mazo-Molina *et al.*, 2020). Ptr1 recognition of AvrRpt2 variants correlates with their ability to eliminate tomato RIN4 proteins (Mazo-Molina *et al.*, 2019). Functional Ptr1 orthologues conferring resistance to AvrRpt2 also occur in *Nicotiana benthamiana* and potato (Mazo-Molina *et al.*, 2019, 2020). Based on the sequential and mechanistic diversity of RPS2, MR5, and Ptr1, these NLRs have probably arisen by convergent evolution to detect AvrRpt2 (Toruño *et al.*, 2016; Mazo-Molina *et al.*, 2020; Prokchorchik *et al.*, 2020).

Detection of HopX1 by ZAR1

A recent systematic study of ETI-inducing effectors revealed that the Arabidopsis CC-type NLR HopZ ACTIVATED RESISTANCE1 (ZAR1) confers immunity against a range of effectors including the HopX1 family (Laflamme *et al.*, 2020) (Fig. 2). HopX1-induced activation of ZAR1 also requires the RLCKs HOPZ-ETI-DEFICIENT1 (ZED1) and SUPPRESSOR OF ZED1-D 1 (SZE1), although cleavage of neither ZED1 nor SZE1 was detected (Martel *et al.*, 2020). As yet, no functional relationship has been established between HopX1-mediated cleavage of JAZ proteins and its activation of ZAR1 (Gimenez-Ibanez *et al.*, 2014; Martel *et al.*, 2020). Notably, *E. amylovora* HopX1 contributes to the onset of HR in cultivated tobacco (*Nicotiana tabacum*), while it suppresses it in *N. benthamiana* (Bocsanczy *et al.*, 2012). Additional experiments suggest that in *E. amylovora*'s native host, apple trees, HopX1 may also trigger HR (Bocsanczy *et al.*, 2012). This is in contrast to the observation that HopX1i (a HopX1 allele from *P. syringae*) does not trigger HR in Arabidopsis, despite the onset of ZAR1-dependent ETI (Laflamme *et al.*, 2020).

Recognition of HopB1 protease activity

HopB1 proteolytic cleavage of BAK1 also appears to be detected by plant NLRs (Fig. 2). ETI responses induced by HopB1 are dependent on the presence of the 'helper' NLR ACTIVATED DISEASE RESISTANCE1 (ADR1) and its paralogues (Wu *et al.*, 2020). Helper NLRs do not directly recognize effectors but are required for the full activity of 'sensor' NLRs (Jubic *et al.*, 2019). These findings suggest that BAK1 may be 'guarded' by an as yet unidentified sensor NLR, particularly as ADR1 and HopB1 do not appear to directly interact (Wu *et al.*, 2020).

Regulation of ETI-related HR by effector proteases

As outlined above, detection of effector proteases by NLRs can trigger ETI-dependent HR. However, some effector proteases also act to repress HR. One such example is AvrPphB, a member of the YopT family of *P. syringae* T3S cysteine

protease effectors (Shao *et al.*, 2002). In addition to triggering RPS5-mediated ETI, AvrPphB also functions to suppress ETI launched upon detection of the effector AvrB (Fig. 3). In the absence of AvrPphB, AvrB recruits the host receptor-like cytoplasmic kinase RPM1-INTERACTING PROTEIN KINASE (RIPK) to induce phosphorylation of RIN4, triggering ETI mediated by the NLR RESISTANCE TO P. SYRINGAE PV. MACULICOLA1 (PRM1) (Mackey *et al.*, 2002; Liu *et al.*, 2011). By directly targeting RIPK for cleavage, AvrPphB prevents phosphorylation of RIN4, thus avoiding RPM1 activation (Russell *et al.*, 2015).

HopN1 (formerly known as AvrPtoN) suppresses HR-related cell death in tobacco and tomato (López-Solanilla *et al.*, 2004) and diminishes defence-associated ROS production and callose deposition in Arabidopsis (Rodríguez-Herva *et al.*, 2012). Using *in vitro* pull-down assays followed by MS, the tomato chloroplast protein PsbQ (PSII oxygen-evolving complex protein 3) was identified as a binding partner of HopN1 (Rodríguez-Herva *et al.*, 2012) (Fig. 3). PsbQ is required for full ROS production and HR in response to bacterial infection. Analysis of thylakoid samples from *N. benthamiana* revealed that degradation of PsbQ in the presence of HopN1 depends on its catalytic site remaining intact (Rodríguez-Herva *et al.*, 2012). This finding highlights the contribution of photosynthetic proteins to the immune response, as well as their vulnerability to effector proteases despite localization in the chloroplast.

Effector protease-mediated manipulation of mammalian innate immune signalling

Some families of T3SE proteases are conserved among plant and animal pathogens, albeit with differences in their substrate proteins (see 'Effector proteases: evolutionary conservation and diversity' below for more details). This conservation, together with the similarities between the innate immune signalling pathways in metazoa and plants (Ausubel, 2005; Jones *et al.*, 2016), makes it interesting to compare T3SE protease function in plants and animals. In both lineages, membrane-bound immune receptors detect PAMPs or DAMPs present in the extracellular environment and relay signals into cells via different signal transduction pathways, including MAPK signalling cascades. This induces a proinflammatory response in animals and PTI in plants (Ausubel, 2005). Similarly, in both animals and plants, a large variety of distinct cytosolic NLR receptor proteins sense pathogen-associated perturbations in the cytosol (Jones *et al.*, 2016). Activated NLRs form higher order oligomers as modular platforms to initiate downstream signalling, including

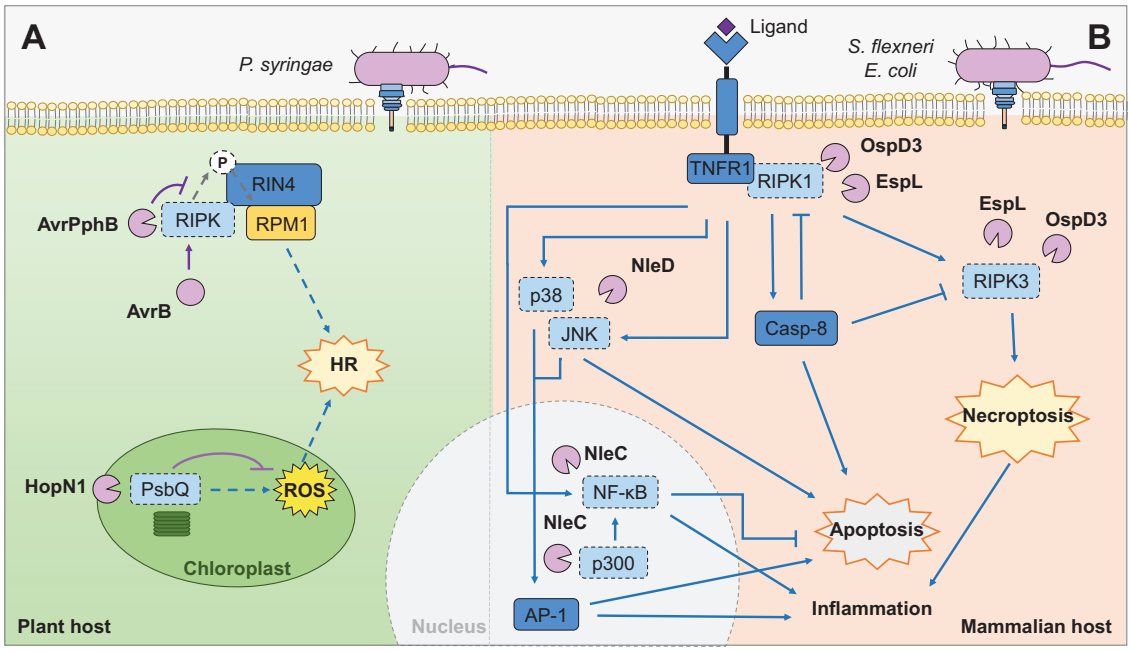


Fig. 3. Bacterial effector proteases interfere with plant and mammalian cell death and pro-inflammatory signalling. (A) *P. syringae* T3SE proteases suppress HR (regulated cell death associated with ETI) in plant cells. AvrPphB cleaves the host kinase RIPK, impeding AvrB-induced phosphorylation of RIN4 to prevent RPM1-mediated HR. In the chloroplast, HopN1 suppresses chloroplast ROS production by cleaving PsbQ. Chloroplast-generated ROS play an important role in establishing HR (Liu *et al.*, 2007; Zurbriggen *et al.*, 2010; Rodríguez-Herva *et al.*, 2012). (B) Perturbations of the extracellular microenvironment are sensed by membrane-bound receptors such as TNF receptor 1 (TNFR1), activating intracellular signalling. Bacterial proteases injected by the T3SS cleave key components of both pro-inflammatory signalling as well as cell death pathways. For details, see main text. Pink pacman, T3SE proteases; pink circle, T3SE; blue, host proteins, with light blue colour and dashed lines indicating T3SE protease targets; yellow rectangle, NLR involved in the detection of T3SE proteases and onset of ETI; dashed lines indicate processes that are disrupted as a consequence of T3SE protease activity.

initiation of cell death programmes (Dangl and Jones, 2019). In plants, cell death triggered by effector recognition is typically categorized as HR, but the mechanisms leading to the onset of cell death are not understood in as much detail as they are in animals (Pitsili *et al.*, 2020). In animals, distinct cell death pathways emitting different signals to the surrounding tissue have been defined (Jorgensen *et al.*, 2017; Galluzzi *et al.*, 2018). Apoptosis can be triggered by perturbations of the extracellular environment that are detected by a variety of PM receptors, including tumour necrosis factor receptor 1 (TNFR1), resulting in activation of the cysteine protease caspase-8 (Fig. 3). Alternatively, apoptosis may be triggered by activation of caspase-9 as a result of mitochondrial outer membrane permeabilization induced by intracellular stress. Both pathways converge on the activation of the effectors caspase-3 and caspase-6, which cleave hundreds of protein substrates to orchestrate an orderly demise of the cell (Crawford *et al.*, 2012). Apoptosis eliminates cells during development or after cellular stress that exceeds the capacity for repair, and is generally considered to be immunologically silent (Bedoui *et al.*, 2020).

Pyroptosis and necroptosis, in contrast, are highly inflammatory forms of cell death leading to immune cell recruitment (Galluzzi *et al.*, 2018; Bedoui *et al.*, 2020; Flores-Romero *et al.*, 2020). Pyroptosis is induced after activation of cytosolic NLRs, which triggers formation of higher order complexes termed inflammasomes that activate caspase-1. Alternatively, intracellular pathogen-derived lipopolysaccharide (LPS) can activate caspase-4 and caspase-5 (Shi *et al.*, 2014). On activation, all three inflammatory caspases cleave a number of substrates including gasdermin-D (GSDMD) (Agard *et al.*, 2010; Shi *et al.*, 2015). The N-terminal fragment of GSDMD oligomerizes and forms pores in the cell membrane, resulting in the release of pro-inflammatory cytokines and subsequent cell death (Bedoui *et al.*, 2020; Flores-Romero *et al.*, 2020). Necroptosis is a caspase-independent pro-inflammatory form of cell death initiated by PM receptors such as TNFR1 and mediated by the receptor-interacting serine-threonine kinases 1 (RIPK1) and RIPK3, which phosphorylates the protein MLKL (mixed-lineage kinase domain-like) (Galluzzi *et al.*, 2018; Bedoui *et al.*, 2020) (Fig. 3). Phosphorylated MLKL assembles into large pore-forming oligomers that cause PM rupture and release of a multitude of pro-inflammatory cellular DAMPs (Flores-Romero *et al.*, 2020).

These cell death pathways are remarkably interconnected, with caspase-8 at the nexus (Fritsch *et al.*, 2019; Bedoui *et al.*, 2020). In the extrinsic pathway of apoptosis, PM receptor stimulation results in activation of caspase-8, which cleaves RIPK1 and RIPK3 and thereby prevents necroptosis. Thus, necroptosis can be considered as a back-up programme to induce cell death when apoptosis to extrinsic stimuli is blocked (Jorgensen *et al.*, 2017; Bedoui *et al.*, 2020). Inactive caspase-8 further triggers inflammasome formation and caspase-1 activation, resulting in cell death by pyroptosis when necroptosis is prevented by RIPK3 or MLKL ablation (Fritsch *et al.*, 2019). Thus, the mammalian cell death pathways guard not only the innate immune signalling pathways, but also each other, against pathogen interference (Jorgensen *et al.*, 2017; Bedoui *et al.*, 2020). Bacterial pathogens therefore must not only prevent pro-inflammatory responses, but also avoid the trip wires of mutually cross-loaded cell death programmes (Fig. 3).

Two examples illustrate how T3SE proteases contribute to overcome this formidable challenge (Table 1). Enteropathogenic *Escherichia coli* (EPEC), an attaching and effacing bacterium that causes persistent diarrhoea primarily in children, uses a variety of T3SEs to simultaneously suppress immune and cell death signalling (Shenoy *et al.*, 2018). This includes two zinc metalloproteases, NleC and NleD (Fig. 3), that interfere with the pro-inflammatory nuclear factor (NF)-κB signalling. Specifically, NleC attacks pro-inflammatory signalling pathways by cleavage and inactivation of three subunits of NF-κB (Yen *et al.*, 2010; Baruch *et al.*, 2011; Pearson *et al.*, 2011), and also cuts the acetyltransferase p300 that acts as a transcriptional co-activator for many genes, including those regulated by NF-κB (Shames *et al.*, 2011). The second metalloprotease, NleD, cleaves and inactivates the MAPKs c-Jun N-terminal kinase (JNK) and p38 that are involved in pro-inflammatory and apoptotic signalling (Baruch *et al.*, 2011). A third T3SE protease, the cysteine protease EspL, targets RIPK1 and RIPK3 to prevent necroptosis (Pearson *et al.*, 2011) (Fig. 3). The Gram-negative bacterium *Shigella flexneri*, which causes diarrhoea in humans, similarly prevents necroptotic cell death by degradation of RHIM-containing proteins, including RIPK1 and RIPK3, with the EspL homologue OspD3 (Ashida *et al.*, 2020).

Comparison of known T3SE protease functions in modulating immune signalling pathways in plants and mammals reveals striking similarities. In both lineages, substrates that allow T3SE proteases to interfere with PRR-activated MAPK signalling pathways have been identified. In plants, numerous substrates of T3SE proteases in PRR-mediated signalling pathways are guarded by NLRs, resulting in HR cell death and a strain-specific response (Pitsili *et al.*, 2020). Similarly, in metazoa, pathogen-mediated manipulation of cellular processes is sensed by cytosolic NLRs, triggering enhanced pro-inflammatory responses including cell death, in analogy to plant ETI and HR (Lopes Fischer *et al.*, 2020). Identification of specific T3SE protease targets in mammals has shown how these effectors allow pathogens to manipulate these cell death-inducing pathways for their benefit. One conspicuous difference in plants is that, in contrast to the wealth of knowledge in mammals, the mechanism(s) by which plant HR cell death is executed remain poorly understood. Several plant proteases of different mechanistic classes, as well as autophagy and the UPS, have been implicated in the onset of regulated cell death induced by different triggers, but so far comparatively few substrates are known (Balakireva and Zamyatnin, 2019; Salguero-Linares and

Coll, 2019). Consequently, the molecular mechanisms of how T3SE proteases, and in fact T3SEs in general, suppress plant HR-related cell death have remained elusive. Indications for such effector-mediated suppression of HR have nevertheless been reported (Jamir *et al.*, 2004; Guo *et al.*, 2009; Wei *et al.*, 2018).

Effector proteases: evolutionary conservation and diversity

Identification of T3SE proteases requires evidence of type III secretion, knowledge of host targets or of effects on the host immune response (e.g. dampening of PTI or ETI activation) (Lindeberg *et al.*, 2005), identification of catalytic residues/triad, as well as *in planta* or *in vitro* evidence of protease activity. Pto DC3000 has been used as a model pathogen for four decades (Xin *et al.*, 2018). As highlighted above, the Pto DC3000 genome is predicted to encode four T3SE proteases: HopB1, HopC1, HopN1, and HopX1 (Table 1). Another potential T3SE protease is HopZ1, although its protease activity appears to be very weak *in vitro* and it may primarily act as an acetyltransferase (Zhou *et al.*, 2011). Other pathovars of *P. syringae* code for additional T3SE proteases, such as, for example, AvrPphB and AvrRpt2, both of which have been among the most studied T3SE proteases, as highlighted above (Table 1).

Analysis of the genomes of 494 *P. syringae* belonging to different pathovar groups (Markowitz *et al.*, 2012; Wattam *et al.*, 2014; Dillon *et al.*, 2019) indicates that HopX1 (formerly known as AvrPphE) homologues are widely distributed across 308 different strains, including a variety of pathovars (Studholme *et al.*, 2009; Dillon *et al.*, 2019). The broad distribution of HopX1 might reflect its importance to facilitate *P. syringae* entry inside host tissue via stomata (Gimenez-Ibanez *et al.*, 2014), an essential first step in the infection process of *P. syringae* (Xin *et al.*, 2018). Interestingly, variations in HopX1 sequences among different races of *P. syringae* pv. *phaseolicola* affect both strain virulence and the host's ability to trigger ETI in bean plants (Stevens *et al.*, 1998). This highlights the fine-tuning mechanisms at play in effector protease sequence, target selection, and recognition by host NLRs.

Other Pto DC3000 T3SE proteases such as HopC1, HopN1, and HopB1 are also broadly distributed among *P. syringae* strains, with putative homologues found in 115, 74, and 66 strains, respectively (Dillon *et al.*, 2019). HopN1 appears to be particularly important as, together with seven other T3SEs, it is part of a so-called minimal repertoire of Pto DC3000 effectors needed to restore virulence of a Pto DC3000 mutant strain in which 28 effectors have been deleted (Cunnac *et al.*, 2011). Although Pto DC3000 codes for all four proteases in its genome, only five other *P. syringae* strains code for the same four effector proteases, including one other Pto strain and some strains of *P. syringae* pv. *maculicola* (Pma). Interestingly, some of these Pma strains are thought to belong to the same phylogenetic group as Pto DC3000 (Clarke *et al.*, 2010), perhaps highlighting that the concept of pathovar does not necessarily correlate with phylogenetic relationship (Berge *et al.*, 2014).

AvrRpt2 (initially isolated from Pto JL1065) is arguably one of the most studied T3SE proteases (Innes *et al.*, 1993). Analysis of the 494 genome sequences of *P. syringae* suggests that only 25 of these strains code for potential AvrRpt2 homologues (Dillon *et al.*, 2019). These 25 strains belong to different pathovar groups, indicating that AvrRpt2 function as a virulence factor is not specific to one host type. Notably, AvrRpt2 is also encoded by the genomes of other plant pathogens (e.g. *R. solanacearum* or *E. amylovora*), as well as symbiotic bacteria (e.g. *Mesorhizobium huakuii* and *Sinorhizobium medicae*) (Eschen-Lippold *et al.*, 2016). AvrRpt2 from *E. amylovora* in particular also acts as a virulence factor during infection of its native host (pear and apple trees) (Zhao *et al.*, 2006; Vogt *et al.*, 2013). However, a single amino acid change (Cys156Ser) found in natural variants of *E. amylovora* AvrRpt2 is sufficient to change its recognition by cognate NLRs in apple (Vogt *et al.*, 2013). Similarly, differences in the substrate specificity of AvrRpt2 homologues found in pathogenic and non-pathogenic bacteria have been found (Eschen-Lippold *et al.*, 2016). These findings highlight how sequence differences among putative AvrRpt2 homologues may be relevant in terms of virulence/avirulence.

Other Pto DC3000 T3SE proteases are also conserved among plant pathogens. For example, HopX1 homologues have been identified in *R. solanacerum*, *Xanthomonas campestris*, and *E. amylovora* (Nimchuk *et al.*, 2007; Bocsanczy *et al.*, 2012). Proteases with sequence similarities to HopX1 are also encoded by animal pathogens such as *Legionella pneumophila* (the causative agent of 'Legionnaire's disease') (Nimchuk *et al.*, 2007). However, it is expected that the substrates of the potential homologues have probably diverged (Nimchuk *et al.*, 2007).

Such widespread distribution of effector proteases across plant and animal pathogens is also found among YopT family members (Table 1), which includes YopT from *Yersinia pestis* (the causative agent of bubonic plague), as well as HopC1, HopN1, AvrPphB (*P. syringae* pv. *phaseolicola*), NopT (*Sinorhizobium fredii* NGR234), and RipT in *R. solanacearum* (Shao *et al.*, 2002; Dowen *et al.*, 2009). All YopT family members have a conserved catalytic triad, as well as similar secondary structures, but are otherwise variable in sequence (Shao *et al.*, 2002; Dowen *et al.*, 2009). YopT family members from plant pathogens exhibit autoproteolytic activity which is essential for virulence (Shao *et al.*, 2002; Dai *et al.*, 2008; Dowen *et al.*, 2009), as well as recognition by cognate NLRs (Shao *et al.*, 2002, 2003; Ade *et al.*, 2007). However, in animal pathogens such as *Y. pestis*, YopT does not undergo self-cleavage (Shao *et al.*, 2002). Similarly to HopX1, it is expected that the substrates of YopT family members have diverged between plant and animal pathogens. This is supported by the fact that (i) expression of

AvrPphB in mammalian cell lines does not trigger the same cytotoxic phenotype as expression of YopT (Shao *et al.*, 2002); and (ii) YopT's main target appears to be RhoA, a member of the GTPase family of proteins (Shao *et al.*, 2002, 2003), while AvrPphB's main target in Arabidopsis is the unrelated protein kinase AvrPphB SUSCEPTIBLE PROTEIN1 (PBS1) (Shao *et al.*, 2003). Furthermore, based on the crystal structure of AvrPphB, some features of the substrate-binding sites of YopT family members are not conserved (Zhu *et al.*, 2004), even within plant pathogens. Hence, YopT family members such as HopC1, HopN1, AvrPphB, and NopT could target different protein substrates (Zhu *et al.*, 2004). Interestingly, symbiotic bacteria (e.g. *Rhizobium* species) also code for YopT family members, but, again, these probably target distinct host proteins. For example, *Sinorhizobium* NopT (formerly known as Y4zC) does not cleave PBS1 (Zhu *et al.*, 2004).

A more contentious case of potential effector proteases found among plant and animal pathogens are members of the YopJ family (reviewed in Ma and Ma, 2016). YopJ family members may act as both or either acetyltransferases and/or proteases. The founding member of this family, YopJ from *Y. pestis*, appears to act, at least in part, by decreasing the amount of ubiquitin and SUMO chains *in vivo*, suggesting that it may have ubiquitin-like protease activity (Orth *et al.*, 2000; Zhou *et al.*, 2005; Sweet *et al.*, 2007). However, it is unclear if this is directly due to YopJ activity as a protease, or if it is a secondary effect of YopJ activity on another substrate, perhaps via acetylation since YopJ acts as an acetyltransferase (Mukherjee *et al.*, 2006; Meinzer *et al.*, 2012; Paquette *et al.*, 2012). Nevertheless, some members of the YopJ family, including HopZ1a and HopZ3 (Table 1) from *P. syringae* pv. *syringae* and pv. *glycinea* (Ma *et al.*, 2006), as well as XopJ from *X. campestris* pv. *vesicatoria* (Üstün and Börnke, 2015), have been shown to exhibit some degree of protease activity (Ma *et al.*, 2006; Zhou *et al.*, 2011).

In sum, the evolutionary conservation of effector protease families such as YopT across animal and plant pathogens is testament to their important roles as effectors. Nevertheless, T3SE proteases are versatile actors in the interplay between pathogenicity and immunity upon infection of a host, as outlined in sections above. Sequence variation and divergence in function enable the recognition of different host substrates, but also allow for differential recognition by hosts who have evolved cognate receptors. Notably, despite the evolutionary relevance of T3SE proteases and mechanistic details underlying their activity and recognition, overall relatively little is still known about their targets at the proteome-wide level (Box 1). In recent years, MS-based methods have emerged as a promising tool to address these questions in a largely unbiased manner (Demir *et al.*, 2018). Specific tools for protease substrate identification have been developed (Box 1), firmly establishing degradomics as a subfield in proteomics (López-Otín and Overall, 2002). In biomedical research, degradomics have been extensively used to define caspase substrates (Agard *et al.*, 2010), caspase specificity (Julien and Wells, 2017), and proteolytic mechanisms underlying cell death (Crawford *et al.*, 2012), and is increasingly used to characterize host manipulation by viral and bacterial proteases (Marshall *et al.*, 2017). However, to the best of our knowledge, no similar applications to define the substrates of plant effector protease have been reported. We anticipate that our understanding of proteolytic processes and protease function in plant cell death and pathology will similarly benefit from degradomics approaches, particularly as recent methodological advances have increased sensitivity and now enable analysis of samples that yield only microgram amounts of proteins (Shema *et al.*, 2018; Weng *et al.*, 2019).

Conclusion

The intriguing mechanisms underlying T3SE protease function highlight how phytopathogenic bacteria deploy these enzymes to undermine plant immunity, indeed cutting the line from signal perception to response. Equally intriguing is how plants guard T3SE protease targets and use decoys to detect T3SE protease activity, setting an emergency 'red' line to swift and massive responses that in turn are targeted by additional effectors including proteases. However, our proteome-wide knowledge of T3SE protease substrate repertoires, and therefore also of their function, is incomplete even in the extensively studied Arabidopsis–*Pseudomonas* model pathosystem. In other plant–microbe interactions, this knowledge is even more fragmented. Similarly to other T3SEs, we are currently lacking information on complete substrate repertoires, enzymatic properties such as sequence specificity, and similarities and differences among homologous T3SE proteases in both pathogenic and non-pathogenic microbes. We believe that sensitive unbiased approaches, including the MS-based techniques outlined in Box 1, will reveal new T3SE protease targets and further inform on plant immune responses (Toruño *et al.*, 2016). In addition, clarification of their substrate specificity will facilitate decoy engineering of host proteins guarded by specific NLRs (Kourelis *et al.*, 2016). For instance, seminal work demonstrated that substitution of the AvrPphB effector cleavage site in *Arabidopsis thaliana* PBS1, which is guarded by the NLR RPS5, enables recognition of other pathogen bacterial and viral effector proteases and thereby confers resistance to new pathogens (Kim *et al.*, 2016). This system has already been translated to soybean as a crop system (Pottinger and Innes, 2020), suggesting that T3SE protease activity can be exploited more widely for engineering disease-resistant crops.

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Conflict of interest

The authors confirm that they have no conflicts of interest.

Author contributions

EG and PFH conceptualized the review; BCM and MM composed the figures and tables; all authors performed literature research, drafted sections of the manuscript, edited and approved the final version.

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