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Review

The YopJ superfamily in plant-associated bacteria

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SUMMARY

Bacterial pathogens employ the type III secretion system to secrete and translocate effector proteins into their hosts. The primary function of these effector proteins is believed to be the suppression of host defence responses or innate immunity. However, some effector proteins may be recognized by the host and consequently trigger a targeted immune response. The YopJ/HopZ/AvrRxv family of bacterial effector proteins is a widely distributed and evolutionarily diverse family, found in both animal and plant pathogens, as well as plant symbionts. How can an effector family effectively promote the virulence of pathogens on hosts from two separate kingdoms? Our understanding of the evolutionary relationships among the YopJ superfamily members provides an excellent opportunity to address this question and to investigate the functions and virulence strategies of a diverse type III effector family in animal and plant hosts. In this work, we briefly review the literature on YopJ, the archetypal member from *Yersinia pestis*, and discuss members of the superfamily in species of *Pseudomonas, Xanthomonas, Ralstonia* and *Rhizobium*. We review the molecular and cellular functions, if known, of the YopJ homologues in plants, and highlight the diversity of responses in different plant species, with a particular focus on the *Pseudomonas syringae* HopZ family. The YopJ superfamily provides an excellent foundation for the study of effector diversification in the context of wide-ranging, co-evolutionary interactions.

INTRODUCTION

The recognition of conserved pathogen-associated molecular patterns (PAMPs) by the host induces a form of innate immunity, termed 'PAMP-triggered immunity' (PTI) in the plant pathology literature (Chisholm *et al*., 2006; Jones and Dangl, 2006). Examples of well-characterized bacterial PAMPs that can induce plant PTI include flagellin, a structural component of the bacterial flagella, and Ef-Tu, a component of the protein translational machinery (Boller and Felix, 2009). Successful phytopathogens must suppress PTI to establish infections and proliferate in the host tissue, and many bacterial pathogens are able to accomplish this via the type III secretion system (T3SS) and its translocated type III secreted effector (T3SE) proteins (Galan and Wolf-Watz, 2006). Plants have responded evolutionarily to this challenge through a second and more directed tier of immunity, termed 'effector-triggered immunity' (ETI), in which certain effectors (or, more typically, the actions of these effectors) induce a defence response that is often accompanied by a localized programmed cell response, termed the 'hypersensitive response' (HR). ETI relies on resistance (R) proteins, which characteristically have either a coiled-coil (CC) or toll-like-receptor (TIR) domain linked to a nucleotide-binding site-leucine-rich repeat (NBS-LRR) (Dangl and Jones, 2001). However, it should be noted that cell death is not an ETI-specific response, as some PAMPs can induce HR-like responses (Thomma *et al*., 2011). In the grand tradition of the classic arms race, some pathogens have been able to counter ETI, either by losing the defence-eliciting T3SE or via the action of other T3SEs that disrupt ETI signalling.

The YopJ superfamily of T3SEs is one of the largest and most widely distributed bacterial effector families. *Yersinia pestis*, the causal agent of the black plague, contains the archetypal member YopJ. YopJ homologues are also found in the animal pathogens *Salmonella* (AvrA), *Vibrio* (VopJ) and *Aeromonas* (AopP), which cause infections ranging from gastroenteritis to typhoid fever and other life-threatening diseases. In plant pathogens, YopJ homologues are found in species of *Pseudomonas* (the HopZ family), *Xanthomonas* (AvrRxv, AvrXv4, AvrBsT and XopJ), *Erwinia* (ORFB) and *Ralstonia* (PopP1 and PopP2), and the plant symbiont *Rhizobium* (Y4LO) (Fig. 1) (Ma *et al*., 2006).

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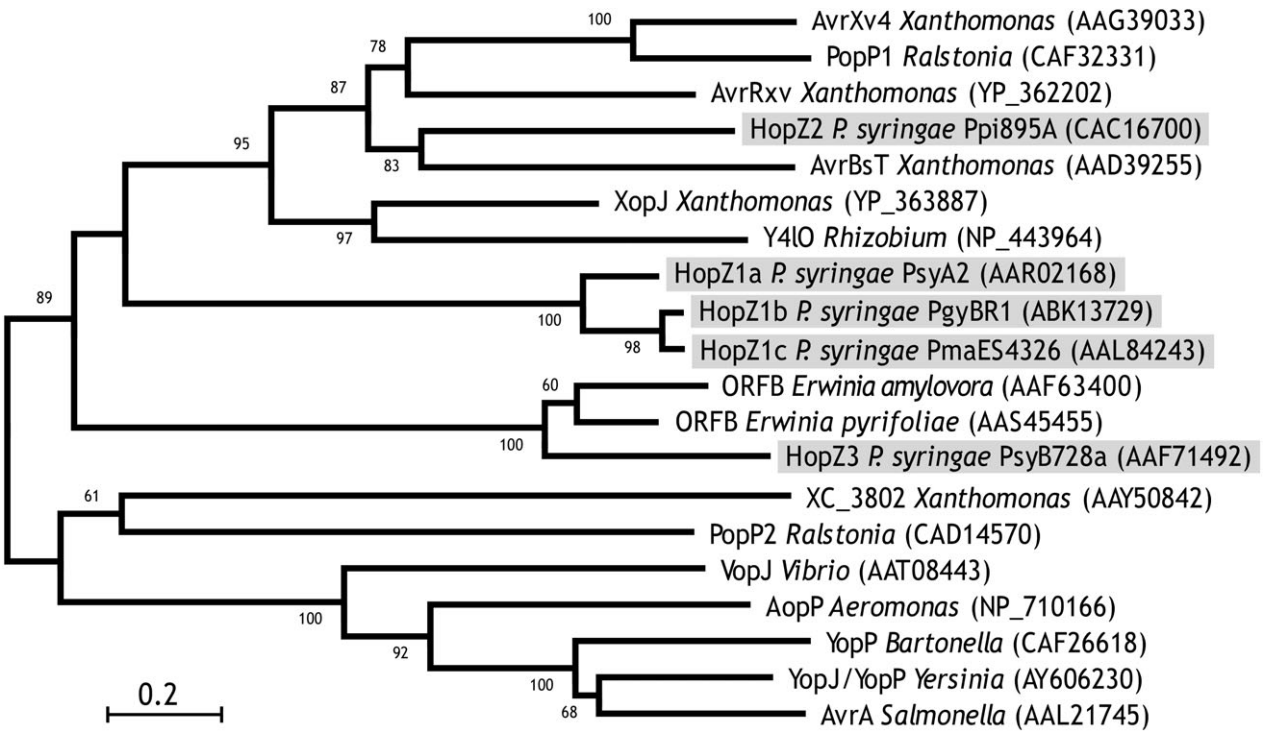

Fig. 1 Phylogenetic relationships of the YopJ superfamily of type III secreted effector (T3SE) proteins. Neighbour-joining tree of YopJ family of T3SE proteins. Bootstrap support is indicated above each node, with only values above 60% being shown. Accession numbers for each protein are presented in parentheses following the protein name and species. Modified from Ma *et al*. (2006). The scale bar indicates the evolutionary distances computed using the JTT substitution matrix (in numbers of amino acid substitutions per site).

In this article, we begin with a brief summary of an extensive body of work on YopJ, followed by a more detailed discussion of the functions of YopJ homologues from phytopathogens, with particular emphasis on the *Pseudomonas syringae* HopZ family (summarized in Table 1 and Fig. 2). Despite the extensive sequence and host-specific diversity within the YopJ superfamily, there appears to be significant similarity in molecular function, suggesting that YopJ may provide a valuable framework to study evolutionary diversification of this type III effector superfamily.

YOPJ FUNCTIONS AND PHENOTYPES

The *Yersinia pestis* and *Y. pseudotuberculosis* T3SE YopJ blocks the mammalian innate immune response by inhibiting the mitogen-activated protein kinase (MAPK) and the nuclear factor kappa B (NFκB) signalling pathways (Mukherjee *et al*., 2006; Orth *et al*., 1999, 2000). Furthermore, YopJ promotes apoptosis in macrophages by preventing the activation of the NFκB pathway (Cornelis and Wolf-Watz, 1997; Monack *et al*., 1997; Zhang *et al*., 2005). YopJ inhibits these signalling pathways by binding directly to the MAPK kinases (MAPKKs) and the inhibitor of the NFκB complex, IκBβ, but not to the upstream MAPK kinase kinases (MAPKKKs) or the downstream MAPKs (Orth *et al*., 1999). The interaction between YopJ and MAPKKs inhibits the phosphorylation of MAPKKs, thus blocking the signal transduction that leads to cytokine production and anti-apoptotic factor expression (Mukherjee *et al*., 2006, 2007; Orth *et al*., 1999, 2000).

YopJ contains a conserved catalytic triad [histidine (His), glutamate (Glu) and cysteine (Cys)], and mutations in these catalytic residues disrupt YopJ's ability to inhibit the MAPK and NFκB pathways (Orth *et al*., 2000). Given that the predicted secondary structure and catalytic domain of YopJ are similar to those of the adenovirus protease (AVP), it was initially hypothesized that YopJ was a cysteine protease (Orth *et al*., 2000). Indeed, experiments have demonstrated de-sumoylating and de-ubiquitinating activity for YopJ (Orth *et al*., 2000; Sweet *et al*., 2007). However, direct targets of these protease activities remain to be identified. In addition, using liquid chromatography-tandem mass spectrometry, Mukherjee *et al*. (2006) showed that YopJ acetylates MKK6 on serine (Ser) and threonine (Thr) residues in its activation loop. An *in vitro* acetylation assay further demonstrated that YopJ requires its catalytic Cys to acetylate MKK6 (Mukherjee *et al*., 2006; Mukherjee and Orth, 2008). In the presence of acetyl-CoA and YopJ, MKK6 or IκBβ could no longer be phosphorylated by the upstream MAPKKK, thus providing an elegant mechanistic explanation for how YopJ inhibits

Table 1 The YopJ superfamily in plant-associated bacteria.

Allele	Pathovar of origin	Enzymatic activity	Phenotype in host	Localization	Reference
YopJ	*Yersinia pestis*, *Y. pseudotuberculosis*	Acetyltransferase, SUMO protease, ubiquitin protease	Inhibition of MAPK and NFκB signalling pathways; macrophage cell death; blocking both innate and adaptive immunity	?	Monack *et al*. (1997); Mukherjee *et al*. (2006); Orth *et al*. (1999, 2000); Sweet *et al*. (2007)
HopZ1a	*Pseudomonas syringae* pv. *syringae*	Protease, acetyltransferase	HR in *Arabidopsis**,†, *Glycine max* (soybean)†,§, *Oryza sativa* (rice)†, *Sesamum indicum* (sesame)†, *Nicotiana benthamiana*†,§,††	Membrane	Lewis *et al*. (2008); Ma *et al*. (2006); Morgan *et al*. (2010); Zhou *et al*. (2009); A. Lee *et al*., unpublished data
HopZ1b	*P. syringae* pv. *glycinea*	Protease?, acetyltransferase?	Weak HR in *Arabidopsis*†,‡‡, HR in *N. benthamiana*§,††, promotes *P. syringae* virulence in *G. max* (soybean)‡	Membrane	Lewis *et al*. (2008); Ma *et al*. (2006); Zhou *et al*. (2009); A. Lee *et al*., unpublished data
HopZ1c	*P. syringae* pv. *maculicola*	Protease, acetyltransferase?	?	Membrane	Lewis *et al*. (2008); Ma *et al*. (2006); A. Lee *et al*., unpublished data
HopZ2	*P. syringae* pv. *pisi*	Protease, acetyltransferase?	HR in *Phaseolus vulgaris* (common bean)†, promotes *P. syringae* virulence in *Arabidopsis*‡	Membrane	Arnold *et al*. (2001); Lewis *et al*. (2008); Ma *et al*. (2006); A. Lee *et al*., unpublished data
HopZ3	*P. syringae* pv. *syringae*	Protease, acetyltransferase?	HR in *Nicotiana tabacum*†† and *Phaseolus vulgaris* (common bean)††, reduces *P. syringae* growth and disease symptoms in *Phaseolus vulgaris*§,¶ and *N. benthamiana*§,¶, suppresses HR induced by AvrPto1††, HopAA1††, HopM1††, HopAE1†† and HopZ1b**,†† in *N. benthamiana*, promotes *P. syringae* virulence in *Arabidopsis*§,¶	Soluble	Lewis *et al*. (2008); Ma *et al*. (2006); Vinatzer *et al*. (2006); Zhou *et al*. (2009); A. Lee *et al*., unpublished data
AvrBsT	*Xanthomonas campestris* pv. *vesicatoria*	?	HR in *Capsicum annuum* (pepper)†,¶,††, *C. pubescens* (tree chilli)†, *N. benthamiana*† and *Arabidopsis* Pi-0†, suppresses AvrBs1 HR in pepper†,¶,††	Nucleus	Cunnac *et al*. (2007); Escolar *et al*. (2002); Minsavage *et al*. (1990); Orth *et al*. (2000); Szczesny *et al*. (2010)
AvrRxv	*X. campestris* pv. *vesicatoria*	?	HR in *Phaseolus vulgaris* (common bean)†, *G. max* (soybean)†, *Vigna sinensis* (cowpea)†, *Medicago sativa* (alfalfa)†, *Zea mays* (corn)†, *Gossypium hirsutum* (cotton)† and *Solanum esculentum* (tomato)†,§,¶,††	Cytoplasm or membrane	Bonshtien *et al*. (2005); Ciesiolka *et al*. (1999); Whalen *et al*. (1988, 1993)
AvrXv4	*X. campestris* pv. *vesicatoria*	SUMO protease	HR in *Solanum pennellii* (wild tomato)† and *N. benthamiana*†,††	Cytoplasm	Astua-Monge *et al*. (2000); Roden *et al*. (2004)
XopJ	*X. campestris* pv. *vesicatoria*	?	HR in *N. benthamiana*†† and *N. clevelandii*††, suppresses PTI in *Arabidopsis*‡‡	Membrane	Bartetzko *et al*. (2009); Thieme *et al*. (2007)
PopP1	*Ralstonia solanacearum*	?	HR in *Petunia*§,¶	Cytoplasm (predicted)	Lavie *et al*. (2002)
PopP2	*R. solanacearum*	Acetyltransferase	HR in *Arabidopsis*§,¶	Nucleus	Deslandes *et al*. (2003); Narusaka *et al*. (2009); Tasset *et al*. (2010)
Y4LO	*Rhizobium* species	?	Contributes to symbiosome differentiation¶	?	Yang *et al*. (2009)

HR, hypersensitive response; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa B; PTI, pathogen-associated molecular pattern (PAMP)-triggered immunity.

*Effector expressed in natural strain.

†Cloned effector expressed in virulent strain lacking any homologue.

‡Cloned effector expressed in nonhost strain lacking any homologue.

§Cloned effector expressed in strain knocked out for same allele.

¶Strain knocked out for effector.

**Cloned effector expressed in virulent strain with different homologue.

††Effector expressed by *Agrobacterium*-mediated transient expression.

‡‡Effector expressed in transgenic plant.

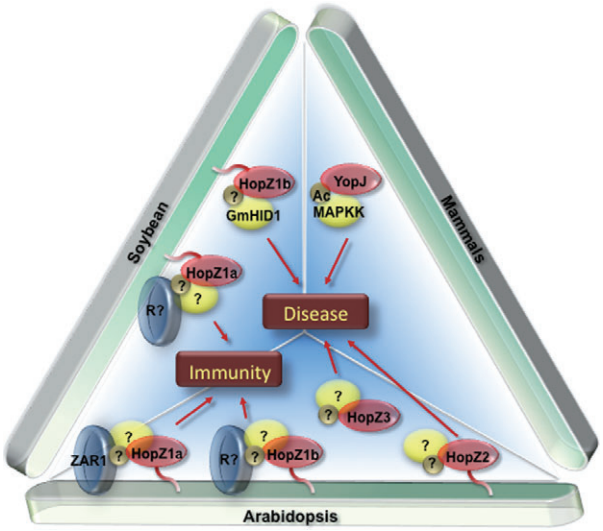

Fig. 2 Functional diversification of the YopJ superfamily of type III secreted effector (T3SE) proteins. The HopZ family of *Pseudomonas syringae* T3SE proteins demonstrates the remarkable functional diversification of the YopJ superfamily in plants. In *Arabidopsis thaliana* ecotype Col-0, HopZ1a and HopZ1b are recognized by two independent resistance (R) proteins resulting in a hypersensitive response (HR) (Lewis *et al*., 2010). Both recognition events require an intact myristoylation sequence and catalytic cysteine for recognition, indicating that it is likely that the enzymatic activity of these effectors on membrane-bound host targets is recognized by their corresponding R proteins (Lewis *et al*., 2008). HopZ2 promotes the growth of *P. syringae* in *Arabidopsis*. This activity requires an intact myristoylation sequence and catalytic cysteine, indicating that HopZ2 targets a membrane-associated protein(s) to promote *P. syringae* virulence (Lewis *et al*., 2008). HopZ3 also promotes the growth of *P. syringae*, but does not possess a myristoylation sequence, suggesting that its targets are soluble proteins (Vinatzer *et al*., 2006). In soybean, HopZ1a induces an HR in cultivars OAC Bayfield and Williams 82, which requires an intact myristoylation sequence and catalytic cysteine (Zhou *et al*., 2009). HopZ2 and HopZ3 also trigger an HR in soybean cultivars OAC Bayfield and Williams 82 (not shown; R. L. Morgan and W. Ma, unpublished data). HopZ1b can promote the growth of *P. syringae* in soybean cultivar OAC Bayfield, and it has recently been demonstrated that this virulence activity partially requires the 2-hydroxyisoflavanone dehydratase (GmHID1), which interacts directly with HopZ1b (Zhou *et al*., 2011). Although all members of the HopZ family possess the catalytic triad present in the archetypical member YopJ, their biochemical function on host targets remains to be demonstrated. YopJ can acetylate and inactivate eukaryotic mitogen-activated protein kinase kinases (MAPKKs), thereby suppressing host immune responses (Mittal *et al*., 2006; Mukherjee *et al*., 2006; Mukherjee and Orth, 2008). Ac, acetyl group.

the MAPK and NFκB signalling pathways. Given the similarity to cysteine proteases, YopJ has been proposed to modify its substrates via a 'ping–pong mechanism', whereby YopJ first forms an acetyl-enzyme covalent intermediate before transferring the acetyl group to the Ser/Thr [or lysine (Lys)] residues of its substrate (Mukherjee *et al*., 2007).

Mittal *et al*. (2006) provided additional evidence that YopJ acetylates other MAPKKs (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2, MEK1/2) in their activation loop, which consequently blocks the phosphorylation of these Ser residues in the kinase. Interestingly, Mittal *et al*. (2006) also observed that, in addition to acetylating its host target MEK2, YopJ appears to autoacetylate. However, autoacetylation does not appear to be required for the acetylation of MEK by YopJ (Mittal *et al*., 2010). The autoacetylation site of YopJ and its functional consequences remain to be determined. One possibility is that autoacetylation represents the acetyl-enzyme covalent intermediate predicted from the ping–pong mechanism of transfer in which the catalytic Cys would be acetylated (Mukherjee *et al*., 2007). YopJ also appears to require a eukaryotic activating factor, inositol hexakisphosphate (IP6), for full activation of its acetyltransferase activity (Mittal *et al*., 2010).

The MAPK signalling pathway is highly conserved across eukaryotes and therefore, perhaps not surprisingly, YopJ can also disrupt the MAPK signalling pathways in *Saccharomyces cerevisiae* (Hao *et al*., 2008; Yoon *et al*., 2003). Expression of wild-type YopJ, but not the catalytically inactive form, in yeast disrupts the MAPK pathways used for pheromone perception (mating pathway) and high osmolarity growth (HOG pathway). Similar to the observation in the mammalian system, YopJ disrupts the mating pathway and the HOG pathway in yeast by preventing the phosphorylation of the MAPK Fus3p and Hog1p, respectively (Yoon *et al*., 2003). Specifically, YopJ blocks the HOG pathway by binding and acetylating Pbs2, a conserved MAPKK, to prevent the activation and phosphorylation of the kinase (Hao *et al*., 2008). YopJ cannot block the MAPK pathway downstream of the activated MAPKK, Pbs2, as phospho-mimic mutants of Pbs2 can grow in the presence of high osmolarity and YopJ. Using a suppressor screen to identify Pbs2 mutants that are insensitive to YopJ inhibition of the HOG pathway, Hao *et al*. (2008) identified a conserved hydrophobic region in the kinase domain of Pbs2 required for YopJ binding. If YopJ is unable to bind to this hydrophobic α-helix in Pbs2 and other MAPKKs, it cannot acetylate these targets. This represents an elegant use of a heterologous system to understand how YopJ interacts with and modifies its targets, the MAPKKs.

The YopJ superfamily provides an excellent opportunity to investigate the diversification of a T3SE family. How can an effector family effectively promote the virulence of pathogens on hosts from two separate kingdoms? Studies on the phytopathogenic members of the YopJ superfamily are beginning to reveal the mechanisms by which an effector family can diversify not only between animal and plant hosts, but also between different plant hosts. Below, we provide an overview of the YopJ family members from phytopathogens on various plant hosts, which highlights the remarkable functional diversity of this T3SE family.

THE *P. SYRINGAE* HOPZ FAMILY

The HopZ family of *P. syringae* comprises three major forms: HopZ1, HopZ2 and HopZ3. Under pressure from the host immune system, HopZ1 diversified into at least three allelic forms (HopZ1a, HopZ1b and HopZ1c) (Ma *et al*., 2006). HopZ1a induces a strong defence response in a wide variety of plant species, including *Arabidopsis thaliana*, soybean, sesame, *Nicotiana benthamiana* and rice (Lewis *et al*., 2008; Ma *et al*., 2006). HopZ1b is weakly recognized in *Arabidopsis* and strongly recognized in *N. benthamiana*, whereas HopZ1c does not induce defence responses in any host tested so far (Lewis *et al*., 2008; Zhou *et al*., 2009). As HopZ1a is most similar to the ancestral HopZ allele in *P. syringae*, it has been hypothesized that members of HopZ1 diversified under pressure from the host immune response (Ma *et al*., 2006). HopZ2 is more closely related to *Xanthomonas* YopJ homologues than the *Pseudomonas* HopZs, and was putatively acquired by horizontal gene transfer, whereas HopZ3, which is quite divergent from HopZ1 and HopZ2, was putatively acquired by horizontal gene transfer from *Erwinia* species (Ma *et al*., 2006). All of the members of the HopZ family, except HopZ3, contain a consensus myristoylation site (Gly2), which is required for membrane localization (Lewis *et al*., 2008; Zhou *et al*., 2009).

Based on their relatedness to YopJ and the conservation of the catalytic triad, members of the HopZ family of effector proteins were originally hypothesized to be cysteine proteases (Ma *et al*., 2006; Orth *et al*., 2000). The HopZ proteins display weak protease activity using a fluorescence-based assay with modified casein as a generic substrate (Ma *et al*., 2006); however, the host targets of this activity remain to be identified. More recently, our data have suggested that HopZ1a can autoacetylate in the presence of eukaryotic cofactors (A. Lee *et al*., unpublished data). This is reminiscent of YopJ, suggesting that the HopZ family may also be cofactor-activated acetyltransferases (Mittal *et al*., 2006, 2010).

Functions of the HopZ family in *Arabidopsis*

HopZ1a is recognized by the CC NBS-LRR R protein ZAR1 in *Arabidopsis* (Lewis *et al*., 2010). Recognition of HopZ1a depends on its catalytic Cys residue (Cys216) as well as the consensus myristoylation site (Gly2), which is required for membrane localization. These data suggest that ZAR1 recognizes the membrane-localized enzymatic activity of HopZ1a (Lewis *et al*., 2008; Ma *et al*., 2006).

In the absence of ZAR1 recognition in *zar1* mutant *Arabidopsis* plants, HopZ1a demonstrates a catalytic Cys-dependent virulence function by promoting the growth of a nonhost *P. syringae* pv. *cilantro* 0788–9 strain (*Pci*0788-9; Lewis *et al*., 2010), that natively carries no HopZ allele, although it is closely related to a virulent, HopZ1c-bearing *P. syringae* pv. *maculicola* strain (Ma *et al*., 2006). These results suggest that ZAR1 may have evolved to monitor and respond to an ancestral virulence function of HopZ1 (Lewis *et al*., 2010). HopZ1a also appears to suppress ETI induced by the T3SEs AvrRpt2, AvrRpm1 and AvrRps4, as shown by a competitive index measure (Macho *et al*., 2009, 2010). AvrRpt2, AvrRpm1 and AvrRps4 are recognized by different R proteins and require different *R* gene signalling components relative to HopZ1a to trigger ETI (Aarts *et al*., 1998; Austin *et al*., 2002; Century *et al*., 1995; Feys and Parker, 2000; Lewis *et al*., 2010; Macho *et al*., 2010; Muskett *et al*., 2002; Tornero *et al*., 2002). It remains to be determined how HopZ1a may suppress ETI from these diverse effector proteins.

Although similar to HopZ1a (72.1% amino acid identity, 80.7% similarity), HopZ1b is only weakly recognized in *Arabidopsis* ecotype Col-0 and causes an HR in approximately 25% of leaves (Lewis *et al*., 2008). The recognition of HopZ1b depends on its catalytic Cys residue (Cys212) and, surprisingly, is independent of ZAR1 (Lewis *et al*., 2008, 2010). Therefore, at least two resistance proteins have evolved in *Arabidopsis* to recognize members of the HopZ family. HopZ1c is almost identical to HopZ1b (97% amino acid identity up to the insertion mutation in *hopZ1c* that results in a 19-amino-acid frameshift and premature stop codon), but is truncated at the C-terminus, resulting in a protein that is about one-third smaller than HopZ1b. HopZ1c retains the catalytic residues, but does not have any observable functions in *Arabidopsis* (or any other plant host) so far (Lewis *et al*., 2008; Zhou *et al*., 2009).

HopZ2 is more similar to the *Xanthomonas* homologues of the YopJ superfamily than to the HopZ1 alleles (Fig. 1; Ma *et al*., 2006). HopZ2 was originally identified because of its avirulence function in *Phaseolus vulgaris* (common bean) cultivars (formerly AvrPpiG; Arnold *et al*., 2001). In *Arabidopsis*, HopZ2 has a virulence function and promotes the growth of the nonhost *Pci*0877-9 strain (Lewis *et al*., 2008). Virulence again depends on the catalytic Cys of HopZ2 (Cys229) and localization to the membrane, indicating that HopZ2 virulence targets are membrane localized (Lewis *et al*., 2008).

HopZ3 is most similar to *Erwinia* members of the YopJ superfamily, which remain uncharacterized at this time (formerly HopPsyV; Deng *et al*., 2003; Ma *et al*., 2006; Fig. 1). HopZ3 differs from the HopZ1 and HopZ2 alleles in that it is not membrane associated (Lewis *et al*., 2008) and the *hopZ3* operon contains a predicted type III chaperone (Ma *et al*., 2006). HopZ3 promotes *in planta* multiplication of *P. syringae* pv. *syringae* strain B728a (*Psy*B728a) in the nonhost *Arabidopsis* (Vinatzer *et al*., 2006).

Functions of the HopZ family in soybean

The cellular functions of HopZ1a and HopZ1b were investigated in *Glycine max* (soybean) after the discovery that all the *P.*

syringae pv. *glycinea* strains, which were largely isolated from soybean, produce functional HopZ1b (Ma *et al*., 2006). Bacterial growth assays demonstrated that HopZ1b can promote *P. syringae* growth in soybean. For example, the kidney bean isolate *P. syringae* pv. *phaseolicola* strain 1302A (*Pph*1302A) carries no *hopZ* homologue and multiplies to only a low level in the soybean cultivar OAC Bayfield, but can grow to a 10-fold higher population density in soybean when transformed with a *hopZ1b* allele (Zhou *et al*., 2009).

Although the wild-type *P. syringae* pv. *glycinea* strain BR1 (*Pgy*BR1), carrying the endogenous *hopZ1b*, is virulent on the soybean cultivars OAC Bayfield and Williams 82, *Pgy*BR1 expressing HopZ1a triggers HR in both cultivars (Ma *et al*., 2006; Zhou *et al*., 2009). As discussed earlier, both the HR-triggering activity of HopZ1a and the pathogen growth-promoting activity of HopZ1b require the catalytic Cys residue, indicating that the enzymatic activities of HopZ1a and HopZ1b are important for their cellular functions. Domain shuffling experiments demonstrated that a central domain upstream of the conserved catalytic Cys residue of HopZ1a and HopZ1b determines the allelic specificity (Morgan *et al*., 2010). Within this domain, a single substitution of Cys141 found in HopZ1a with Lys found at the corresponding position in HopZ1b (Lys137) abolishes HopZ1a-triggered HR in soybean. As this position is under strong positive selection, the Cys141/Lys137 mutation might represent a key step during HopZ1 evolution to evade host recognition. Protein structure modelling analysis indicates that the Cys141/Lys137 residue may play a role in substrate binding (Morgan *et al*., 2010). Indeed, characterization of HopZ1a- and HopZ1b-interacting proteins in soybean revealed both common and distinct proteins associating with these two alleles (Zhou *et al*., 2011). These data suggest that sequence diversification allows altered substrate-binding specificity, which then leads to different cellular functions of HopZ1 alleles.

HopZ1a and HopZ1b both interact with 2-hydroxyisoflavanone dehydratase (GmHID1), which is an enzyme involved in soybean isoflavone biosynthesis (Zhou *et al*., 2011). Silencing of GmHID1 leads to increased susceptibility of soybean to *P. syringae* infection, as well as a compromised HopZ1b-mediated increase in *P. syringae* growth, indicating that this enzyme plays a role in host defence and HopZ1b-mediated virulence. Furthermore, isoflavone levels in soybean leaves inoculated with *P. syringae* pv. *glycinea* strains producing functional HopZ1b are significantly lower than in leaves inoculated with *P. syringae* pv. *glycinea* carrying the catalytic mutant of HopZ1b. These data, taken together, suggest that HopZ1 suppresses plant defence by inhibiting isoflavone production (Zhou *et al*., 2011).

HopZ2 and HopZ3 both trigger HR in soybean cultivars OAC Bayfield and Williams 82 (R. L. Morgan and W. Ma, unpublished data); yet, it remains to be determined whether the predicted catalytic residues are required for HopZ2- and HopZ3-triggered HR in soybean. HopZ1c does not have any observable phenotype in soybean so far.

Functions of the HopZ family in *N. benthamiana*

The functions of the HopZ effector proteins have been investigated in *N. benthamiana* using *Agrobacterium*-mediated transient expression. Both HopZ1a and HopZ1b trigger a catalytic Cys-dependent HR-like programmed cell death in *N. benthamiana* (Lewis *et al*., 2008; Ma *et al*., 2006; Zhou *et al*., 2009). Moreover, the C-terminal domain downstream of the catalytic core, which is lacking in HopZ1c, is also required for HopZ1b to induce HR in *N. benthamiana* (H. B. Zhou and W. Ma, unpublished data). This C-terminal domain might be important for maintaining the protein structure necessary for the enzymatic activity of HopZ1b.

Although both HopZ1a and HopZ1b trigger HR-like cell death in *N. benthamiana*, they may be differentially recognized. The N-terminal myristoylation enhances the HopZ1b-triggered HR, but does not affect the HopZ1a-triggered HR. The cell death symptoms elicited by the mutant HopZ1a(G2A) were indistinguishable from those of the wild-type HopZ1a, whereas HopZ1b(G2A) triggered a weaker cell death symptom, which was also delayed compared with the wild-type HopZ1b (Zhou *et al*., 2009).

HopZ2 also triggers an HR-like programmed cell death when it is transiently expressed in *N. benthamiana*. Similar to HopZ1a and HopZ1b, the catalytic Cys residue is also required for this activity (Lewis *et al*., 2008; Ma *et al*., 2006). To date, it is not known whether the potential myristoylation site, which is conserved between HopZ1 and HopZ2, is important for the HopZ2-triggered HR in this host.

Transient expression of HopZ3 in *N. benthamiana* blocks the HR-like cell death elicited by effectors AvrPto1, HopAA1, HopM1 and HopAE1 (Vinatzer *et al*., 2006). HopZ1b-triggered cell death is also suppressed by HopZ3 (Zhou *et al*., 2009). Furthermore, the catalytic Cys residue of HopZ3 is required to block HR triggered by HopZ1b, indicating that HR suppression by HopZ3 depends on its enzymatic activity. Although it is possible that HopZ3 suppresses the HopZ1b-triggered HR by competing for binding to similar substrates, it is more likely that HopZ3 targets a common signal transduction component downstream of the initial recognition of these different effectors. This study also demonstrated that HopZ3 can induce an HR-like cell death response in *Phaseolus vulgaris* (snap bean) and *Nicotiana tabacum* (tobacco) using *Agrobacterium*-mediated transient expression. Interestingly, HopZ3 negatively contributes to the epiphytic growth of *Psy*B728a in *N. benthamiana* despite a lack of cell death induction (Vinatzer *et al*., 2006). It will be interesting to investigate the potential role of HopZ3 in preinvasive immunity.

Much of the data obtained for the HopZ family in *N. benthamiana* have relied on transient expression using strong promoters. As this approach results in the over-expression of these proteins relative to the levels delivered by bacteria, it will be important to demonstrate that the functions ascribed using transient expression translate to functions when delivered by the T3SS.

THE *XANTHOMONAS* YOPJ HOMOLOGUES

Xanthomonas campestris pathovars contain several YopJ homologues, including AvrBsT, AvrRxv, AvrXv4 and XopJ. This list is likely to expand as more *Xanthomonas* species and pathovars are characterized and/or sequenced. Unlike the HopZ family in *P. syringae*, a single *Xanthomonas* pathovar can carry multiple, different YopJ homologues (Szczesny *et al*., 2010). As in *P. syringae*, the *Xanthomonas* YopJ homologues display extensive functional divergence.

AvrBsT was originally described because of its avirulence function in *Capsicum annuum* (pepper) isogenic lines carrying the *Bs1 R* gene (Minsavage *et al*., 1990). It also causes an HR in *C. pubescens* (Escolar *et al*., 2002) and *N. benthamiana* (Orth *et al*., 2000). The avirulence function in *N. benthamiana* (and presumably other species) depends on its catalytic triad (Orth *et al*., 2000). AvrBsT can also cause an HR in the *Arabidopsis* ecotype Pi-0 when it is expressed and delivered by the *P. syringae* T3SS (Cunnac *et al*., 2007). AvrBsT-induced resistance is impaired in *R* gene signalling mutants, suggesting that the *Pseudomonas*-delivered AvrBsT is recognized in an *R* gene-mediated fashion in *Arabidopsis* (Cunnac *et al*., 2007). Unlike HopZ1a-induced resistance, AvrBsT-mediated resistance is dependent on certain known signalling components (i.e. *NDR1, EDS1, PAD4, SID2, NPR1*), which suggests that the *R* gene that recognizes AvrBsT differs from *ZAR1*, which recognizes HopZ1a (Cunnac *et al*., 2007; Lewis *et al*., 2010). Using *P. syringae* to deliver AvrBsT, Cunnac *et al*. (2007) designed a genetic screen based on the natural genetic variation in AvrBsT-induced resistance between the resistant Pi-0 and susceptible Col-0 ecotypes, and found that the AvrBsT-induced HR in Pi-0 was caused by a recessive mutation in *SOBER1*. SOBER1 demonstrates phospholipase activity *in vitro* (Kirik and Mudgett, 2009). Functional SOBER1 abrogates the AvrBsT-induced HR in *Arabidopsis* and prevents the accumulation of phosphatidic acid (Cunnac *et al*., 2007; Kirik and Mudgett, 2009). In other systems, homologues to SOBER1 have been implicated in cellular signalling as lipid secondary messengers (Cunnac *et al*., 2007). Kirik and Mudgett (2009) propose that SOBER1 phospholipase activity suppresses downstream phospholipid stress signalling in response to AvrBsT.

A virulence function has not yet been demonstrated for AvrBsT. Knocking out AvrBsT in *X. campestris* pv. *vesicatoria* strain 85–10 does not change the virulence of this strain in tomato; however, this strain also contains two other YopJ homologues, AvrRxv and XopJ, which may confer virulence (Szczesny *et al*., 2010).

AvrBsT is able to suppress ETI induced by AvrBs1, a non-YopJ effector protein (Szczesny *et al*., 2010). Other YopJ homologues in *X. campestris* pv. *vesicatoria* strain 85–10 (AvrRxv and XopJ) cannot suppress the AvrBs1-induced HR, suggesting that their functions have diverged. The ETI suppression function of AvrBsT depends on its catalytic Cys residue (Cys222), and occurs within the plant cell, as demonstrated by *Agrobacterium*-mediated transient expression of AvrBsT and AvrBs1 in pepper leaves. The ETI suppression activity of AvrBsT may indicate that AvrBsT and AvrBs1 target a conserved host protein. AvrBsT interacts with an SnRK1 kinase in yeast two-hybrid assays and by bimolecular fluorescence. The AvrBs1 HR is reduced when SnRK1 is silenced, but it remains to be determined whether SnRK1 is required for AvrBsT-mediated suppression of the AvrBs1 HR (Szczesny *et al*., 2010).

AvrRxv from *X. campestris* pv. *vesicatoria* induces an HR in some cultivars of *Phaselous vulgaris* (common bean), *G. max* (soybean), *Vigna sinensis* (cowpea), *Medicago sativa* (alfalfa), *Zea mays* (corn) and *Gossypium hirsutum* (cotton) (Whalen *et al*., 1988), as well as *Solanum esculentum* (tomato) (Wang *et al*., 1994; Whalen *et al*., 1993). Loss of the HR was observed when each residue of the catalytic core (His180, Glu200, Cys244) was independently mutated, indicating that the catalytic function of AvrRxv is necessary for recognition (Bonshtien *et al*., 2005; Whalen *et al*., 2008). AvrRxv does not contain a consensus myristoylation sequence and appears to localize to the cytoplasm or the membrane (Bonshtien *et al*., 2005). AvrRxv interacts with a 14-3-3 protein in yeast two-hybrid assays and *in vitro* (Whalen *et al*., 2008). 14-3-3 proteins function in protein–protein interactions in diverse cellular activities. It remains to be determined how the AvrRxv–14-3-3 interaction contributes to avirulence in tomato.

AvrXv4 from *X. campestris* pv. *vesicatoria* has an avirulence function in *Solanum pennellii* (wild tomato) (Astua-Monge *et al*., 2000) and *N. benthamiana*, which is dependent on its catalytic Cys (Cys219) and His (His155) residues (Roden *et al*., 2004). In susceptible hosts, AvrXv4 makes a small contribution to virulence (Roden *et al*., 2004). AvrXv4 has small ubiquitin modifier (SUMO) protease activity and causes a reduction in sumoylated proteins in *N. benthamiana* and *C. annuum* (Roden *et al*., 2004). The specific targets of AvrXv4 activity and the role of sumoylation in pathogenicity remain unanswered; however, it is likely that the targets are cytoplasmic, where AvrXv4 is localized in plant cells (Roden *et al*., 2004).

XopJ causes an HR in *N. benthamiana* and *N. clevelandii* (Thieme *et al*., 2007). Induction of the HR depends on the plasma membrane localization of XopJ, which probably occurs by myristoylation (Thieme *et al*., 2007). In *Agrobacterium*-mediated transient expression experiments in *N. benthamiana*, XopJ is found

at the plasma membrane and in vesicles that colocalize with a Golgi marker, suggesting that it trafficks through the secretory pathway. Interestingly, catalytic mutants of XopJ are only found at the plasma membrane (Bartetzko *et al*., 2009). In susceptible hosts, the enzymatic activity of XopJ is necessary for XopJ to block secretion to the apoplast (Bartetzko *et al*., 2009). In addition, XopJ can partially suppress PTI, and it is possible that the block in secretion contributes to the impairment of PTI.

THE *RALSTONIA* POPP FAMILY

Ralstonia solanacearum, the bacterial wilt pathogen, has at least two T3SEs that are part of the YopJ superfamily. PopP1 is believed to have been acquired by horizontal gene transfer and has an avirulence function in *Petunia* (Lavie *et al*., 2002). Interestingly, the *Rhizobium* homologue Y4LO and the *Xanthomonas* XopJ effector protein are part of the same clade. A second *R. solanacearum* homologue, PopP2, forms a clade with an uncharacterized *Xanthomonas* homologue (Ma *et al*., 2006). PopP2 causes an HR in *Arabidopsis* and is recognized by the cooperative action of the RRS1 and RPS4 R proteins (Deslandes *et al*., 2002; Narusaka *et al*., 2009). RRS1 is an atypical TIR-NBS-LRR R protein with a C-terminal WRKY domain found in plant transcription factors (Deslandes *et al*., 2002), whereas RPS4 is a TIR-NBS-LRR R protein originally characterized as recognizing the unrelated *P. syringae* effector AvrRps4 (Gassmann *et al*., 1999). Recognition of PopP2 requires its catalytic activity (Tasset *et al*., 2010) and occurs by direct interaction between PopP2 and RRS1 in the nucleus (Deslandes *et al*., 2003). RPS4 has also been shown to be nuclear localized, which is necessary for AvrRps4-triggered defence responses (Wirthmueller *et al*., 2007). However, it is unknown whether RPS4 interacts directly or indirectly with PopP2 and where this interaction occurs.

Tasset *et al*. (2010) demonstrated that PopP2 is an acetyltransferase that is autoacetylated at K383, a modification necessary for RRS1 recognition. PopP2 appears to stabilize the RRS1 protein and to prevent its degradation by the proteasome (Tasset *et al*., 2010). In addition, RRS1 resistance requires RD19, a cysteine protease, whose expression is induced on infection with *R. solanacearum* (Bernoux *et al*., 2008). RD19 is relocalized to the nucleus when co-expressed with RRS1 (Bernoux *et al*., 2008). RD19 interacts with RRS1 but not PopP2 (Bernoux *et al*., 2008). As a result, when PopP2 is knocked out in *R. solanacearum*, the strain is highly virulent in *Arabidopsis* (Narusaka *et al*., 2009).

THE *RHIZOBIUM* Y4LO HOMOLOGUE

Rhizobium species are usually considered to exist in symbiosis with their plant hosts, where they contribute to nitrogen fixation, and the *Rhizobium* T3SS can contribute to nodulation in some host plants (Marie *et al*., 2001). A YopJ homologue, Y4LO, has been identified in *Rhizobium* species (Astua-Monge *et al*., 2000; Ciesiolka *et al*., 1999; Ma *et al*., 2006). Y4LO is most closely related to the XopJ homologue from *Xanthomonas* (Ma *et al*., 2006). Y4LO can be delivered by the *P. syringae* T3SS into plants (J. Chang, Oregon State University, Corvallis, OR, USA, personal communication). In *Tephrosia vogelii, Phaseolus vulgaris* and *Vigna sinensis*, Y4LO contributes to the production of effective nitrogen-fixing nodules and normal nitrogen content in the plant (Yang *et al*., 2009). Strains of *Rhizobium* with Y4LO disruptions still form nodules; however, differentiation into the symbiosome, with the subsequent ability to fix nitrogen, does not appear to complete normally (Yang *et al*., 2009).

CONCLUSIONS AND FUTURE DIRECTIONS

The YopJ superfamily shows remarkable diversification for a T3SE family, with homologues in animal pathogens, plant pathogens and plant symbionts (Figs 1 and 2; Table 1). In plants, members of the YopJ superfamily have been demonstrated to contribute to either disease or immunity in a host-dependent manner (Fig. 2). In animals, members of the YopJ superfamily have well-characterized, disease-promoting roles; however, it remains to be established whether they can also limit bacterial virulence as observed in plants. Interestingly, the YopJ homologue AvrA has been suggested to limit virulence in vertebrates, indicating that the YopJ superfamily may also display avirulence activities outside of the plant kingdom (Collier-Hyams *et al*., 2002).

All of the YopJ homologues described so far require a common catalytic triad, despite different enzymatic activities. YopJ and PopP2 are acetyltransferases and HopZ1a also shows evidence of this function (Mittal *et al*., 2006; Mukherjee *et al*., 2006; Tasset *et al*., 2010; A. Lee *et al*., unpublished data). Other members are SUMO proteases (AvrXv4), and weak protease activity has been shown for the HopZ family (Ma *et al*., 2006; Roden *et al*., 2004). However, the host targets of these enzymatic activities are still unknown in many cases. It will be critical to demonstrate enzymatic activity on host substrates to confirm their function. It is possible that these effector proteins are bifunctional enzymes with distinct enzymatic functions encoded by the same catalytic triad, as observed with cysteine proteases and acetyltransferases (Mukherjee *et al*., 2007). Neofunctionalization of enzymatic function might be more likely in strains of *Xanthomonas*, where multiple YopJ homologues are found in a single strain.

The targets of phytopathogenic YopJ homologues remain unidentified and are of great interest. Will the plant YopJ homologues also target kinases, as does YopJ? MAP kinases are targeted by several unrelated effector proteins, for example HopAI1 and HopF2, to suppress PTI (Lewis *et al*., 2009; Wang *et al*., 2010; Zhang *et al*., 2007). A SnRK kinase has been shown to interact with AvrBsT. However, the enzymatic activity of AvrBsT and the resultant modification of its targets remain to

be determined (Szczesny *et al*., 2010). It remains to be seen whether disruption of kinase signalling is a general immune suppression technique of the YopJ superfamily. Given the diverse subcellular localization patterns of the YopJ homologues, they probably employ an array of molecular strategies to promote immunosuppression and pathogenesis.

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