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Effectors of Filamentous Plant Pathogens: Commonalities amid Diversity

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SUMMARY Fungi and oomycetes are filamentous microorganisms that include a diversity of highly developed pathogens of plants. These are sophisticated modulators of plant processes that secrete an arsenal of effector proteins to target multiple host cell compartments and enable parasitic infection. Genome sequencing revealed complex catalogues of effectors of filamentous pathogens, with some species harboring hundreds of effector genes. Although a large fraction of these effector genes encode secreted proteins with weak or no sequence similarity to known proteins, structural studies have revealed unexpected similarities amid the diversity. This article reviews progress in our understanding of effector structure and function in light of these new insights. We conclude that there is emerging evidence for multiple pathways of evolution of effectors of filamentous plant pathogens but that some families have probably expanded from a common ancestor by duplication and diversification. Conserved folds, such as the oomycete WY and the fungal MAX domains, are not predictive of the precise function of the effectors but serve as a chassis to support protein structural integrity while providing enough plasticity for the effectors to bind different host proteins and evolve unrelated activities inside host cells. Further effector evolution and diversification arise via short linear motifs, domain integration and duplications, and oligomerization.

KEYWORDS plant pathology

INTRODUCTION

Filamentous pathogens (fungi and oomycetes) are the causative agents of some of the world's most notorious plant diseases. Left unchecked, they can devastate crop harvests, destroy managed and wild forests, affect the supply of ornamental plants, and disturb natural ecosystems (1–3). Perhaps the most famous plant disease outbreak was caused by the oomycete *Phytophthora infestans*, which spread to Europe and triggered the 19th-century Irish potato famine (4). This pathogen remains relevant in agriculture today, infecting potato and tomato crops throughout the world (5). Diseases caused by fungal pathogens, such as rice and wheat blast and wheat stem and stripe rust, are of immediate concern for global food security (1, 6, 7). Major factors in the ability of these filamentous microbes to cause disease on their hosts are effectors, pathogen-encoded proteins that are secreted to either the apoplast or specialized biotrophic interfaces (both are spaces outside plant cells) or are translocated inside host cells (8–11).

Effectors act to modulate host cell physiology to promote susceptibility to pathogens. In turn, plants have evolved cell surface and intracellular receptors to detect the presence of pathogen signatures and mount an immune response to restrict the progression of disease. Cell surface receptors typically recognize microbe-associated molecular patterns (MAMPs), derived from abundant structural components of microbes' cell walls, or secreted proteins that function as virulence effectors. Intracellular receptors respond to the presence of translocated effectors and/or their activity on host cell targets. These intracellular receptors are nucleotide-binding domain- and leucine-rich repeat-containing (NLR) proteins that mediate innate immunity to pathogens in both plants and animals (recently reviewed in reference 12).

One of the defining features of effector proteins, be they of bacterial or filamentous pathogen origin, is the lack of clear sequence similarity to proteins of known function. This is thought to be the consequence of evolutionary pressure that drives the rapid diversification of effector activities in host cells to optimize function and/or avoid recognition by the innate immune system. The frequent difficulty in recognizing common motifs that indicate the function or activity of effectors may be due to few of them having enzymatic activity or the absence of known domains for direct interaction with host factors. In addition, many effectors are small proteins of <15 kDa, and thus, their rapid diversification would result in a loss of sequence similarity. With a few notable exceptions (the RXLR motif of effectors in some oomycetes being the most prominent), this sequence diversity has meant that it is challenging to confidently produce catalogues of effectors from filamentous plant pathogen genomes despite many of these now being available. In some cases, bioinformatic approaches have been useful in predicting and classifying candidate effectors from filamentous plant pathogens (13–23) (Table 1). However, it can be challenging to pick the most relevant proteins to select for further investigation from these lists. These bioinformatic approaches use some of the commonalities identified among effectors from different organisms, such as genomic context, the presence of a secretion signal, the absence of predicted transmembrane domains, expression patterns, and the lack of similarity to known protein domains. Recent advances in the computational prediction of effectors have employed machine-learning approaches, which are proving useful for prioritizing effectors for further study (24). There are also examples of effectors of filamentous plant pathogens that share common sequence motifs with known enzymes, enzyme inhibitors, sugar-binding proteins, and toxins, with some being shown to possess such activities.

It is well established that protein structure is more conserved than amino acid sequence, and in many cases, this is due to the evolutionary relationship between structure and function (25). The fact that structural conservation can be a powerful method for the functional annotation of proteins is a fundamental concept that has driven the development of structure determination as a tool to understand the effector

TABLE 1 Effectors of filamentous plant pathogens that have sequence similarities with enzymes or enzyme inhibitors

Effector class	Hyphal pathogen	Example(s)	Reference(s)
Chorismate mutases	*Ustilago maydis*	Cmu1	45
Lipase effector	*Fusarium graminearum*	FGL1	112
Enzyme inhibitors			
Protease inhibitors	*Cladosporium fulvum*	Avr2	41
Cystatin-like protease inhibitor domains	*Phytophthora infestans*	EPIC1, EPIC2B	42
Chitinase inhibitor	*Cladosporium fulvum*	Avr4	56
Proteases and peptidases			
Proteases	*Zymoseptoria tritici* (*Mycosphaerella graminicola*)		33
	Colletotrichum sp.		34
Secreted peptidases	*Zymoseptoria tritici* (*Mycosphaerella graminicola*)	Astacin (peptidase family M12A), serine carboxypeptidase S28	113
Serine protease	*Fusarium oxysporum* f. sp. *lycopersici*	Sep1	35
Alkaline serine protease alp1	*Sclerotinia sclerotiorum*	Peptidase inhibitor I9	23
Metalloproteases			
Zinc metalloprotease	*Magnaporthe oryzae*	AVR-Pita (*AVR2-YAMO*)	36, 114
Deuterolysin metalloprotease	*Sclerotinia sclerotiorum*	Deuterolysin metalloprotease (M35) family (PF02102) homolog of *M. oryzae* AVR-Pita	23
Metalloprotease	*Fusarium oxysporum* f. sp. *lycopersici*	Mep1	35
Nudix hydrolases	*Phytophthora sojae*	Avr3b	46
	Colletotrichum truncatum	CtNUDIX	115
	Melampsora lini	AvrM14	48
Crinklers			
Kinase activity	*Phytophthora infestans*	CRN8	50

biology of both mammalian and plant pathogens (26, 27). In particular, this has been important where the lack of sequence similarity to known functional proteins has prevented the prediction of the molecular mechanism.

In this review, we focus on recent advances that highlight commonalities shared by effectors of filamentous plant pathogens, focusing on functional similarities with known proteins, on effectors that cluster into large structurally common but sequence-divergent families comprising novel folds, or on those that share structural similarity with proteins of known function. It is timely to review progress in this area in light of new insights. We conclude that there is emerging evidence for multiple pathways of evolution of effectors of filamentous plant pathogens, including that some families appear to have evolved from a common ancestor by duplication and diversification in the pathogen.

EFFECTORS OF FILAMENTOUS PLANT PATHOGENS THAT ENCODE ENZYMES AND PROTEASE INHIBITORS

Structural studies of a number of bacterial plant-pathogenic type III secreted effectors (T3SEs) have revealed similarity with proteins of known function, which suggested both how these proteins act and experiments to test mechanisms (28–31). Remarkably, many of these proteins appear to be enzymes with the potential to catalyze a wide variety of different reactions, such as E3 ligation, ADP ribosylation, and proteolysis. In several cases, specific enzymatic activities have been demonstrated for these proteins (32). In contrast, a number of effectors of filamentous plant pathogens have been predicted to have enzymatic activity, but only a few have had such activities confirmed experimentally. To date, there are no structures of enzymes of effectors of filamentous plant pathogens, so these predictions typically rely primarily on sequence comparisons.

Proteases and Protease Inhibitors

Analyses of fungal genomes, including those of *Zymoseptoria tritici* (33), *Colletotrichum* sp. (34), and *Sclerotinia sclerotiorum* (23), identified families of secreted proteases whose expression pattern supports a putative role as effectors, to promote the colonization and growth of the pathogen. *Fusarium oxysporum* f. sp. *lycopersicum* secretes a serine protease, Sep1, and a metalloprotease, Mep1, that act synergistically to cleave host chitinases, preventing their activity in degrading fungal cell walls (35). A double mutant of Sep1 and Mep1 showed reduced disease on tomato, highlighting the importance of these proteins for full virulence.

The rice blast fungus *Magnaporthe oryzae* produces AVR-Pita, an effector with features typical of zinc metalloproteases, including conserved residues known to mediate zinc coordination and catalysis in homologues from other organisms (9, 36). However, to date, actual protease activity for AVR-Pita has not been demonstrated.

A remarkable case is the glucanase inhibitor proteins (GIPs), which are proteins secreted by *Phytophthora* spp. to inhibit the degradation of pathogen β-1,3/1,6-glucans and the release of defense-eliciting oligosaccharides by host β-1,3-endoglucanases (37, 38). GIPs share significant sequence similarity with trypsin serine proteases but are predicted to be proteolytically nonfunctional because they carry mutated catalytic residues.

Interestingly, filamentous plant pathogens also secrete protease inhibitors, which act on host pathogenesis-related proteases to prevent their activities. Examples include EPI1 and EPI10 of *P. infestans*, which carry multiple domains with similarity to the Kazal family of serine protease inhibitors (39, 40). In addition, the Avr2 effector of the fungal pathogen *Cladosporium fulvum* (41) and the *P. infestans* effectors EPIC1 and EPIC2 (42) are unrelated in sequence but have convergently evolved to target the same host proteases (43, 44). The oomycete EPIC family of protease inhibitor effectors has similarity to the widespread cystatin domain (42), whereas *C. fulvum* Avr2 is a small cysteine-rich protein without any notable sequence similarity to other proteins (41).

Fungal Cmu1, an Enzyme Interfering with Metabolic Flux

The maize smut fungus *Ustilago maydis* translocates a chorismate mutase, Cmu1, into plant cells. Cmu1 appears to benefit the pathogen by redirecting the metabolic flux of chorismate away from the biosynthesis of salicylic acid, suppressing the accumulation of this defense-related hormone during infection. Intriguingly, there is evidence to suggest that Cmu1 can move out of infected cells into neighboring cells, where the enzyme's activity can "prime" the host tissue for infection (45).

Translocated Oomycete Effectors Include Enzymes

Oomycete plant pathogens encode putative enzymes in their effector repertoires. *Phytophthora* species have ~300 to 550 RXLR-type effectors that rarely have sequence similarity to know enzyme folds. However, *P. infestans* and *Phytophthora sojae* contain a sequence signature suggestive of Nudix hydrolase (phosphorylase) activity. The *P. sojae* effector Avr3b has been shown to possess ADP-ribose/NADH pyrophosphorylase activity when expressed and epitope purified from plant tissue (46). Furthermore, the virulence activity of Avr3b was dependent on the conserved Nudix motif. Interestingly, the activity of Avr3b as a Nudix hydrolase is dependent on its modification by plant cyclophilins; when produced in *Escherichia coli*, the protein is not active (47). Recently, a putative Nudix hydrolase effector (AvrM14) was identified in the flax rust fungus *Melampsora lini* (48), but catalytic activity for this protein has yet to be shown.

In addition to RXLR effectors, *Phytophthora* species also contain hundreds of "Crinkler" effectors (CRNs) (13, 16, 49). CRNs are modular proteins, some of which induce cell death upon expression in plant cells (13, 16). One C-terminal CRN domain has significant sequence similarity to protein Ser/Thr kinases of the RD (arginine-aspartate) class. Indeed, *P. infestans* CRN8 was shown to be an active kinase present in an autophosphorylated state in plant cells (50). *In planta* expression of CRN8 enhanced the growth
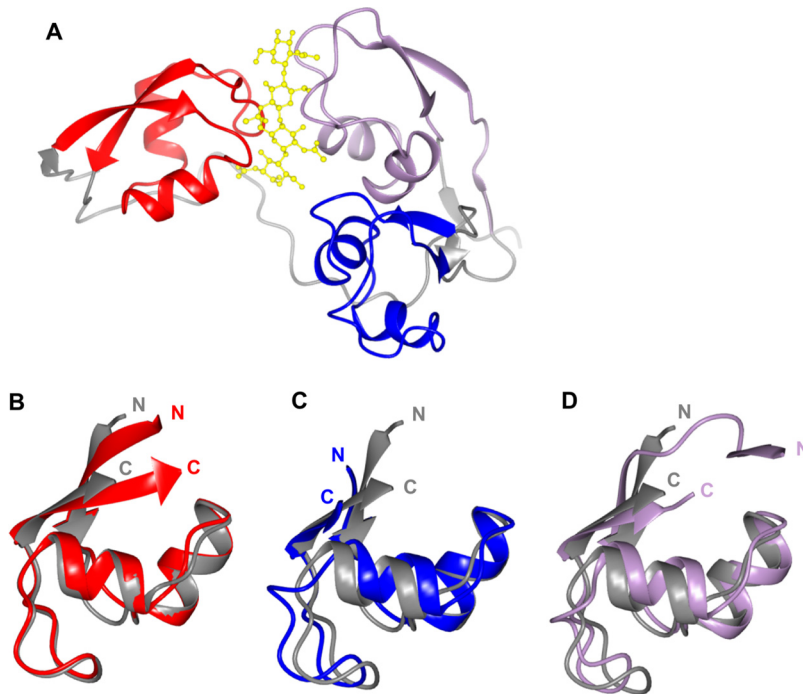
FIG 1 The crystal structure of the LysM effector Ecp6 shows how modularity can be used by effectors to generate new functions (the three LysM domains are shown in red, blue, and lilac, respectively). (A) Two Ecp6 LysM domains combine to bind to a chitin oligomer (shown in yellow). (B to D) Superposition of the Ecp6 LysM domains on the plant (rice) LysM receptor protein MoCVNH3 (in gray) (LysM domains are colored as described above). The amino (N) and carboxyl (C) termini of the proteins are labeled.

of *P. infestans*, and this required the intact RD motif, suggesting that the enzymatic activity of this kinase is relevant for virulence.

EFFECTORS OF FILAMENTOUS PLANT PATHOGENS CAN SHARE FOLDS WITH FUNCTIONALLY SIMILAR PROTEINS

Chitin-Binding LysM Effectors

Chitin is a major component of fungal cell walls, and the detection of this homopolymer in the apoplast is used by plants as a strategy for initiating immune responses (51). Plants detect chitin-derived oligosaccharides via cell surface receptors that contain extracellular lysine motif (LysM) domains. Plant LysM domains comprise ~50 amino acids and adopt a $\beta\alpha\alpha\beta$ structural fold (52, 53) (Fig. 1). To protect themselves from detection by the plant immune system, fungi use LysM effectors to sequester chitin oligomers in the apoplast, outcompeting binding by host receptor domains. The crystal structure of *Cladosporium fulvum* Ecp6 confirmed that this protein contained 3 modular LysM domains (54) (Fig. 1 and Table 2). In a strategy to deliver high-affinity ligand interactions, two of the Ecp6 LysM domains (LysM1 and LysM3) dimerize to "sandwich" a chitin oligomer in a groove via multiple hydrogen bonds and hydrophobic interactions (Fig. 1A). To date, this ligand-induced LysM dimerization to increase binding affinity is unique to Ecp6 and highlights the propensity of pathogen effectors to adapt protein folds to acquire new activities (51). Interestingly, the ligand-binding capability of the LysM2 domain of Ecp6 was also shown to interfere with chitin-triggered immunity *in planta*, but the underlying mechanistic basis remains unclear (55).

Multidomain LysM effectors are also found in other fungal plant pathogens, including the wheat pathogen *Zymoseptoria tritici* and the rice blast pathogen *Magnaporthe oryzae*, suggesting that they represent a widespread mechanism for the suppression of detection by the plant immune system. However, unlike Ecp6, *Z. tritici* LysM effectors protect fungal hyphae against hydrolysis by host chitinases, although the mechanism by which they achieve this is not understood (55).

TABLE 2 Effectors of filamentous plant pathogens that have had their structures determined

Protein	Origin	Immune receptor(s)	Targeted process[c]	Fold	Comparison to known structure			
					RMSD (Å) (no. of residues in overlay)[a]	Sequence identity (%)	PDB accession no.	Reference
Avr3a11	P. capsici		Unknown	WY	ND	ND	3ZR8	74
Avr3a4	P. capsici		Unknown	WY	1.26 (42)	79.0	2LC2	77
PexRD2	P. infestans		MAPKKɛ-mediated immune signaling	WY	1.41 (40)	27.8	3ZRG	74
PexRD54	P. infestans		Autophagy	WY	1.73 (41)	20.0	5L7S	78
ATR1	H. arabidopsidis	RPP1	Unknown	WY	2.37 (36)	23.7	3RMR	76
AvrL567-D	M. lini	L6	Unknown	ToxA-like	2.74 (82)	22.2	2QVT	105
AvrL567-A	M. lini	L5 and L6	Unknown	ToxA-like	2.58 (81)	19.7	2OPC	105
avrM	M. lini		Unknown	WY-like	ND	26.1	4BJM	106
AvrM-A	M. lini	M	Unknown	WY-like	ND	23.9	4BJN	106
AVR-PikD (in complex)	M. oryzae	Pik1/Pik2	Unknown	MAX	ND	ND	5A6W	82
Avr1-CO39	M. oryzae	RGA5/RGA4	Unknown	MAX	1.36 (55)	17.2	2MYV	80
AVR-Pia	M. oryzae	RGA5/RGA4	Unknown	MAX	2.24 (52)	16.4	2MYW	80
AVR-Pizt	M. oryzae	Piz-t	E3 ligase-mediated immunity	MAX	2.33 (58)	15.6	2LW6	84
Avr4	P. fuligena	Cf-4	Chitin-mediated immunity/fungally derived chitin perception	CBM14-like	1.98 (52)	22.2	4Z4A	61
Ecp6	C. fulvum		Chitin-mediated immunity/fungally derived chitin perception	LysM1	0.8 (45)	35.9	4B8V	54
Ecp6	C. fulvum			LysM2	1.17 (43)	37.1	4B8V	54
Ecp6	C. fulvum			LysM3	1.51 (45)	20.8	4B8V	54
AvrLm4-7	L. maculans	Rlm4 and Rlm7	Production of plant hormones and hydrogen peroxide/plant hormone-mediated immunity	Unique	ND	ND	4FPR	110
ToxA	P. tritici-repentis	Tsn1[b]	Photosynthesis	ToxA-like	ND	ND	1ZLE	103
ToxB	P. tritici-repentis		Photosynthesis	MAX	2.25 (58)	25.4	2MM0	81
toxb	P. tritici-repentis		Inactive allele	MAX	2.33 (57)	19.7	2MM2	81
NLP	P. aphanidermatum		Plasma membrane integrity	Actinoporin-like	2.34 (68)	21.9	3GNZ	64
NLP	M. perniciosa		Plasma membrane integrity	Actinoporin-like	2.24 (68)	19.3	3ST1	70

[a]Template proteins used for comparison are Avr3a11 (WY and WY-like), AVR-PikD (MAX), tachycitin (CBM14-like), MoCVNH3 (LysM), ToxA (ToxA-like), and sticholysin II (actinoporin-like). RMSD, root mean square deviation; ND, not determined (either to avoid comparison with self or because the comparison is not meaningful).

[b]Tsn1 is a susceptibility factor.

[c]MAPKKɛ, mitogen-activated protein kinase kinase kinase ɛ.
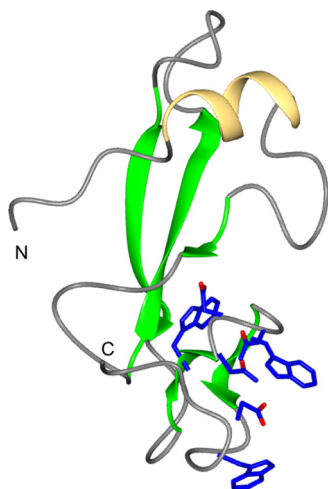
FIG 2 CBM14 family structure of *P. fuligena* Avr4. The structures comprise an alpha helix (yellow) and five beta strands (green). The residues predicted to be involved in the interaction with chitin are shown in blue.

CBM14-Like Avr4 Effectors

In a second strategy to evade chitin-mediated recognition by the plant immune system, fungi can secrete effector proteins that bind to chitin in their cell wall and prevent the action of host chitinases in generating chito-oligosaccharide fragments. The *Cladosporium fulvum* effector Avr4 was predicted to adopt a carbohydrate-binding module family 14 (CBM14)-like structure, based on its disulfide bond pattern, and *in vitro*, Avr4 protects chitin from hydrolysis by plant chitinases (56, 57). CBM14 proteins are defined as having chitin-binding activity, with one being characterized as having antimicrobial properties (58). The structure of the CBM14 member tachycitin, from the horseshoe crab *Tachypleus tridentatus*, revealed a distorted β-sandwich fold flanked by short loops and turns, stabilized by disulfide bonds (59). Tachycitin was described as sharing some structural similarity to a domain found in the plant chitin-binding protein hevein (60).

Avr4 homologues are found in a number of plant-pathogenic fungal species. Recently, the crystal structure of Avr4 from the tomato pathogen *Pseudocercospora fuligena* confirmed that the Avr4 family of effectors adopts the CBM14-like fold (Fig. 2), and this enabled the investigation of structure-function relationships in chitin binding by these proteins (61). As predicted for tachycitin, the chitin-binding site of Avr4 is located between two β-strands and the connecting β-hairpin and is mediated by aromatic amino acids and adjacent polar residues (Fig. 2).

The evolutionary dynamics of CBM14 family proteins are complex (62). While chitin binding is a critical feature of this fold for fungal defense against the plant immune system, it is clear that other functions can be attributed to the wider family given that CBM14 proteins occur in nonpathogenic species and were previously shown to have antimicrobial properties.

NLPs

NLPs (necrosis- and ethylene-inducing peptide 1-like proteins) are a large family of secreted proteins found in plant-associated fungi, oomycetes, and bacteria. NLPs were initially characterized by their ability to induce necrotic cell death in dicotyledonous plants (63), which is thought to be dependent on toxin-induced host cell damage (64). However, it is now well established that not all NLPs share this activity (65, 66). Despite this, both cytotoxic and noncytotoxic NLPs can trigger cell surface-dependent immune responses in plant cells, and this activity has been localized to a 24-amino-acid peptide (67, 68) recognized by a receptor complex comprising RLP23/SOBIR-1/BAK1 (69). Clues to the mechanism of the cytolytic activity of NLPs came from the crystal structures of
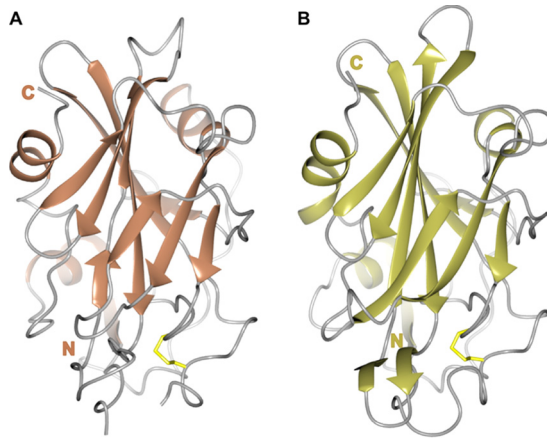
FIG 3 Crystal structures of the NLP family members NLP$_{Pya}$ (A) and MpNEP2 (B), showing the central β-sandwich surrounded by 3 helices. The conserved structural elements are shown in a cartoon representation, with residues contributing to disulfide bridges shown as sticks (in yellow) and loops shown in gray.

NLPs from *Pythium aphanidermatum* and *Moniliophthora perniciosa* (Fig. 3), which showed that this family of proteins shares a fold with the actinoporin pore-forming toxin stichoysin (64, 70). However, there is no experimental evidence for pore-forming activity by NLPs, and their toxicity may be the result of the NLP-induced release of membrane damage factors that are then sensed by the plant (68). Interestingly, the 24-amino-acid peptide, which acts as a MAMP for the activation of plant immunity, is largely buried within the core of the intact structure, with only a small number of residues being displayed on the surface (67). This suggests that the protein is probably unfolded and/or digested for recognition by the receptor.

THE THREE-DIMENSIONAL STRUCTURES OF EFFECTORS OF FILAMENTOUS PLANT PATHOGENS SHOW CONSERVED FOLDS WITHIN FAMILIES

Oomycete Effectors and the WY Fold

The RXLR class of host-translocated oomycete effector proteins is defined by the presence of a conserved N-terminal RXLR motif and a diverse C-terminal domain that exerts effector activity inside the host cell (16, 71, 72). Analysis of the sequences of the RXLR repertoires of *Phytophthora sojae* and *Phytophthora ramorum* identified conserved motifs, which were named "W" (Trp), "Y" (Tyr), and "L" (Leu), after the single-letter amino acid code for a highly conserved residue in each sequence (73). Protein structural analysis subsequently revealed that the amino acids at the conserved W and Y positions were buried in the hydrophobic core of a three-α-helical bundle and stacked against one another in an energetically favorable interaction (74) (Fig. 4). Intriguingly, except for the *Hyaloperonospora arabidopsidis* effector ATR13 (75), all of the structures of oomycete RXLR effectors that have been determined to date adopt the "WY domain" fold. Nonetheless, these proteins display significant primary sequence differences. They also show diverse structural adaptations, including N- and C-terminal extensions, loop regions, and domain duplication, that give rise to very different overall structures (74, 76–78) (Fig. 4). Hidden Markov model (HMM) sequence searches, based on the knowledge of the WY domain structure, predicted that nearly half of the RXLR effector complement of *Phytophthora* species would adopt this fold (74).

The structure of the *P. infestans* effector PexRD2 is comprised of five α-helices, three of which contribute to the WY domain three-α-helical bundle (Fig. 4A). The additional helices (present between two helices of the core WY domain) are instrumental in forming an extensive homodimeric interface in the PexRD2 structure, consistent with the observation that PexRD2 self-associates *in planta*. The structures of *Phytophthora capsici* AVR3a4 and AVR3a11 comprise monomeric four-helical bundles (Fig. 4B), with an N-terminal helical extension to the WY domain fold (74). It is possible that the
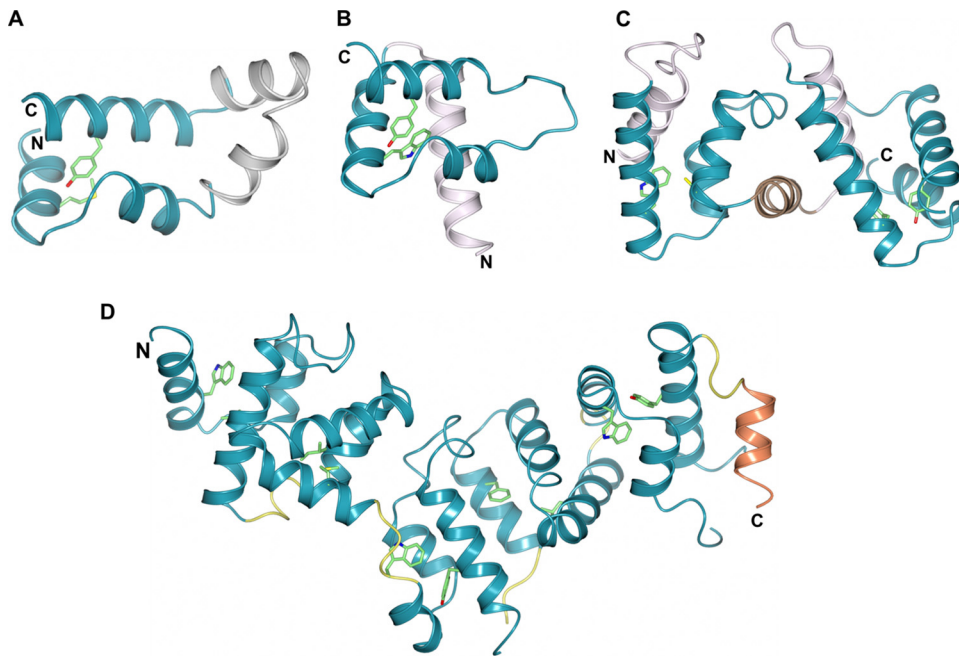
FIG 4 The structures of oomycete WY domain effectors reveal how modularity and domain repeats give rise to different overall structures. For each panel, the region of the protein comprising the WY domain fold is shown in blue, and the residues at the W and Y positions are shown as sticks (green carbon atoms). Shown are PexRD2 (monomer) (A), Avr3a11 (Avr3a4 is essentially identical and not shown) (B), ATR1 (the region toward the N terminus that does not form a WY domain is not shown) (C), and PexRD54 (D), with amino (N) and carboxyl (C) termini labeled. Avr3a11/4 and ATR1 carry an additional N-terminal helix (pink). The tandem WY domains of ATR1 and PexRD54 are separated by a helix (brown) in ATR1 and loops (yellow) in PexRD54. PexRD54 carries a short helix (coral) at the C-terminal end prior to the ATG8-interacting motif (AIM) (not shown, as it was disordered in the crystals). All structure figures were prepared with ccp4 mg (111).

N-terminal helix is important for maintaining the stability of monomeric, single-WY-domain proteins, although this has not been explicitly tested.

The HMM-based sequence searches mentioned above revealed that these effectors could also comprise tandemly repeated WY domains encoded by a single gene. The first crystal structure of a tandem WY domain effector was that of ATR1 from *Hyaloperonospora arabidopsidis* (76) (Fig. 4C). In ATR1, two WY domains (each with an N-terminal helical extension) are connected through an additional helix, which acts as a linker. Recently, the crystal structure of PexRD54 revealed how five WY domains can pack together in a stable structure with diverse domain-domain interactions (78) (Fig. 4D). Within each of these tandem WY domain structures, the individual domains can be overlaid with high confidence despite limited sequence identity (76, 78). Interestingly, PexRD54 employs a short linear motif known as the ATG8-interacting motif (AIM) to engage a host protein and to exert its virulence activity (79). The AIM is presented at the C terminus of PexRD54 and is linked to the last WY domain via a short helix. The structure of PexRD54 suggests that one function of tandem WY domains is to serve as a scaffold to present functional motifs for interaction with host proteins.

The WY domain fold serves as a chassis for the evolution of novel functions in oomycete effectors while maintaining their structural integrity. The fold presents a flexible platform that supports effector evolution and diversification via the acquisition of short linear motifs, domain duplications, and dimerization. Thus, the WY domain structure is not predictive of the precise function of the effectors but appears to provide enough plasticity for the effectors to bind different host proteins and evolve unrelated activities inside host cells.

MAX Effectors of *Magnaporthe*

Recently, a new family of effectors of filamentous plant pathogens has been described, which also shares a conserved common structure but displays a diverse
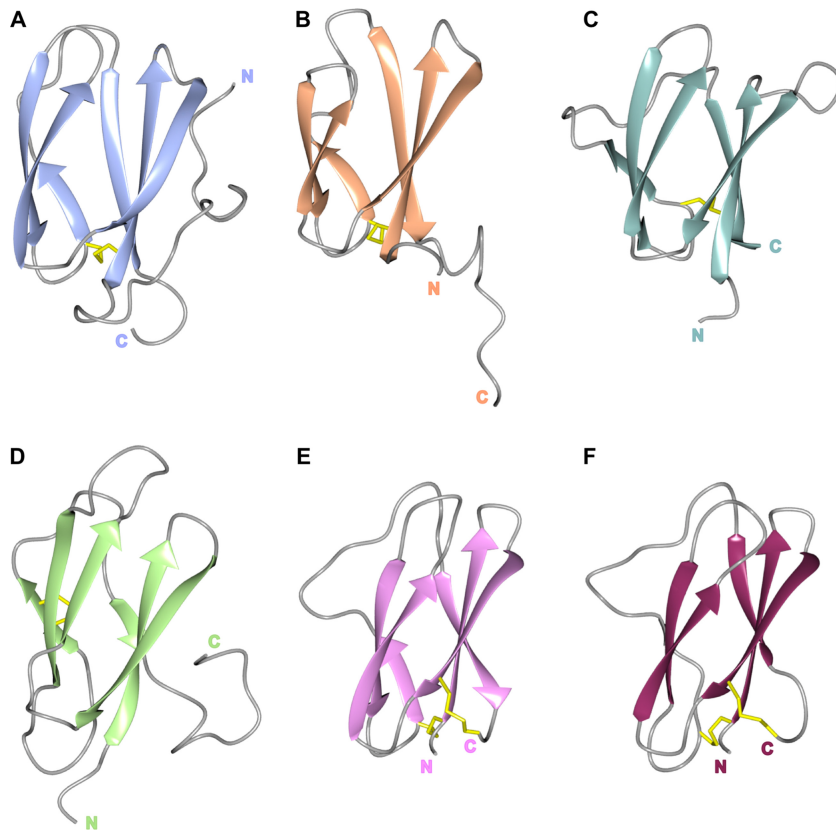
FIG 5 The structures of MAX effectors reveal the shared β-sandwich fold. The conserved β-strands are shown in a cartoon representation for each protein, with residues contributing to disulfide bridges shown as sticks (in yellow) and loops in gray. Shown are AVR-PikD (A), AVR1-CO39 (B), AVR-Pia (C), AVR-Pizt (D), ToxB (E), and toxb (F), with amino (N) and carboxyl (C) termini labeled.

protein sequence. The *Magnaporthe* Avrs and ToxB-like (MAX) family was defined following structural work on effectors from the fungal pathogen *M. oryzae*, the causal agent of rice blast disease (80). Despite typically sharing less than 25% sequence identity, each member of this family that has had a structure determined (80–84) shares a characteristic six-stranded β-sandwich fold (Fig. 5). This fold is stabilized by at least one disulfide bond, generally with Cys residues present in β1 and in, or immediately before, β5. In most cases, one of the β-sheets is formed by strands β1, β2, and β6, and the second is formed by strands β3, β4, and β5. The length and orientation of the different structural elements are variable, in particular for strand β5 and for the various connecting loops, giving rise to proteins with distinct shapes and surface properties (80). In addition, the *M. oryzae* effector AVR-PikD contains an N-terminal extension to the six-stranded β-sandwich structure (Fig. 5A), and this region contains polymorphic residues that contribute to the evasion of recognition by the plant innate immune system (82, 85). Interestingly, the *M. oryzae* effectors AVR-Pik, AVR-Pia, and AVR1-CO39 all bind to heavy metal-associated (HMA) domains that have been integrated in intracellular plant immune receptors (NLRs) throughout evolution. This suggests that the conserved MAX effector family fold is well suited to interact with such domains and may suggest a putative virulence target in host cells for these effectors.

Intriguingly, the MAX effector family includes ToxB, a proteinaceous toxin from the fungus *Pyrenophora tritici-repentis* (86). This toxin shares the common three-dimensional structure of MAX effectors (Fig. 5E and F), but its mode of action is unclear, and no interacting partner has been identified. However, the N-terminal region of ToxB has been shown to be essential for activity, while both the central and C-terminal parts are required for full activity (87), suggesting that the conserved structure is important

for function. A naturally occurring nontoxic version of ToxB (toxb) shares 78% sequence identity with the active protein. These proteins share essentially the same structure, although toxb may overall be less stable than ToxB (81).

PSI-BLAST followed by HMM-based profile searches revealed that the majority of MAX effectors are found in *Magnaporthe* species (80). However, a small number of hits were detected in other fungal species such as *Colletotrichum* (80). Thus, the discovery of the MAX effectors enables a more robust prediction of candidate effectors in these fungal pathogens.

RALPH Effectors of Powdery Mildew

Nearly 500 candidate effectors of the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* were predicted from the genome sequence using bioinformatic tools by searching for genes with characteristics of effectors, particularly those encoding small secreted proteins. Many of these candidate effectors have been shown to be expressed during infection (88–90).

To further characterize *B. graminis* candidate effectors, their sequences were subjected to structural annotation using protein fold recognition methods. A subset of these candidate effectors are predicted to have structural similarities with ribonucleases and were named RALPHs (RNase-like proteins expressed in haustoria) (91). Although confirmation that RALPHs adopt RNase-like folds awaits the determination of an experimentally derived structure, it is intriguing that many *B. graminis* effectors may share a structural scaffold with each other, a feature common in other families of effectors of filamentous plant pathogens. In another parallel with the MAX effectors, RALPHs have been predicted to contain a disulfide bond, with Cys residues being largely conserved toward both the N terminus (contained within a "YxC" motif) and the C terminus of the proteins.

Recently, data have emerged showing that RALPH effectors function as both virulence and avirulence determinants in *B. graminis*-barley and -wheat interactions. Using host-induced gene silencing, five RALPHs were shown to be involved in the formation of haustoria (92, 93). AVR_{A1} and AVR_{A13} were shown to be required for disease resistance in barley mediated by the powdery mildew resistance loci Mla1 and Mla13, respectively (94), and AvrPm2 was recently cloned as the cognate effector of the wheat *Pm2* gene (95). Furthermore, the *B. graminis* f. sp. *tritici* effector SvrPm3$^{a1/f1}$ (formerly Bcg1^{vir}) has been shown to suppress avirulence triggered by the interaction of effector AvrPm3$^{a2/f2}$ (svrPm3$^{a1/f1}$, formerly Bcg1^{avr}) with its receptor Pm3a/f (96, 97). As with other host-translocated effectors, the ability of RALPHs to activate plant immune responses may help explain the strong diversifying selection seen in these proteins.

STRUCTURES OF OTHER NOTABLE EFFECTORS OF FILAMENTOUS PLANT PATHOGENS

Flax Rust Effectors Show Divergent Structures

Melampsora lini causes rust disease on crop plants such as flax and linseed. Genomic analyses of *M. lini* predicted that this fungus has a large repertoire of putative effector proteins (22). Unlike oomycete RXLR and CRN effectors, but similar to effectors from other fungal species, no widely conserved sequence-based motifs have been identified for flax rust effectors thus far. To date, six *M. lini* effector proteins have been validated experimentally, based on their avirulence activity (AvrL567, AvrM, AvrP4, AvrP123, AvrL2, and AvrM14) (48, 98–101). These effectors trigger specific immune responses mediated by NLRs in the host cell. AvrL567, AvrM, and their cognate NLRs exhibit polymorphisms giving rise to allelic variants of the effector and receptor with specific recognition profiles (98, 102). For example, AvrL567-A is recognized by the NLRs L5 and L6, whereas AvrL567-D is recognized by L6 but not L5.

Crystal structures of the AvrL567 alleles AvrL567-D and AvrL567-A revealed that the two proteins share the same architecture, adopting a β-sandwich fold comprising seven antiparallel β-strands (Fig. 6A). Interestingly, the structures share some homology with ToxA (103), a host-selective toxin of *Pyrenophora tritici-repentis* which induces cell
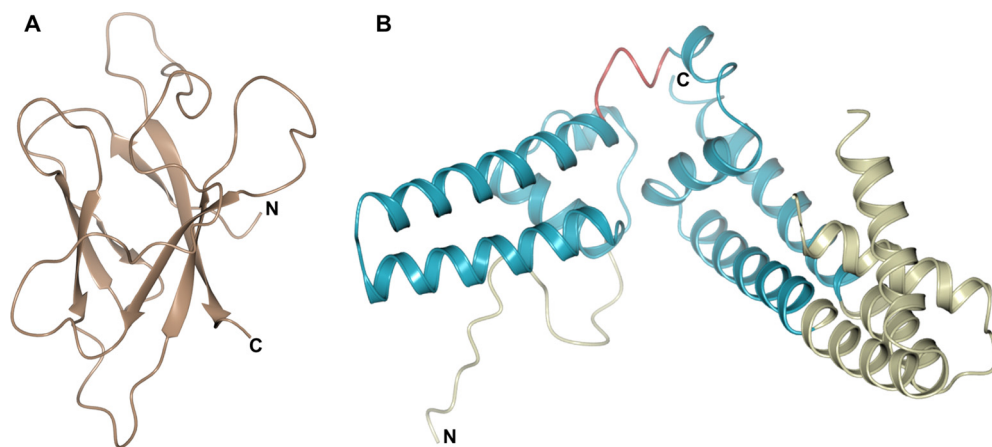
FIG 6 Divergent structures obtained for flax rust effectors. (A) Cartoon representation of AvrL567-A (the D allele is essentially identical and not shown), showing the β-sandwich fold. (B) Cartoon diagram of avrM, where the helical repeats, which have some resemblance to the oomycete WY domain fold, are shown in blue and separated by a loop (red). The amino (N) and carboxyl (C) termini of the proteins are labeled.

death in sensitive wheat cultivars. ToxA was described as having a distant relationship with mammalian fibronectin proteins, and an Arg-Glu-Asp (RGD) motif was found in a loop region of the protein that may mediate interactions with plant cell integrin-like receptors (103). This motif was subsequently shown to be required for protein internalization (104), although the precise mechanism remains unclear. AvrL567 lacks the RGD motif, implying that it is internalized by a different mechanism. Both AvrL567-D and -A display two positively charged patches on the protein surface and have been shown to bind nucleic acid *in vitro* (105). However, the biological relevance of nucleic acid binding remains unknown. Structure-led mutagenesis revealed that multiple contacts mediate the interaction between AvrL567 alleles and their cognate receptors (105).

Crystal structures of C-terminal domains of two allelic variants of AvrM (AvrM-A and avrM) revealed an L-shaped α-helical fold comprising two helical repeats (106) (Fig. 6B). The structural repeat, another example of modularity in effectors of filamentous plant pathogens, was not evident from sequence analysis and was revealed only after the structure was determined.

AvrLm4-7, a Lone Effector Structure with a Novel Fold

AvrLm4-7 is a Cys-rich protein that is recognized by oilseed rape cultivars harboring Rlm4 and Rlm7 resistances (107). The loss of AvrLm4-7 in the pathogen strongly impacts pathogen fitness (108, 109). The crystal structure of AvrLm4-7 does not share significant homology with other structures in the Protein Data Bank, and as such, it has proven challenging to infer putative protein function (110). The crystal structure identified the positions of the four disulfide bonds in the protein, which, as for other effectors, are probably involved in stabilizing the structure. In addition, a strongly positive patch was identified on the protein surface, which may represent a functionally relevant surface of the protein, although it has not been possible to show that this region binds a negatively charged ligand. A single amino acid polymorphism that perturbs the recognition of the effector by Rlm4 is located on a loop of the protein, exposed to the surface. It is therefore unlikely that this polymorphism affects the overall structure of the protein, but it may be important for a specific recognition site.

CONCLUSION

The high complexity of the secretomes of filamentous plant pathogens points to a multitude of independent evolutionary pathways to generate effector proteins that target a diversity of host molecules and processes. However, despite this extraordinary sequence diversity, it is now evident that some conserved protein folds, such as the WY

and MAX domains, define widespread families of effector proteins that occur across different plant pathogen taxa. There are both practical and theoretical implications of this finding. Structure-guided sequence similarity searches enable more precise and sensitive annotation of effector catalogues, notably of fungal effectors, which have proven more difficult to annotate than their oomycete counterparts. This should enable prioritization of effectors for further study, thus accelerating their functional characterization. In addition, the conserved structures provide a framework to unravel how the rapid evolution of effector proteins has resulted in new host targeting activities and tease out the physical and physiological constraints that these proteins face. In this regard, the next phase of research should go beyond the analyses of individual structures of effectors of individual filamentous pathogens and consider the structures of effectors in complex with host proteins (78, 82). In the future, we need to further improve our understanding of the biophysical properties of effector-host protein complexes to gain comprehensive knowledge of effector structures and functions.

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