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Pathogen virulence factors as molecular probes of basic plant cellular functions

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Summary

To successfully colonize plants, pathogens have evolved a myriad of virulence factors that allow them to manipulate host cellular pathways in order to gain entry into, multiply and move within, and eventually exit the host for a new infection cycle. In the past few years, substantial progress has been made in characterizing the host targets of viral and bacterial virulence factors, providing unique insights into basic plant cellular processes such as gene silencing, vesicle trafficking, hormone signaling, and innate immunity. Identification of the host targets of additional pathogen virulence factors promises to continue shedding light on fundamental cellular mechanisms in plants, thus enhancing our understanding of plant signaling, metabolism and cell biology.

Introduction

Nutrient-rich plant tissues provide one of the most important niches for survival and proliferation of microbes. Over time, plants have evolved a complex and multilayered immune system that is effective in warding off most microbial infections [1–3]. Nonetheless, numerous microbes - such as bacteria, fungi, oomycetes and viruses - have evolved the ability to cause disease in plants. To complete their infection cycles, pathogens need to evade or suppress host defenses and to manipulate host cellular functions to their advantage. This is achieved through a wide array of virulence strategies, relying on sophisticated molecular mechanisms that we are only beginning to understand.

Decades of plant pathology studies have uncovered a remarkable assortment of proteins and toxins used as virulence factors by plant pathogens. Gram-negative bacterial pathogens such as *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas* and *Erwinia spp.* use the conserved type III secretion system (TTSS) to translocate virulence-mediating “effector” proteins into the host cell [4]. TTSS effectors collectively participate in causing disease [5–8]. *Agrobacterium tumefaciens*, the causal agent of crown gall disease, injects into the plant cell a fragment of its plasmid DNA (T-DNA) and several virulence (Vir) proteins via a type IV secretion system (TFSS) [9]. The T-DNA, encoding genes necessary for tumor induction and disease development, is imported into the plant cell nucleus and integrated in the host genome, where these genes are expressed [9]. Bacterial as well as fungal pathogens of plants also produce a variety of phytotoxins, which are often key determinants of pathogenicity (the ability to cause disease) or virulence (the degree of pathogenicity) [10–12]. Viruses, being obligate parasites, depend entirely on the host cell. They utilize the plant machinery for their nucleic

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acid and protein synthesis and they take advantage of the plant's transport system to spread, locally through plasmodesmata and systemically through the phloem [13].

Elucidating the mechanisms by which pathogens suppress host immunity and exploit eukaryotic processes/pathways to promote disease is currently a principal objective in molecular plant pathology. Not surprisingly, most of the host processes known to be targeted by pathogen virulence factors are, directly or indirectly, involved in plant immune responses. However, investigating the molecular bases of plant-pathogen interactions often uncovers novel aspects of plant cell biology and signaling mechanisms. In this review, we highlight several pathogen virulence factors for which cellular targets in the plant host cell have been recently identified. We also speculate on how investigation of such host targets might augment our understanding of fundamental cellular processes in plants.

Post-transcriptional gene silencing (PTGS) and viral effectors

RNA silencing, an important mechanism for gene regulation in plants, is also critical for establishing innate immunity against viral and bacterial infection [14,15**]. RNA silencing is invariably initiated by double-stranded (ds) RNAs that are cleaved into 21- to 24-nucleotide short interfering (si) RNA duplexes by Dicer-like RNases (DCLs). The siRNAs are subsequently incorporated into an RNA-induced silencing complex (RISC), which includes the Argonaute (AGO) enzyme, ultimately responsible for siRNA-guided degradation of specific target RNA substrates, including viral RNAs [14].

To circumvent the RNA interference (RNAi)-based host defense mechanism, successful viral pathogens employ proteinaceous virulence effectors known as suppressors of silencing. These viral suppressors target the core components of the plant RNA-silencing machinery. *Turnip crinkle virus* P38 capsid protein, for instance, suppresses silencing by targeting primarily DCL4 and, in the absence of DCL4, DCL2 activity [16**]. Several structurally unrelated viral suppressors of silencing, such as p19 of tombusviruses, p21 of closteroviruses and HC-Pro of potyviruses bind to and sequester double-stranded siRNA molecules, thus preventing assembly of the RISC [17**]. *Cucumber mosaic virus* 2b protein directly interacts with Arabidopsis AGO1 *in vivo* and *in vitro*. This interaction specifically inhibits AGO1 cleavage activity in assembled RISCs [18**]. In short, the current evidence suggests that viral suppressors of silencing act via direct protein-protein or protein-RNA interactions to inhibit the plant RNA-silencing process. Although all the host targets identified so far are known components of the RNA-silencing machinery, characterization of additional viral suppressors may unveil novel components involved in the regulation or execution of RNA silencing.

MAPK signal transduction and bacterial effectors

In the plant immune response, perception of pathogen-associated molecular patterns (PAMPs) such as flg22 (a peptide derived from bacterial flagellin) by plasma membrane-localized receptors rapidly activates a signal transduction cascade, involving the MPK3 and MPK6 mitogen-activated protein (MAP) kinases [19]. Recent studies show that this cascade is inhibited by several bacterial TTSS effectors that have virulence functions. For example, the *P. syringae* effector AvrPto had been shown previously to suppress plant basal defense [20]. He *et al.* [21] recently showed that AvrPto and a functionally related effector, AvrPtoB [22], inhibit the MAP kinase signaling cascade by blocking the activation of MPK3 and MPK6 in Arabidopsis cells. Both effectors appear to act, by mechanisms not yet understood, upstream of MAPKKK [21].

Another *P. syringae* TTSS effector, HopAI1, belongs to a newly characterized family of bacterial virulence factors acting as phosphothreonine lyases, which remove the phosphate group from phosphothreonine to inactivate MAP kinases [23]. HopAI1 was recently shown to

directly interact with *Arabidopsis* MPK3 and MPK6 [24**]. Transgenic overexpression of HopAI1 in *Arabidopsis* suppresses endogenous MPK3 and MPK6 activation by flg22 and dampens PAMP-triggered immune response [24**].

Besides playing an important role in plant immune response, MPK3 and MPK6 also participate in other plant cellular processes such as stomatal differentiation and abiotic stress response [25,26]. MPK3 and MPK6 appear to perform overlapping functions in *Arabidopsis*. Simultaneous mutation of MPK3 and MPK6 is embryo-lethal [25], presenting a challenge to rigorous genetic analysis of the biological roles of these kinases throughout the plant developmental cycle. Further elucidation of the mechanisms and specificities by which HopAI1, AvrPto, and AvrPtoB inhibit the MAPK cascade may lead to alternative methods of studying the function of MPKs through conditional and/or cell-type-specific expression of these effectors.

Cellular trafficking and viral and bacterial effectors

Inter- and intra-cellular trafficking of macromolecules are fundamental processes in plants. Viruses are well known for manipulating host cell functions for cell-to-cell and long-distance trafficking [13,27,28]. For instance, virus-encoded movement proteins (MPs) facilitate the passage of viruses through plasmodesmata [28]. Plasmodesmata also control the movement of important endogenous signaling molecules [29], many of which are RNAs and/or proteins, including the flowering-induction signal florigen [30,31]. It is not clear exactly how MPs promote the movement of viruses across plasmodesmata; however, elucidating how MPs modulate the plasmodesmatal channel is likely to contribute to our understanding of the transport mechanisms across these unique plant intercellular gateways.

Increasing evidence indicates that the intracellular vesicle trafficking and polarized secretion pathways are important for plant immunity against fungal and bacterial pathogens [32–36] and that pathogen virulence factors may be targeting intracellular trafficking to suppress host immunity [37**]. For example, the *P. syringae* effector protein HopM1 was recently shown to target AtMIN7, one of the eight guanine nucleotide exchange factor (GEF) proteins that activate ARF GTPases in *Arabidopsis* [37**]. HopM1 physically interacts with AtMIN7 and mediates its degradation through the 26S proteasome. Importantly, *atmin7* mutant plants are compromised in host immunity and are more susceptible than wild-type *Arabidopsis* to a bacterial mutant lacking HopM1 [37**].

ARF-GEF proteins are important regulators of intracellular vesicle traffic in eukaryotic cells, although little is known about functional specificity of the various family members. The best-characterized plant ARF-GEF is GNOM, which controls polarized subcellular localization of the auxin efflux transporter PIN1 protein [38]. *P. syringae* HopM1 targets AtMIN7, but not GNOM, suggesting a certain level of specificity [37**]. However, various HopM1 alleles from *P. syringae* strains may exhibit different ranges of specificity towards ARF-GEFs. If so, HopM1 alleles may be engineered for use as potential inhibitors of various ARF-GEF proteins, enabling the study of different vesicle traffic pathways in plants.

Nuclear gene transcription and bacterial transcription-activator-like (TAL) effectors

Large-scale changes in host gene expression associated with susceptibility to bacterial pathogens have been explored in the past few years [39–41]. Aside from global changes in the plant transcriptome during pathogen infection, changes in expression of several host genes are found to be specifically associated with certain bacterial effectors. For instance, the TAL effectors AvrBs3 and AvrXa27 of the bacterial pathogen *Xanthomonas* carry eukaryotic

nuclear localization signals, are translocated into the host nucleus and are responsible for induction of target host genes [42,43]. In particular, Yang *et al.* [44*] reported that expression of the *Os8N3* gene of rice is highly and specifically up-regulated by PthXo1, a TAL effector of the rice pathogen *X. oryzae* pv. *oryzae*. *Os8N3* and *pthXo1* are both required for disease compatibility, providing the first example of a strain-specific host susceptibility gene that is transcriptionally regulated in response to an individual virulence effector [44*]. Future research should determine whether *PthXo1* and other TAL effectors directly target the promoter regions of the cognate host target genes. If so, elucidating how these bacterial effectors interact with the promoter elements, and/or exploit the host transcription machinery, may lead to new insight into transcriptional regulation mechanisms in plants.

Plant RNA-binding proteins and bacterial mono-ADP-ribosyltransferase (ADP-RT) effectors

A novel virulence mechanism was recently identified for the *P. syringae* effector HopU1, which acts as an ADP-RT on plant protein substrates [45**]. While numerous animal pathogens were known to utilize ADP-RTs to activate or inactivate host pathways to their advantage [46], this enzymatic activity had not been described yet in either a plant pathogen, or the plant cell. HopU1 targets glycine-rich RNA-binding proteins (GR-RBPs), such as AtGRP7 [45**]. As a group, GR-RBPs are poorly understood in terms of RNA-binding specificity and biological functions in plants. Therefore, further study of HopU1 and its target GR-RBPs is likely to provide new information about the function of this family of plant proteins in host immunity and other basic cellular processes.

26S Proteasome-mediated degradation and bacterial and viral virulence factors

A major mechanism of targeted proteolysis in eukaryotic cells is ubiquitin-mediated degradation via the 26S proteasome, essential for development as well as for stress responses. Proteins to be degraded via this pathway are typically ubiquitinated by specific E3 ligases and subsequently degraded by the proteasome. An increasing number of pathogen virulence factors appear to exploit the host cell ubiquitin/proteasome pathway to promote disease by removing specific host proteins [47]. Several bacterial and viral effector proteins contain an F-box motif, typical of certain components of eukaryotic E3 ubiquitin ligases: among these are VirF of *A. tumefaciens* [48], the silencing suppressor protein P0 of poleroviruses [49], and seven related TTSS effectors (GALA effectors) of *R. solanacearum* [50*]. VirF targets the host protein VIP1 for proteasome-dependent degradation. VIP1 is a basic leucine zipper motif-containing protein required for nuclear import and genomic integration of the T-DNA. VirF-mediated degradation of VIP1 is proposed to be involved in the uncoating of the T-DNA complex prior to or during T-DNA integration into the host genome [48]. The specific substrate proteins of the polerovirus P0 suppressor and of *R. solanacearum* GALA effectors remain to be identified.

Besides the ability to suppress the activation of the MAPK cascade in Arabidopsis, the N-terminal portion of the *P. syringae* effector AvrPtoB triggers host immunity in specific tomato genotypes that contain the Fen protein kinase. Interestingly, the C-terminal portion of AvrPtoB acts as a ubiquitin ligase on Fen, targeting it for proteasome-mediated degradation and effectively eliminating the N-terminus-mediated host immunity [51,52]. The intramolecular “battles” of the AvrPtoB effector presumably reflects the evolutionary race between tomato and *P. syringae*, manifested on the same effector protein. Analogous “battles” between effectors and host targets were previously described for the *P. syringae* effectors AvrRpt2 and AvrRpm1 and their common target protein RIN4 in Arabidopsis (reviewed in [8]).

Not only proteinaceous effectors act on the host ubiquitin/proteasome pathway, but also plant pathogen-derived toxins can exploit the activity of E3 ubiquitin ligases, to promote disease. The most notable example is coronatine (COR), which is produced by several pathovars of *P. syringae* [11]. In Arabidopsis, COR promotes stomatal opening [53], enhances bacterial multiplication and symptom development in infected leaves [54–56], and induces susceptibility to *P. syringae* throughout the infected plant [57]. COR bears a striking structural similarity to the plant hormone jasmonate (JA), especially the jasmonoyl-isoleucine (JA-Ile) conjugate, which is involved in many aspects of plant biology. The action of COR and JA is dependent on the F-box protein COI1 [58]. Recent results suggest that a COI1-substrate (e.g., JAZ1) complex is a possible receptor site for JA-Ile [59,60]. Thus, by mimicking JA-Ile, COR may directly target the COI1-specific E3 ubiquitin ligase complex to perform its virulence functions.

Interestingly, phytopathogenic bacteria also target SUMO (small ubiquitin-related modifier)-mediated processes. Specifically, the XopD effector of *Xanthomonas campestris* exhibits an isopeptidase activity that reduces the amount of SUMO protein conjugates [61].

In the future, it will be interesting to identify the host targets of P0 viral suppressor, GALA effectors and XopD, to gain further insights into gene silencing, SUMOylation, and possibly other cellular processes targeted by these particular effectors. Further study of how COR manipulates COI1 activity should enhance our understanding of the JA-signaling pathway in plant development and defense response. Finally, HopM1 is a novel protein which likely manipulates the plant ubiquitination/proteasome in a unique manner [37**]. Further study of HopM1-mediated degradation of the AtMIN7 ARF-GEF should improve our knowledge of vesicle trafficking, as discussed above, and provide insight into a potentially novel mechanism of regulation of the plant ubiquitin/proteasome pathway.

Chloroplast function and bacterial and fungal effectors

The *P. syringae* effector HopI1, when expressed in the plant cell, is targeted to the chloroplast, where it induces remodeling of the thylakoid structure and suppresses salicylic acid accumulation [62*]. HopI1 is characterized by a C-terminal J domain, necessary for virulence activity and thylakoid remodeling. J domains typically mediate interaction with 70-kDa heat-shock proteins (Hsp70s), suggesting that chloroplast Hsp70 may be the target of the HopI1 virulence function [62*]. Interestingly, HopI1-expressing plants have increased heat tolerance.

A fungal virulence protein, ToxA, produced by the wheat pathogen *Pyrenophora tritici-repentis*, also appears to target chloroplast function. ToxA is internalized in mesophyll cells of sensitive, but not insensitive, wheat cultivars and localizes to the cytosol and the chloroplasts [63]. A yeast two-hybrid screen was used to identify a ToxA-interacting novel chloroplast protein, ToxABP1 [64], a homologue of which is necessary for thylakoid membrane organization in Arabidopsis [65].

Further investigation of the virulence activities of HopI1 and ToxA may help our understanding of chloroplast-based heat stress response, chloroplast structure and membrane dynamics.

Concluding remarks

Study of plant-pathogen interactions has a history of providing not only useful insights into pathogenesis and other plant cellular mechanisms, but also precious experimental tools. Several research technologies of outstanding importance for the study of plant biology make use of plant pathogens and/or their virulence mechanisms. For example, *Agrobacterium*-mediated gene expression has become established in the past two decades as the tool of choice for genetic transformation of plants [66]. Viral suppressors of RNA silencing are commonly used to enhance transgene expression in transient assays [67]. Virus-induced gene silencing (VIGS)

has emerged as a powerful technique for transient knock-down of one or multiple genes *in planta* [68–70]. By highlighting several recent studies describing the host targets of various plant pathogen virulence factors, we hope that this review will convey a sense of continuing excitement in the field of molecular plant pathology. Study of the molecular action of pathogen virulence factors should continue to contribute to our knowledge of fundamental plant cellular processes and may generate additional breakthrough technologies useful for plant biology research.

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Figure 1. Virulence factors of diverse plant pathogens act inside the plant cell by manipulating a variety of host pathways

Bacterial (red, and brown for *Agrobacterium*), fungal (purple) and viral (green) virulence factors are depicted in this diagram along with the host cellular targets (yellow). The bacterial effector HopI1 and the fungal virulence protein ToxA are targeted to the chloroplast. HopU1 of *P. syringae* targets plant GR-RBPs, which are involved in RNA metabolism. Several *Xanthomonas* effectors localize in the plant cell nucleus: XopD, which affects SUMOylated proteins stability [71], and the PthXo1 and AvrBs3 effectors, which stimulate transcription of specific host genes. AvrPphB acts as a protease on the host substrate PBS1 [72]. AvrPto, AvrPtoB and HopAI1 interfere with signal transduction initiated by the flagellin receptor FLS2. *P. syringae* AvrRpt2, AvrB and AvrRpm1 target RIN4, a negative regulator of plant defense [73,74]. A variety of pathogen effectors (AvrPtoB, VirF, HopM1 and GALA effectors of bacterial origin, and viral P0) use the plant proteasome to promote degradation of host proteins involved in immunity and other cellular functions. Viral movement proteins (MPs) participate in viral particles movement between cells. Several viral suppressors of RNA silencing (P38, P19, P21, HC-Pro, 2b) directly target various components of the plant's RNA silencing machinery.