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REVIEW

Arms race: diverse effector proteins with conserved motifs

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ABSTRACT

Effector proteins play important roles in the infection by pathogenic oomycetes and fungi or the colonization by endophytic and mycorrhizal fungi. They are either translocated into the host plant cells *via* specific translocation mechanisms and function in the host's cytoplasm or nucleus, or they reside in the apoplast of the plant cells and act at the extracellular host–microbe interface. Many effector proteins possess conserved motifs (such as the RXLR, CRN, LysM, RGD, DELD, EAR, RYWT, Y/F/WXC or CFEM motifs) localized in their N- or C-terminal regions. Analysis of the functions of effector proteins, especially so-called "core effectors", is crucial for the understanding of pathogenicity/symbiosis mechanisms and plant defense strategies, and helps to develop breeding strategies for pathogen-resistant cultivars, and to increase crop yield and quality as well as abiotic stress resistance. This review summarizes current knowledge about these effector proteins with the conversed motifs and their involvement in pathogenic or mutualistic plant/fungal interactions.

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Introduction

Mutual symbiosis and commensal parasitism between plants and microbes are initiated by recognition mechanisms prior to infection and colonization of the plant cells by the microbes. The molecular crosstalks show a common pattern: pattern recognition receptors (PRRs) sense and recognize the microbe-associated molecular patterns (MAMPs), and then PRRs bind to MAMPs. Downstream receptor activation, signal transduction processes reprogram the plant cell in response to the fungi and oomycetes. Before the physical interaction, signal molecules from both partners initiate the molecular crosstalk: Myc factors are released by mycorrhizal fungi,[1-5] Nod factors by rhizobacteria,[6-10] peptidoglycans by bacteria,[11,12] and strigolactones by plants.[13-15] In addition, fungi, oomycetes, and bacteria release small secreted proteins (SSP), which are important for the fate of the symbiotic interaction and pathogenicity, such as MiSSPs (Mycorrhiza-induced Small Secreted Proteins) which are secreted by *Laccaria biocor*,[16,17] Ecp6 (Extracellular protein 6) by *Cladosporiium fulvum*,[18-20] Mg3LysM (*Mycosphaerella graminicola* LysM) by *M. graminicola*,[21,22] or Slp1 (Secreted LysM Protein 1) by *Magnaporthe oryzae*.[23]

SSPs either reside in the host apoplast and act at the extracellular host–microbe interface (i.e., apoplastic effector proteins), or they travel across the host plasma membrane into plant cells and target intracellular proteins or DNA (i.e., cytoplasmic effector proteins).[24-36] For example, the *Fusarium oxysporum* Avr2 effector selectively inhibits the apoplastic proteases PIP1 and Rcr3 in tomato.[25,26] *Ustilago maydis* secretes Cmu1 (chorismate mutase 1) which changes metabolic pathways inside the host cell.[29] Tin2 interacts with and stabilizes the cytoplasmic protein kinase ZmTTK1 in maize.[31] The RXLR effector Pi22926 from *Phytophthora infestans* enters the plant cell and accumulates in the nucleus during infection.[37] Various effector proteins occur in oomycetes and fungi,[34] but they do not share evident sequence similarity or conserved sequence motifs, which has been attributed to the adaptation of the host plant to environmental changes and the rapid evolution/diversifying selection of the effector proteins in the arms race between microbes and hosts.[38-40] However, some SSPs with either apoplastic or cytoplasmic functions contain specific motifs which target host proteins with distinct roles in the infection process, and control virulence or mutual benefits by alterations of physiological and molecular activities in the hosts. The discovery of these motifs initiated a new direction in the research of plant–microbe interaction, since they play an important role in the "arms race" between hosts and microbes.[41] Since the last review published by Kale and Tyler,[42] great progresses has occurred, especially, genome sequences of many microorganisms greatly promote insights into the functions of effector proteins and the defense responses of host plants to microbial infection or symbiosis. These microorganisms include ectomycorrhizal fungi (e.g., *Laccaria bicolor*),[16] pathogens of important crops and trees (e.g., *Phytophthora sojae*,[43] *P. ramorum*,[43] *P. infestans*,[44] *P. capsici*,[45] *P. ipomoeae, P. mirabilis* and *P. phaseoli*,[46,47] *P. lateralis*,[48] *P. plurivora*,[49] *P. cactorum*,[50] *Melampsora lini*,[51] *Plasmopara halstedii*,[52] *P. viticola*,[53] *Peronospora tabacina*,[54] and

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clubroot pathogen *Plasmodiophora brassicae*,[55] endophytic fungi (e.g., *Piriformospora indica*[56] and *Trichoderma* species,[57] and arbuscular mycorrhizal fungi (e.g., *Rhizophagus irregularis*.[58] Since it is a great challenge to elucidate the role of effector proteins in plant-microbial interactions and to promote plant health,[35] more and more efforts are necessary. Here we summarize the new advances, with the special focus on the roles of specific motifs in oomycete and fungal SPPs.

RXLR effectors

Oomycetes and fungi are classified into different biological kingdoms, but they share similar strategies in infecting their different host plants, presumably as a result of convergent evolution.[59] One of the conserved strategies utilizes the RXLR motif for entry of their effector proteins into host plant cells. RXLR and RXLR-like motifs occur in many effector proteins secreted by different oomycetes[50,60-70] and fungi.[35,56,57,71-76] The RXLR motif is named after a conserved Arg-X-Leu-Arg sequence located in the N-terminal regions of the effector proteins which is often followed by a dEER motif 5 to 20–25 amino acids downstream.[41,56,77,78] The RXLR motif is similar to the host-targeting signal (RXLXE/A) that is in charge of the transport of secreted malaria proteins across the parasitiphorous vacuolar membrane into the cytoplasm of erythrocytes.[79,80] The KRLTG sequence in the rust effector protein Ps87 from *Puccinia striiformis*[75] and the QLLR and GKLR sequences in effector proteins of some downy mildew pathogens[81] are examples for variations of the RXLR motif. Other fungal effectors carry even more degenerated N-terminal RXLR-like motifs ([RHK]X[LMIFYW]).[73]

The numbers of effector proteins with the RXLR motifs can vary substantially in oomycetes and fungi. *Phytophthora sojae* contains 385 putative RXLR effector proteins,[38] sequences for 563 are found in the *P. infestans* genome[44], and more than 400 in the *P. capsici* genome.[45,82,83] *P. parasitica* contains 172,[69] *Hyaloperonospora arabidopsidis* 134,[84] *Plasmopara viticola* at least 100[64] and *Plasmopara viticola* more than 100 predicted effector proteins with such a motif.[53,70,85] The number of effector proteins with RXLR or RXLR-like motifs is much lower in investigated biotrophic pathogens, such as *Albugo candida*,[86] *Albugo laibachii*,[87] *Bremia lactucae*[88] or *Pseudoperonospora cubensis*.[89] In oomycete pathogens, 50 RXLR effector proteins are found in *Plasmopara halstedii* and *P. viticola*[90] and 25 in *Albugo laibachii*.[87] Furthermore, *Pythium ultimum* and *Aphanomyces euteiches*, two species with a necrotrophic lifestyle, contain only two or no RXLR motifs, respectively.[91,92] Furthermore, *P. infestans* isolates show RXLR effector protein diversity[67] and the avirulence locus Avr3c encodes a multi-copy RXLR effector with sequence polymorphisms among pathogen isolates.[93] These quite huge differences in the numbers of RXLR effector proteins reflect different strategies used by pathogens to infect host plants.

Compared to the role of the RXLR effector proteins from pathogens, little attention has been paid to those from mycorrhizal and endophytic fungi. The genome of the well-studied endophytic fungus *P. indica* (named later to *Serendipita indica*[94]) contains 12 predicted effector proteins with the RXLRX-EER and 155 proteins with the RXXLRX-EER motifs, but only 5 effectors with the latter sequence combination contain signal peptides.[56] Since *P. indica* shows very strong colonization capacity with a wide range of host plants,[95] it has been speculated that the flexible feature of RXLR motifs might help the fungus to escape the immune response of host plants.

RXLR effector proteins are modular molecules. A canonical RXLR effector protein usually contains a signal peptide at the N terminal region for the secretion of the protein from the microbe, followed by one or two RXLR motifs and a more variable second dEER motif at varying distances from the end of RXLR motif to the C terminus of the protein.[41,56,96] The RXLR motif can be part of an uptake signal for the translocation of the effector proteins from oomycetes and fungi into the cytoplasm of host plant cells.[97-100] To investigate the function of the two RXLR (RXLR1 and −2) and dEER motifs of the Avr1b effector, Dou et al. generated transgenic *P. sojae* strains in which mutations in either or both of the RXLR motifs and the dEER motif were introduced.[99] Five independent transgenic lines expressing an RXLR2^{AAAA} mutant showed no gain of avirulence against soybean Rps1b cultivars, despite the presence of abundant mRNA from the transgene. However, avirulence was lost in the RXLR1^{AAAA} RXLR2^{AAAA} double mutants. In addition, a mutation in the dEER motif abolished avirulence in two independent transformants.[99] The results showed that the RXLR and the dEER motifs are required for Avr1b function.[99] The ATR5^{Emoy2} (avirulence protein from the downy mildew pathogen *Hyaloperonospora arabidopsidis* isolate Emoy2) and ATR5L (ATR5-like) proteins possess signal peptides, a canonical EER motif, and an RGD motif located in their C-terminal regions, but they lack the canonical translocation motif RXLR.[101] For both proteins, the signal peptides and N-terminal regions, including the EER motif of ATR5^{Emoy2}, are not required to trigger an RPP5-dependent immune response.[101] The results suggest that signal peptides and RXLR motifs in the N-terminal regions of the proteins are in charge of secretion and translocation of RXLR effector proteins into host cells, and that the EER motif mediates translocation when RXLR motifs are lacking. Furthermore, the two RXLR motifs in the effector protein Avr1b showed the functional difference in avirulence. For instance, based on studies with the RXLR2^{AAAA} mutant which contains a functional RXLR1 motif, the motif seems to be nonfunctional.[99]

The detailed mechanism of how the RXLR motif mediates the translocation of effector proteins into host plant cells is still unclear. However, some cues might help to understand the mechanism. The oomycete and fungal RXLR motifs enable binding to the phospholipid, phosphatidylinositol-3-phosphate (PI_3P), which is abundant on the outer surface of plant cell plasma membranes.[73] The RxLR domain of Nuk10, an effector of the human malaria parasite *Plasmodium falciparum*, was also shown to bind to PI_3P with high affinity.[102] The C-terminal regions of RXLR effectors also play an important role in binding to PI_3P. Yaeno et al. investigated the lipid-binding properties of three RxLR effectors (*P. infestans* Avr3a and *P. capsici* Avr3a4 and Avr3a11) and found that the basic region in the effectors' C-terminal domain rather than the RxLR motif was critical for PI_3P recognition and that PI_3P recognition may be associated with the intracellular virulence-promoting activity.[103] Another research strengthened the finding. Sun et al. characterized

structurally and functionally the RXLR effector Avh5 from *P. sojae* for its entry into host plant cells and found Avh5 is helical with a long N-terminal disordered region.[104] Their results showed that a C-terminal lysine-rich helical region was the principal lipid-binding site, while the N-terminal RXLR motif (RFLR) played only a minor role. However, the RXLR motif is still necessary for entry of Avh5 into host plant cells, because mutations in this motif or in the basic region significantly reduced protein entry into plant cells, and both regions independently mediated cell entry *via* a PI_3P-dependent mechanism.[104] No matter what region is used to bind lipids, the translocation of RXLR effector proteins into host plant cells suggests that it is mediated by PI_3P, i.e., an endocytosis process that resembles those mediated *via* lipid rafts in vertebrates.

Other studies demonstrated that RXLR motifs are not required for the activities of the effector proteins.[98,105] Bos et al. found that mutation of the $AVR3a^{KI}$ RXLR sequence in *P. infestans* did not interfere with induction of R3a hypersensitivity when the protein is directly expressed in the leaves of *Nicotiana benthamiana*. In addition, deletion analyses of $AVR3a^{KI}$ indicated that 75 amino acids in the C-terminal region of AVR3a, which excludes the RXLR domain but includes the two polymorphic amino acids K^{80} and I^{103} (which were mutated in the nonfunctional allele) were sufficient for avirulence function when expressed directly inside plant cells.[105] In the modular RXLR proteins, the C-terminal regions endow the proteins with the physiological functions: both as activator and suppressor of plant immunity.[106]

The progress in obtaining genome sequence information from microbial species uncovers more and more putative secreted proteins with RXLR or RXLR-like motifs. Win et al. proposed an algorithm for *ab initio* mining of RXLR effectors from oomycete genomes.[107] In the first step, SignalP is used for the identification of the RXLR effectors in the microbial genomes. The sequences should be picked out with the conditions of an HMM score of >0.9 and a predicted amino acid cleavage site within a 10–40 amino acid region. Secondly, three additional conditions must be fulfilled: the position of the RXLR motif must be located in the first 30–60 amino acids of the protein sequence, the RXLR motif position must be located downstream of the signal cleavage site and a length of the SignalP v2.0 NN predicted cleavage site of <30 amino acids.

In general, the numbers of RXLR effector proteins are more than those of any other types of effector proteins in pathogens, thus more attention have been paid to these effector proteins. Although the functional redundancy causes severe difficulties in the experiments,[108] more and more effector proteins were discovered and their functions were elucidated. RXLR effector proteins can be classified into two types: one type induces cell death, such as PITG_22798 from *P. infestans*[63] and Avh238 from *P. essential*[109]; another type blocks cell death, such as the RXLR-WY effector protein AVR3a from *P. infestans*.[110] No matter which type they belong to, they target different proteins or compounds in host plant cells and promote pathogenicity or symbiosis. Therefore, the RXLR effectors show various distinct functions in host plants.

Pathogens can escape, suppress, or alter the recognition event caused by MAMPs in ways that allow them to grow and reproduce,[111] resulting in pathogenicity. Eight RXLR-type SFI effectors (i.e., PITG_04097, PITG_04145, PITG_06087, PITG_09585, PITG_13628, PITG_13959, PITG_18215, and PITG_20303, named as SFI1 to SFI8, respectively) from *P. infestans* significantly suppressed flg22-triggered immune responses,[112] and SFI5 suppresses MAMP-triggered immunity (MTI) by interacting with host calmodulins.[113] NRL1 is a predicted CULLIN3-associated ubiquitin E3 ligase and transient overexpression of NRL1 results in the suppression of INF1-mediated cell death and enhanced colonization *P. infestans*.[114] Yang et al. and He et al. showed that NRL1 is a susceptibility factor which suppresses INF1-triggered cell death, and that it interacts with SWAP70, a guanine nucleotide exchange factor and a positive regulator of immunity.[114,115] The RXLR effector Pi02860 from *P. infestans* interacts with the host protein NRL1 and enhances the association between NRL1 and SWAP70 to promote proteasome-mediated degradation of SWAP70, which results in the suppression of immunity.[115] Interestingly, the effector does not attenuate cell death triggered by Cf4/Avr4 coexpression,[114] showing that it does not suppress all cell death responses activated by cell surface receptors. In Arabidopsis, the protein FKBP15-2 possesses PPIase activity and is involved in ER stress sensing and ER stress-mediated immunity. When *Phytophthora capsici* infects Arabidopsis, the RXLR effector protein PcAvr3a12 targets and inhibits FKBP15-2 and thus suppresses ER-mediated host immunity.[116] The RXLR effectors HaRxL23 from *Hyaloperonospora arabidopsidis* and PsAvh73 from *P. sojae* suppress PAMP-triggered immunity (PTI) in *N. benthamiana* and effector-triggered immunity (ETI) in soybean.[65] The RXLR effector Avh238 from *P. sojae* not only contributes to pathogen virulence but also triggers host cell death.[109] The 79th residue (histidine or leucine) of Avh238 determined its cell death-inducing activity and 53 amino acids in the C-terminal region are responsible for promoting *Phytophthora* infection.[109] Thus, pathogens can escape recognition by the host plants by mutating one nucleotide site in Avh238, and can suppress host immune response to enhance pathogenicity. Additional studies support the important role of RXLR effector proteins in the suppression of host immune responses.[117,118] Other RXLR effectors trigger host immune response and induce host cell death. For example, Avh241 from *P. sojae* requires plasma membrane localization to induce host cell death.[119] The above-mentioned PITG_22798 from *P. infestans* and Avh238 from *P. essential* induce also host cell death, but the mechanisms are not well understood.[109,120]

The RXLR-WY effector AVR3a from *P. infestans* is translocated into host cells and occurs in two forms, AVR3a(KI) and AVR3a(EM). AVR3a(KI) is detected by the potato resistance protein R3a and strongly suppresses INF1 (a PAMP elicitin in *P. infestans*)-triggered cell death (ICD), whereas AVR3a(EM), which evades recognition by R3a, weakly suppresses host ICD.[105,121] The RXLR protein AVR3a suppresses CMPG1 (an U-Box ubiquitin E3 ligase)-dependent cell death.[110] A model proposes that AVR3a binds and stabilizes CMPG1[110] and suppress BAK1/SERK3-regulated immunity triggered by INF1.[122] The suppression is mediated by the C-terminal region of AVR3a, because this region is sufficient to trigger AVR3a-mediated hypersensitivity and suppresses INF1-induced cell death in *N. benthamiana*.[105] $AVR3a^{KI\text{-}Y147del}$, a mutant with a deleted C-terminal tyrosine residue, fails to suppress INF1-mediated cell death.[121] Another study showed that AVR3a is

a multifunctional effector protein that can suppress BAK1/SERK3-mediated immunity through at least two different pathways.[123] Similarly, expression of the RXLR effector Pi17316 from *P. infestans* attenuates cell death induced by INF1 and suppresses pattern-triggered immunity, but Pi17316 does not attenuate cell death triggered by a range of resistance proteins.[68] The RXLR effector PITG_14736/PexRD8 also suppressed INF1-mediated programmed cell death (PCD).[124] PpRxLR2 from *P. parasitica* is able to completely suppress INF1-induced cell death, whereas PpRxLR3 and PpRxLR5 moderately suppressed *N. benthamiana* immunity in a less-extensive manner.[69] In *P. infestans*, two other effector proteins can also suppress cell death. The cell death triggered by the RXLR effector protein PITG_22798 is suppressed by the effector AVR3b,[120] and SNE1 acts broadly as the suppressor of PCD,[125] but it is still unclear whether they are RXLR proteins. Over 100 candidate RXLR effector proteins were identified in biotrophic oomycete *P. viticola*,[53,85] and the experimental results from Liu et al. showed that 52 effectors could completely suppress cell death triggered by elicitins (INF1 and BAX) and 10 effectors could partially suppress cell death.[70] Xiang et al. also reported an RXLR effector protein which triggers the immune response in *P. viticola*.[62] These examples demonstrate that RXLR effectors can specifically suppress cell death processes, although it appears that quite different mechanisms are involved in the scenario.

Some pathogens use RXLR effectors to inhibit secretion of antimicrobial proteins and defense proteases. Several host plants possess antimicrobial proteins to withstand the invasion of pathogens. For example, potato (*Solanum tuberosum*) contains the antimicrobial protein AP_1,[126] and the pepper antimicrobial protein CaAMP1 shows multiple functions in ABA signaling, as well as salt and drought tolerance.[127] Pathogens can inhibit secretion of antimicrobial proteins of host plants, thereby promoting infection. The RxLR24 effector from *P. brassicae* interacts with host RABA-type GTPases to inhibit vesicle-mediated secretion of antimicrobial proteins PR-1, PDF1.2 and possibly other defence-related compounds.[66] The RXLR effector Pi04314 from *P. infestans* targets plant protein phosphatase 1 catalytic (PP1c) isoforms and promotes late blight disease.[61] It enhances leaf colonization *via* activity in the host nucleus and attenuates induction of jasmonic acid (JA) and salicylic acid (SA)-responsive genes. As mentioned above, AP_1 is an antimicrobial protein, and it has an ATP-binding domain at the C-terminus. The N-terminus shows 58% identity with the acid phosphate from *Mesorhizobium loti*.[126] AP_1 functions in relation to phosphorylation and energy metabolism of plants. It was hypothesized that PP1c acts as an antimicrobial protein and that Pi04314 targets PP1c in order to regulate phosphorylation and energy metabolism of host plants for *P. infestans* infection. The RXLR effector protein AVRblb2 from *P. infestans* associates with papain-like cysteine protease C14 from *N. benthamiana* and tomato, and the overexpressed protein prevents secretion of the plant defense protease C14 in *N. benthamiana* and tomato, which enhances susceptibility of *N. benthamiana* plants to *P. infestans*.[128]

Plants possess two major types of small RNAs, i.e., microRNAs (miRNAs) and small interfering RNAs (siRNAs), and both of them have strong effects on numerous physiological processes. Among others, small RNAs also play important roles in host defense against pathogen infection.[129] Some RXLR effectors affect the biogenesis of small RNAs. The RXLR effector PSR1 interacts with Arabidopsis PINP1,[130] and strongly inhibit the biogenesis of siRNAs.[48] When overexpressed, it enhances susceptibility of *N. benthamiana* to potato virus X and *P. infestans*[48] and susceptibility of Arabidopsis to *P. capsici*.[130] Similarly, the RXLR effector PSR2 from *P. sojae* also inhibits the biogenesis of small RNAs; when silenced, it reduces the virulence of *P. sojae* on soybean plants,[48] but its host target is not known yet. A PsPSR2-like effector (PiPSR2) was identified in *P. infestans*, and PiPSR2 can also suppress RNA silencing in plants and promote *Phytophthora* infection.[131] All these results suggest that the PSR effector family has conserved functions in plant hosts by modulating small RNAs.

RXRL effectors also regulate host transcription by targeting transcription factors to promote pathogenicity. In *P. infestans*, the RXLR effector protein Pi03192 prevents re-localization of two host plant NAC TFs (NTP1 and NTP2) at the ER membrane, stopping entry of the NAC TFs into the nucleus, and finally promote pathogenicity.[132] The RXLR effector HaRxL44 from *H. arabidopsidis* localizes to the nucleus of plant cells, and causes degradation of Arabidopsis MED19a, an important mediator in the interaction between transcriptional regulators and RNA polymerase II, thereby attenuating SA-triggered immunity in Arabidopsis by shifting the balance from transcription of SA-responsive to JA/ET-responsive genes.[133] If manipulatable by RXLR effector proteins, such alterations in the defense response can have enormous effects for the hosts since it interferes with the response pattern to biotrophic and necrotrophic microbes. In addition, the RXLR effector Pi04089 from *P. infestans* interacts with StKRBP1, a putative potato RNA-binding protein with a KH domain.[134] The interaction may affect the expression of genes regulated by StKRBP1.

RXLR effectors also manipulate host MAPK signaling.[135-137] During arms race, pathogens have evolved various strong effectors to manipulate the host MAPK signaling pathway involved in pathogenicity. The RXLR effector PexRD2, a virulence factor of *P. infestans*, interacts with the kinase domain of the host MAPKKKε to suppress MAPKKKε-dependent phosphorylation of MPKs.[138] The RXLR effectors SFI5, SFI6 and SFI7 from *P. infestans* suppress flg22-induced MAP kinase activation in tomato.[112] The RLXR effector Pi17316 from *P. infestans* interacts directly with potato StVIK, a predicted MAP3K.[68] Virus-induced gene silencing of StVIK in *N. benthamiana* attenuated colonization of *P. infestans*, whereas transient overexpression of StVIK enhanced colonization of the pathogen. It would be interesting to elucidate the direct and indirect downstream components of StVIK signaling to understand how the effector controls innate immune responses in the host. The RXLR effector protein AvrLm1 from *Leptoshaeria maculans* possesses kinase activity. It interacts with the *Brassica napus* MAP9, and increases its accumulation and phosphorylation.[139] The RXLR effector Avh331 from *P. sojae* also suppresses the Arabidopsis MAPK-based plant defence to promote pathogenicity.[140] Similarly, in bacteria, some effectors manipulate the MAPK signaling pathway. For example, the *Pseudomonas syringae* effector HopAI1 targets MPK3 and MPK6 and inactivates their kinase function to suppress plant defense responses.[141] The *P. syringae* effector HopF2 targets MKK5 and inactivates MKK5 *via* ADP-ribosylation of its C terminus *in vitro*.-[142] The *P. syringae* effector AvrB interacts with MPK4 to perturb

hormone signaling and to promote infection.[143] All these findings shed light on the importance of MAPK signaling pathways as targets of RXLR effector proteins. Besides interfering with MAPK signaling, AvrLm1 provides an example for an effector which triggers expression of the blackleg resistance gene *LepR3* encoding a receptor-like protein in *B. napus*,[144] and another receptor-like kinase SOBIRI in *B. napus* interacts with LepR3 and is required for AvrLm1-triggered immunity.[145]

From the above summary, it is obvious that RXLR effectors also manipulate the host defense hormone levels. For example, the RXLR effector HaRxL44 from *H. arabidopsidis* attenuates SA-triggered immunity in Arabidopsis and shifts the balance of defense transcription to JA/ET signaling.[133] The RXLR effector HaRxL62 from *H. arabidopsidis* suppresses response to SA[146]; the RXLR effector Pi4314 attenuates both JA- and SA-mediated transcriptional responses of the host plant and promotes late blight disease.[61] It is interesting that RXLR effectors are affecting the phytohormone auxin. The RXLR effector PSE1 from *P. parasitica* can perturbate Arabidopsis development by modulating auxin concentrations at the root apex, and PSE1 increases auxin-dependent Arabidopsis susceptibility to *P. parasitica*.[147] Similarly, AvrRpt2 from *P. syringae* stimulate auxin-dependent protein turnover in Arabidopsis and promotes pathogenicity.[148]

In addition, other functions of RXLR effectors are used to facilitate infection and virulence. For example, the *P. sojae* RXLR effector PsAvr3b functions as an ADP-ribose/NADH pyrophosphorylase and promotes virulence by its enzyme activity.[149] PsAvr3b also acts a Nudix hydrolase, and its activation by plant cyclophilin is required for Nudix hydrolase activity.[150] Avr3b might be delivered into host cells as a Nudix hydrolase to impair host immunity.[151] The RXLR effector PsAvh262 from *P. sojae* stabilizes ER-luminal binding immunoglobulin proteins (BiPs), which act as negative regulators of plant resistance to *Phytophthora*.[152] By stabilizing BiPs, PsAvh262 suppresses ER stress-triggered cell death and facilitates infection of *P. sojae*.

Taken together, RXLR effectors possess various functions. The functional diversity is related to the intrinsic disorder of structures of RXLR effectors, especially great diversity in their C-terminal regions because at least in oomycetes, the intrinsic disorder is a common structural characteristic of RXLR effectors.[153] Therefore, more attention should be paid on structural features of RXLR effectors, because RXLR effectors show functional redundancy,[112] resulting in difficulties in analyzing individual members of the RXLR effector families.

CRN effectors

Like RXLR motifs, CRN (for CRinkling and Necrosis) motifs occur in effector proteins across several biological kingdoms, especially oomycetes.[50,60,64,83,154-157] CRN motif-containing effector proteins are the second largest class of cytoplasmic effectors in oomycetes and an ancient conversed family.[44,156]

The CRN protein family was originally identified in *P. infestans*.[24] They are ubiquitous in plant pathogenic oomycetes and symbiotic fungi, but their numbers in various microbes differ greatly: 45 genes in *Pythium* sp.,[158] about 60 genes in *Plasmopara halstedii* and *P. viticola*,[90] 84 genes in *P. capsici*,[83] approximately 200 genes in *P. infestans*[44] and *P. sojae*.[159] The genome of the endophytic fungus *P. indica* contains few *CRN* genes.[56] Lin et al. found that 42 LxLFLAK-containing proteins are encoded in the genome of the arbuscular endomycorrhizal fungus *Rhizophagus irregularis*.[160] Interestingly, some land plant species also posses CRN proteins, such as *Selaginella moellendorffii, Physcomitrella patens, A. thaliana, Vitis vinifera, Theobroma cacao, S. tuberosum*, and *S. lycopersicum*.[155] The CRN proteins in plant species (such as *V. vinifera* and *T. cacao*) possess Myb domains in the N-terminal regions, which are the important cores in Myb proteins. Most Myb proteins function as TFs with varying numbers of Myb domain repeats for DNA binding, thus controlling plant development, metabolism, and responses to biotic and abiotic stresses.[161,162] Therefore, the occurrence of Myb domains in CRN proteins in land plants suggests that they function in plant development and responses to biotic and abiotic stresses. At present, no evidence shows this for land plants.

Similar to RXLR proteins, the members in CRN protein family are also modular molecules. They carry a signal peptide, conserved N termini, and highly diverse C-terminal domains, and potentially undergo variation *via* recombination of their N- and C-terminal regions.[44,83,159,163] The entry of these proteins into host plant cells depends on the CRN motifs located in their N terminal regions.[154] In a canonical CRN effector protein, there is a distinct structure that possesses a highly conserved N terminal amino acid motif: LxLFLAK followed by the conserved DWL domain that ends in a conversed HVLVVVP motif at the end of CRN N-terminus. This sequence is thought to be a hotspot for recombination events.[28,44,156] After analysis on the domain architectures of CRN proteins, a common "syntax" for CR proteins occurred: CR-NTD[i]+CR-toxin [j, k, l…]; i.e. one of several CR-NTD (Crinkler-RHSP-type N-terminal domain) followed by one or more Crinkler-RHSP-type toxin domains.[155] Maybe it is more suitable to name the protein family as CR family.

To test the functions of CRN motifs for the protein entry into host cells, Schornack et al. constructed CRN2:AVR3a and CRN16:AVR3a derivatives with mutations in the LXLFLAK motif (to LXAAAA) and introduced these constructs into *P. capsici*.[154] Their results showed that *P. capsici* transformants expressing the mutated CRN–AVR3a fusions had no changes in their virulence on the leaves of R3a-transgenic *N. benthamiana*, compared with the wild-type CRN-AVR3a constructs, and that development of disease lesions was evident on the leaves of R3a *N. benthamiana* inoculated with the mutated CRN–AVR3a strains, indicating absence of R3a-mediated resistance. Therefore, they came to the conclusion that the LXLFLAK motif (i.e., CRN motif) is required for effector targeting and translocation.[154] Interestingly, in the oomycete *H. arabidopsidis*, there is a CRN motif overlapping with the RXLR motif, i.e. RXLRLFLAK.[107,154] This overlap implies a function of LXLFLAK motifs in translocation of CRN effector proteins into host plant cells. As mentioned above, the dEER motif has a role in Avr1b function,[99] however, the role of DWL domains in N-terminal regions of CRN proteins is still not clear.

The C-terminal regions in CRN proteins are diverse, and the functions of CRN proteins depend on their C-terminal regions.[82,154-156,164-167] Zhang et al. found that the majority of CRN effectors display either of two architectural types: (i) a P-loop NTPase domain coupled with a nuclease domain of the restriction endonuclease (REase) superfamily. This architectural type accounts for a little over one-fourth of the total CR proteins. (ii) A REase superfamily domain combined with a eukaryote-type protein kinase domain. This type accounts for a little over one-sixth of the total CR proteins.[155] The two architectural types endow CRN effector proteins with important functions. If an effector protein containing a CRN motif does not induce any cell death in host plants, it certainly is not a defining feature of this protein family.[156]

CRN effectors exhibit various pathogenic functions, including induction of PCD and suppression of PCD through PAMP-triggered immunity or/and effector-triggered immunity.[159] In *P. sojae*, PsCRN63 and PsCRN115 share close protein sequence similarity, but they show contrasting and apparently opposite responses when expressed in *N. benthamiana* or soybean: PsCRN63 induces PCD, while PsCRN115 blocks PCD *in the plant*.[164] The results suggest that the two CRN effector proteins can possess distinct and even opposite functions. Furthermore, PsCRN63 alone or PsCRN63 and PsCRN115 together might suppress the immune responses of *N. benthamiana*, and the two cytoplasmic effectors interact with catalases from *N. benthamiana* and *Glycine max*.[168] The two CRN effector proteins regulate PCD and H_2O_2 homeostasis through direct interaction with catalases *in planta*, overcoming host plant immunity and carrying out infection. Further results showed PsCRN63 does not suppress upstream signaling events including flg22-induced MAPK activation and BIK1 phosphorylation, indicating that it acts downstream of MAPK cascades.[169] At the same time, Li et al. found that PsCRN63 forms a dimer that is mediated by inter-molecular interactions between N-terminal and C-terminal domains in an inverted association manner. Astonishingly, the N- and C-terminal domains required for the dimerization are widely conserved among CRN effectors.[169] PiCRN8 from *P. infestans* has a serine/threonine RD kinase domain in the C-terminal regions and it autophosphorylates depending on the presence or absence of an intact catalytic site.[163] Further studies showed that PiCRN8 forms a dimer or multimer. Since homo-/hetero-dimerization often occurs among CRN effectors, it is spectulated that PsCRN63 functions in the formation of homodimers or heterodimers with PsCRN115 or PsCRN79 to balance the relationship between PCD and suppression of host immunity.

CRN effectors induce host cell death, and the induction is related to their localization in the host nucleus. PcCRN4 from *P. capsici* localized to the plant nucleus, and the localization was required for both its cell death-inducing activity and virulent function.[165] Silencing of PcCRN4 in *P. capsici* reduced the ability to suppress plant defenses. PsCRN70 from *P. sojae* was localized to the host nucleus and suppressed cell death triggered by cell death-inducing proteins, including BAX, PsAvh241, PsCRN63, PsojNIP and R3a/Avr3a.[170] Aforementioned PsCRN63, PsCRN115, and PiCRN8 localize to the nuclei where they are functional.[163,168] PsCRN108 from *P. sojae* contains a putative DNA-binding helix-hairpin-helix (HhH) motif and acts in the host cell nucleus.[166] PsCRN108 targeted HSP promoters in an HSE- and HhH motif-dependent manner and could inhibit the association of the HSE with the plant heat shock TF AtHsfA1a, which initializes heat shock protein gene expression in response to stress.[166] All the results suggest that PsCRN108 acts as a nucleomodulin in down-regulating the expression of plant defense-related genes by directly targeting specific promoters of host DNA, just like some bacterial effectors.[171] The C-terminal regions of some CRN effectors in *Plasmopara halstedii* and *P. viticola* show similarity to serine proteases,[90] however, their host targets remain unknown.

CRN effectors induce crinkling and necrosis, but not all CRN effectors induce cell death. Out of 10 *P. sojae* CRN effectors tested, only one of them (PsCRN172-2) induced cell death when overexpressed in *N. benthamiana* leaves, and the remaining 9 CRN effectors suppress cell death caused by PsNIP; 8 CRN effectors by PsCRN63; 5 CRN effectors by combined treatment of Avr3a and R3a; and 3 CRN effectors by Avh241.[159] Together, the results indicate that CRN effectors from fungal and oomycete pathogens seem to act as cell death regulators rather than inducers to balance cell death and suppression of host immunity.

CRN effectors are also found in mycorrhizal and endophytic fungi, such as *P. indica*,[56] *R. irregularis*[155,172] and *L. bicolor*,[155] but little attention were paid to their functions in symbiosis and mutualism. RiCRN1 from *R. irregularis* accumulates during symbiosis establishment.[172] Expression of *RiCRN1* in *N. benthamiana* leaves and *Medicago truncatula* roots suggests that the effector is not involved in cell death processes. Like other CRN effectors, RiCRN1 dimerizes and localizes to nuclear bodies. Downregulation of *RiCRN1* expression impaired the symbiosis in *M. truncatula* and lower *MtPT4* expression, and ectopic expression of *RiCRN1* led to a drastic reduction in arbuscule size.[172] All these results suggest that RiCRN1 plays an important role in symbiosis progression and the proper initiation of arbuscule development. MiSSP7 is vagarious secreted protein in *L. bicolor*, because its activation is not ectomycorrhizal (ECM) host plant specific.[173] MiSSP7 is produced in *L. bicolor* upon receipt of diffusible signals from plant roots throughout the development of the mycorrhizal root tips. Importantly, MiSSP7 can be imported into the host plant cells and targets to host nucleus to promote the formation of the Hartige net. Although it is not clear whether MiSSP7 is a CRN effector, it shows some features of CRN effectors: it is targeted to the host nucleus[173] and represses host immune response *via* stabilizing the *Populus* JAZ6 protein, a negative regulator of JA-induced gene regulation.[174] In view of the functions of mycorrhizal and endophytic fungi in the acquisition of water and mineral nutrition and resistance to abiotic and biotic stresses, more investigations on CRN effectors in these systems is required.

RGD effectors

In 1984, the Arg-Gly-Asp (RGD) motif was identified as the amino acid sequence within fibronectin that mediates cell

attachment.[175] This motif is present in a large number of proteins that reside in the extracellular matrix (such as vitronectin), and the RGD proteins play a role in cell adhesion and cell growth.[176-178] The human integrins are targeted by pathogenic proteins with RGD motifs which attach to the cell surface. For example, the opportunistic pathogen *Pseudomonas aeruginosa* possesses the type IV pilus (tfp)-associated protein PilY1, which contains an integrin-binding RGD motif, and purified PilY1 binds integrin *in vitro* in an RGD-dependent manner.[179] Similarly, in plants, synthetic peptides and proteins containing the RGD motif disrupt the adhesion between plasma membrane and cell wall[180-182] which leads to altered physiological processes affecting plant development,[183-186] gravity sensing,[187] and the interaction between plants and microbes.[188-194] Also, the RGDBP and VPS9 effector proteins from *Puccinia graminis* are located in the apoplast of the plant cell[35] and initiate signaling events which lead to Rpg1-mediated stem rust resistance.[195] Shenchou et al. identified an 80-kDa plasma membrane protein as receptor for the RGD-containing protein IPI-O from *P. infestans*, which promotes the disruption of the cell wall-membrane contacts in Arabidopsis.[182]

While these examples demonstrate apoplastic and/or plasma membrane-associated functions, RGD proteins are also imported into plant cells. The above-mentioned ATR5^{Emoy2} and ATR5L proteins from *H. arabidopsidis* with RGD motifs posses encrypted translocation signals, such as KIFK and RIL [G/D] sequences at the N-terminal regions. For ATR5^{Emoy2}, these sequences mediate entry of into host plant cells.[101] The role of the RGD motif for the translocation process was not investigated. However, the RGD motif in the *Pyrenophora tritici-repentis* ToxA protein, which is located more downstream in the protein sequence region than the motif in ATR5^{Emoy2}, is required for entry of the ToxA protein into host plant cells.[81,194] Apparently, more studies are required to clarify the role of the RGD motifs for host cell entry.

DELD effectors

The *P. indica* genome encodes 867 secreted proteins, including 386 small secreted proteins, including a novel SSP effector family "DELD", 25 members of which have conserved novel seven-amino acids (RSIDELD) motifs at the C-terminal regions.[56] All DELD proteins have a similar size between 101 and 135 amino acids without obvious functional protein domains.[56,196] The structure of DELD proteins shows approximately 30% similarity to HRPII, a well-studied effector protein family from *P. falciparum*,[196] although the relationship between the two protein classes, if any, is not clear. Among the analyzed microorganisms, *P. indica* possesses the most number of DELD proteins, one was found in the *Coprinopsis cinereus* genome, while the genome of many microorganisms, including *Laccaria bicolor, Trichoderma atroviride, T. reesei, Tuber melanosporum, Puccinia graminis, M. oryzae, Fusarium oxysporum* and *Ustilago maydis* lack sequences for DELD proteins.[56] This also holds true for the genomes of *Pyrenophora teres* f. *teres* 0-1, *Pyrenophora tritici-repentis, Ustilago hordei, Aspergillus oryzae, Bacillus amyloliquefaciens, Bacillus subtilis, Mesorhizobium loti*, and *P. syringae* pv. *syringae* B728a (http://pedant.helmholtz-muenchen.de/genomes.jsp?category=al). Compared to other fungi, *P. indica* harbors only a few RXLR and CRN proteins, and one might speculate that the greater number of DELD proteins in *P. indica* might be compensatory. Whether this is related to the wide host range of the endophyte,[95] requires further investigations.

The function of DELD motif-containing proteins is unknown. Based on the conserved sequence motif in proteins which otherwise lack obvious sequence similarities, it is reasonable to assume that the motif has a physiological or biochemical function. Its potential involvement in the translocation into the host cells as well as its functional role can be easily investigated with the available molecular tools.

LysM effectors

The lysin motif (LysM) was originally described as a small protein domain and it was often found in repeats of lytic enzymes from bacteria and bacteriophages (such as *Bacillus phage* f29 and *Streptococcus* (*Enterococcus*) *faecalis*[197-200]), but not in archaeal proteins.[201] LysM motifs are carbohydrate-binding domains found in almost all life kingdoms. They bind N-acetylglucosamine (GlcNAc)-containing carbohydrates, such as chitin, chitiooligosaccharides (including lipochitiooligosaccharides) and peptidoglycan.[201-203] In various fungi, LysM motifs occur predominantly in subgroup C chitinases[204,205] and LysM effector proteins.[206] Their occurrence supports the fungal life.[207]

Fungal LysM effector proteins have no catalytic domains, and in enzymes, LysM motifs mediate the attachment to insoluble carbon sources. Proteins with LysM motifs also occur in plant species, such as NFR1 and NFR5 in *Lotus japonicus*,[208,209] LYK3 and LYK4 in *M. truncatula*,[210] and LYM1, LYM2 and LYM3 in *A. thaliana*.[211] Although receptor-like kinases with LysM motifs are under evolutionary constraints, the motif is conserved in life kingdoms.[212,213]

LysM motifs have 40–65 amino acids located in C-terminal regions and show a βααβ secondary structure, with the two α-helices packed on the same side of the two-stranded antiparallel β-sheet.[201,206,214] Some proteins contain also more than one LysM motif, for example, 2 in Blys5 and 5 in Blys2 of *Beauveria bassiana*,[215] 3 in Ecp6 of *C. fulvum*,[18] 6 in a peptidoglycan hydrolase (NCBI: AAO80613) of *Enterococcus faecalis*,[201] 7 in TAL6 from *Trichoderma atroviride*[216] and 12 in a chitinase (NCBI: O16237) of *Caenorhabditis elegans*.[201] These LysM motifs are often separated by Ser-, Pro-, or Thr-rich regions that can form a flexible region between the conserved LysM motifs.[217,218] Proteins often contain tandem LysMs that can assemble into quaternary structures.[219]

GlcNAc-containing carbohydrates (such as chitin, chitiooligosaccharides or peptidoglycans) derive from the cell walls of bacteria, oomycetes, and fungi and act as signal molecules in the crosstalk between microbes and host plants. Therefore, LysM motifs play an important role in masking the presence of fungi, oomycetes or bacteria. For instance, the *C. fulvum* effector protein Ecp6 with three LysM motifs binds chitooligosaccharides released from the fungal cell wall by plant chitinases, thereby preventing a chitin-triggered immune response in host plants.[19] Similarly, Mg3LysM from *Mycosphaerella graminicola* and Slp1

from *M. oryzae* inhibit immune responses in wheat and rice, respectively.[22,23] Ecp6 possesses a high affinity for chitooligosaccharides of various lengths, whereas Avr4, a chitin-binding lectin from *C. fulvum*, does not contain LysM motifs, and binds preferably to polymeric chitin. This results in the protection of fungal hyphae against chitinases, in contrast to Ecp6 which does not exhibit this property.[19] An additional lineage-specific LysM effector is encoded in the genome of *Verticillium dahliae* strain VdLs17, but not in any other *V. dahliae* strain. This LysM effector binds chitin, is able to suppress chitin-induced immune responses and protects fungal hyphae against hydrolysis by plant hydrolytic enzymes.[220]

Some LysM effectors possess distinct features. Eleven LysM effector proteins in *Penicillium expansum* possess signal peptides,[221] suggesting that they secreted by the fungus. Blys2 and Blys5 from *B. bassiana* binds chitin, Blys5 chitin, chitosan and cellulose, and both effectors are required for full fungal virulence.[215] The LysM protein TAL6 from *T. atroviride* binds different polymeric chitins, but not chito-oligosaccharides.[216] Further, TAL6 was shown to specifically inhibit germination of *Trichoderma* spp., but interestingly not of other fungi. Therefore, the results suggest that fungal LysM proteins are also involved in the self-regulation of fungal growth and development.[216]

Besides suppressing chitin-triggered immunity, some LysM effectors also control appressoria formation. ChELP1 and ChELP2 from *Colletotrichum higginsianum* are transcriptionally activated during the initial intracellular biotrophic phase of infection.[222] Further investigation showed that ChELP2 was concentrated on the surface of bulbous biotrophic hyphae at the interface with living host cells but is absent from filamentous necrotrophic hyphae. Both proteins suppress the chitin-triggered activation of two immune-related MAPKs in the host Arabidopsis. ChELP1 and ChELP2 are essential for fungal virulence and appressorium-mediated penetration of both Arabidopsis epidermal cells and cellophane membranes *in vitro*.[222] These results confirm the dual role of these LysM proteins.

LysM effector proteins also play an important role in the symbiosis between fungi and host plants. The endophytic fungus *P. indica* possesses some LysM motif-containing effector proteins, such as PIIN_00867, PIIN_08721, and PIIN_08723.[56] PIIN_00867 and PIIN_08721 contain signal peptides but no transmembrane regions or other known structural domains (www.ncbi.nlm.nih.gov), and PIIN_08723 has none of these domains (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t65672_Pir_indic_v2). The involvement of *P. indica* or mycorrhizal LysM proteins in symbiosis has not yet been demonstrated. Therefore, two key questions should be in the main focus: (1) Which plant components in or at the plasma membrane sense and bind the fungal LysM effector proteins? (2) Since the activated complexes initiate rapid $[Ca^{2+}]_{cyt}$ spiking responses in the host cells, how are they translated into appropriate host cell responses?

CFEM effectors

CFEM (common in several fungal-specific eight-Cys-containing domain of extracellular membrane proteins) motifs contain eight conserved cysteine residues[223,224] with the consensus sequence PxC[A/G] x_2Cx_8-$12Cx_{1\text{-}3}$[x/T]$D_{x2\text{-}5}CxCx_{9\text{-}14}Cx_{3\text{-}4}$ $Cx_{15\text{-}16}$ (where x is any residue, and its range is indicated[224]). This conserved consensus sequence is unique to fungi.[224] The lengths and the location of the cysteine residues in the CFEM motifs are similar to those of epidermal growth factor (EGF)-like domains which function as extracellular receptors or sensors.[223,225] CFEM motifs are found in fungal proteins with proposed roles in pathogenicity, such as from *Melampsora lini*,[226] *Aspergillus fumigatus*,[227] *Microbotryum lychnidis-dioicae* [228] and *M. silenes-dioicae*,[229] *Fusarium oxysporum*,[230] *Sclerotinia sclerotiorum*,[231] *Mycosphaerella fijiensis*,[232] *Botrytis cinerea*[233] and *Magnaporthe grisea*.[234] Some plant species, such as *Populus* spp., *Sorghum bicolor* and *Zea mays* contains also proteins with CFEM motifs.[223,224]

In the genome of *M. grisea* (http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/), at least eight CFEM-containing proteins have been identified, including ACI1 and Pth11.[16,234] ACI1, an adenylate cyclase (MAC1)-interacting protein, may play a role in the infection by *M. grisea*, because MAC1 controls appressorium formation, growth, and development by interacting with ACI1 which results in the production of cAMP, a key regulator of appressorium development.[235] Pth11, an important G-protein-coupled receptor located in plasma membrane mediates appressorium differentiation and formation as well as pathogenicity.[234,236] Kou et al. investigated the role of the CFEM motif in the putative surface sensing/response function of Pth11. They showed that increased/constitutive expression of CFEM resulted in precocious, albeit defective, appressorium formation in wild-type *M. oryzae*. The Pth11C63A/C65A mutant, probably with disrupted disulfide bonds in the CFEM, showed delayed appressorium formation and reduced virulence.[234]

The CFEM motif-containing protein BcCFEM1 from *B. cinerea*.[233] is significantly up-regulated during an early stage of *Phaseolus vulgaris* leaf infection and induces chlorosis in *N. benthamiana* leaves after *Agrobacterium* infiltration. Targeted deletion of BcCFEM1 in *B. cinerea* affected virulence, conidial production and stress tolerance, but not growth rate, conidial germination, colony morphology, and sclerotial formation.[233] The protein has a putative GPI-anchored site at the C-terminal region, thus it might not be secreted and translocated into host plant cells. Therefore, its function should occur at the early stage of infection by *B. cinerea*. CgCcw14, a cell wall structural protein with CFEM motif, is essential for the maintenance of the intracellular iron content, adherence to epithelial cells and virulence of *Candida glabrata*.[237] However, not all CFEM proteins participate in fungal virulence. An example provides the three CFEM-motif GPI-anchored proteins $Cfm_{A\text{-}C}$ from *Aspergillus fumigatus*, which affect cell wall stability, but not fungal virulence.[227]

The *P. indica* genome habors three proteins with CFEM motifs (i.e., PIIN_03540, PIIN_05622, and PIIN_08499). PIIN_03540 has no signal peptide and transmembrane region, PIIN_05622 has a signal peptide and no transmembrane region, and PIIN_08499 has a signal anchor and one transmembrane region. Thus, PIIN_08499 appears to be an integral membrane protein. When hyphae of *P. indica* invade into a plant cell, the hyphal tip forms a small inconspicuous appressorium on the surface of the outer wall of the rhizoids or root cells.[238] Since the

role of appressoria for *P. indica* colonization is not clear, the investigation of the role of the three CFEM proteins might be an interesting tool for further investigation of the root colonization process by the endophyte.

The MiSSPs (mycorrhiza-induced small secreted proteins) of the ectomycorrhizal fungus *L. bicolor* contain a family of secreted proteins with CFEM motifs, such as Lac310796, Lac296573, and Lac296572.[16] Based on the predicted signal peptides, the proteins could be translocated into host plant cells. Further investigations are important to understand their functions. Interestingly, the protein HESP178 (haustorially expressed secreted proteins) from *M. lini* possesses a CFEM motif.[16] Although its function has not been investigated yet, the identification of the protein suggests a function in haustorium formation during pathogen infection.[37] The described examples highlight that future research on CFEM-containing proteins may unravel novel processes in appressorium/haustorium formation, which is important for microbial colonization and pathogenicity.

Effectors with other conversed motifs

Several effector proteins contain other conserved motifs. For instance, the eukaryotic transcriptional repressor motif EAR (ethylene-responsive element binding factor-associated amphiphilic repression) is involved in the recruitment of transcriptional co-repressors.[239-242] This motif is also present in the effector protein PopP2 from *Ralstonia solanacearum* and required for avirulence and stability of PopP2.[243]

A strong candidate for convergent evolution amongst intracellular non-necrotrophic fungi is the degenerate Y/F/WxC motif. Such a motif is present in the N-terminal regions of effector candidates from barley powdery mildew (*Blumeria graminis*), wheat stem rust (*Puccinia graminis*) and the wheat leaf rust fungi (*Puccinia triticina*).[244-246] Also, these fungi harbor 107, 178 and 57 such Y/F/WXC-proteins, respectively,[244] their detailed functions in haustorium formation and virulence is still unclear.

The effector protein AvrLm6 contains an RYWT motif in the N-terminal region which is required for effector-GFP accumulation in root cells and re-entry in bombardment assays. The related RIYER motif in the effector protein Avr2 showed a similar function.[73] However, the ubiquity of the two motifs among oomycetes and fungi is not clear.

CHXC motifs were identified in the N-terminal regions of a class of predicted secreted proteins from the obligate oomycete pathogen *Albugo laibachii*.[87] This motif has functional similarities to the RXLR motif. For instance, the N-terminal domain of CHXC9 carries the C-terminus of *P. infestans* Avr3a into *N. benthamiana* cells when expressed in *P. capsici*, and mutation of the CHXC motif to AAAA eliminated most of the translocation activity.[87] Interestingly, two conserved sequences, KYLG and RLYW, lie very close to the CHXC motif in CHXC9: it will be interesting to investigate whether either of them contribute to CHXC9 translocation process, just like the EER motifs which are located downstream of the RXLR motifs. So far, no reports show CHXC motif functions in other oomycetes and fungi, although CHXC motifs were identified in the N-terminal regions of several small fungal proteins, such as RCO7_07428, RCO7_08487 and RCO7_09455 in *Rhynchosporium commune*; SCHCODRAFT_37592, SCHCODRAFT_31636 and SCHCODRAFT_34289 in *Schizophyllum commune* H4-8; TRIVIDRAFT_55621, TRIVIDRAFT_60504 and TRIVIDRAFT_51004 in *Trichoderma virens* (http://pedant.helmholtz-muenchen.de/genomes.jsp?category= fungal).

All 21 flax (*Linum usitatissimum*) rust HESPs have no RXLR motifs. Since they enter the host cells, the translocation mechanism must be different. The N-terminal regions of AvrL567 (GYTR), AvrM (GFLR) and AvrP4 (GFSR) and the C-terminal region of AvrP123 (GIAR) contain the conserved consensus sequence Gx1x2R (x1 is a hydrophobic or aromatic amino acid, x2?) which are common to these avirulence proteins.[226] Mutations of the motif did not affect the recognition of secreted or non-secreted versions of AvrM or AvrP4 *in planta*.[226] A hydrophobic surface patch in AvrH is required for internalization into plant cells, whereas the C-terminal coiled-coil domain mediates interaction with the resistance protein M in flax.[247] Likewise, the *AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells.[248] It has been proposed that the proteins enter the host cells using the hydrophobic surface patch of the Gx1x2R amino acid region, similar to AvrH.[226]

Conserved motifs have also been reported for effector proteins other species, such as [L/I]XAR in *M. oryzae*,[249] YXSL[R/K] in a family of putative secreted effectors from *Pythium ultimum*,[92] [R/K]VY[L/I]R from *Blumeria graminis*[250] and [SG]-P-C-[KR]-P in various *Fusarium* effector proteins.[40] However, none of these motifs have been functionally characterized yet.

Concluding remarks and future prospective

Pathogenicity and mutualism in plant/fungus interactions depend on the functions of multifarious effector proteins. Some of them are translocated into host plant cells using different mechanisms. During the translocation, some conserved motifs, such as RXLR, CRN, and CFEM, are common to oomycetes and fungi. However, as outlined above, other entry mechanisms are also known. Understanding of the scenario will allow us to manipulate these processes, and opens promising avenues for disease control and mutualism. Effector proteins from pathogens target different host plant proteins, and independently evolved virulence effectors converge onto hubs in a plant immune system network.[251,252] The rapid increase in knowledge about plant innate immunity will help us to understand the function and targets of microbial effector proteins. In particular, more information is required about the structural basis for their recognition.[253-257] Bioinformatic tools will allow the identification of common structural features in effector proteins from different life kingdoms.[96] In addition, the effects of small RNAs in plant defense[36,129,130,258] are targets of effector proteins, and it would be interesting to know how they cooperate. Furthermore, most of the knowledge from effector function derive from pathosystems, while much less is known about their role in beneficial or mutualistic interactions. For instance, no effector protein has been identified in endophytic bacteria.[16,17,172,174]

Finally, elucidation of effector functions will be helpful for the identification of targets for resistance breeding strategies against biotrophic, hemibiotrophic and necrotrophic plant pathogens,[259] as well as for crop improvement. Besides resistance breeding, investigations on effector functions, especially so-called "core effectors", will greatly promote the development of new strategies for resistance breeding. Bart et al. used a genome sequencing strategy to search for conserved effector genes in the 65 bacterial pathogen strains of *Xanthomonas axonopodis* pv. *Manihotis* and found a set of conserved effectors (i.e., core effectors).[260] They could be used as ideal targets for developing resistance strategies. Dangl et al. reviewed the principle of core effectors and proposed an improved practice to breed durable resistance *via* genomic strategies by the identification of core effectors,[261] and Fawke et al. reviewed the infection strategies of pathogenic oomycetes and the major principles of host resistance.[262] Considering the enormous loss of *Capsicum annuum* caused by *P. capsici*, Barchenger et al. proposed novel breeding approaches and strategies, and carried out researches on race characterization of *Phytophthora* root rot on *Capsicum* as a basis for anticipatory resistance breeding.[263,264] It became clear that the identification of core effectors and their detailed functions are important future breeding tools to increase host resistance. Extensive collaborations between plant breeders and plant phythologists are important and necessary.

In nature, plants are surrounded by symbionts and pathogens, and might suffer from both types of microbes, in particular when they approach the plants simultaneously. Both symbionts and pathogens secrete effector proteins to facilitate hyphae entry into host cells. How do the host plants recognize the effectors from the friends and foes and which strategies do they use to defend pathogens and accept symbionts? The arms race between hosts and the microbes stimulated the evolution of effector proteins and shaped them; however, we are only at the beginning to understand how plants discriminate between effectors from pathogens and friendly symbionts.

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