

# Effector biology during biotrophic invasion of plant cells

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Several obligate biotrophic phytopathogens, namely oomycetes and fungi, invade and feed on living plant cells through specialized structures known as haustoria. Deploying an arsenal of secreted proteins called effectors, these pathogens balance their parasitic propagation by subverting plant immunity without sacrificing host cells. Such secreted proteins, which are thought to be delivered by haustoria, conceivably reprogram host cells and instigate structural modifications, in addition to the modulation of various cellular processes. As effectors represent tools to assist disease resistance breeding, this short review provides a bird's eye view on the relationship between the virulence function of effectors and their subcellular localization in host cells.

## Introduction

Being sessile organisms, plants are constantly challenged by their environment, and their situation is compounded by biotic stresses. A number of plant pathogens, such as fungi, oomycetes, bacteria, viruses, nematodes, etc., pose serious threats to the plant well-being. Nonetheless, over the course of evolution, plants have acquired a refined, two-layered immune system to respond to pathogen attack.<sup>1</sup> The first line of plant immunity, thought to be the most ancient, relies on the recognition of evolutionarily-conserved pathogen molecules known as PAMPs (pathogen-associated molecular patterns), and is therefore referred to as PAMP-triggered immunity (PTI).<sup>2–4</sup> Pattern recognition receptors (PRRs) are plant components responsible for the detection of PAMPs<sup>5</sup> and for activating the immune machinery of plants. One of the best characterized PRRs in plants is FLAGELLIN SENSITIVE 2 (FLS2), a receptor kinase that activates PTI upon perception of flagellin, a conserved protein found in bacterial flagellum.<sup>6,7</sup>

To gain greater access to plant resources for subsequent colonization, plant pathogens, just like their animal equivalents, deploy an arsenal of highly-sophisticated molecules known as effectors. These molecules greatly augment the pathogen's capacity to propagate on its host by interfering with various cellular

processes, including PTI. Fortunately, plants monitor the presence of some effectors through their resistance (R)-proteins, which constitutes the second line of defense, also known as effector-triggered immunity (ETI).<sup>1</sup> ETI typically results in a strong hypersensitive response, characterized by cell death, which shows some mechanistical similarities with apoptosis in animals.<sup>8</sup> It is regulated by direct physical interaction between a R-protein and its corresponding effector (ligand-receptor model) or between a R-protein and a host-protein modified by an effector (guard model). Resistance thus depends on the presence of both the R-protein and its corresponding effector, a situation depicted by Flor's gene-for-gene model.<sup>9,10</sup>

For pathogens to succeed, proper delivery of these effectors is as crucial as the molecule itself. The bacterial type three secretion system (T3SS), one of many secretion systems deployed by *Pseudomonas syringae*, is well-characterized and has been studied in great detail. The syringe-like T3SS provides bacteria with a robust mechanical structure which enables it to inject key molecules involved in pathogenicity directly into host cells.<sup>11</sup> Obligate biotrophic, filamentous pathogens, such as many fungi and oomycetes, are devoid of such secretion systems. Instead, they invaginate within host cells to form particular infection structures called haustoria.<sup>12,13</sup> To accommodate haustoria, host cells are forced to greatly expand their plasma membrane, and it is plausible that pathogens drive this process for their own benefit.

Filamentous pathogens have a large suite of predicted, secreted proteins, which could act early during infection to suppress PTI as the pathogens are establishing themselves and, at later stages, to rewire host cellular activities to meet the pathogen's metabolic needs. It has been proposed that protein trafficking from haustoria allows pathogens to hijack host cells for their own purposes. However, the precise mechanism governing effector translocation from the extra-haustorial space to host cells has eluded scientists thus far.<sup>14</sup> For the purpose of this review, we have classified effectors into three types based on the subcellular compartment they target: apoplasmic effectors, cytoplasmic effectors and nuclear effectors. Apoplasmic effectors can be secreted by appressoria and/or hyphae invading the intercellular space where they remain outside the cells. This class of effectors includes proteins with inhibitory functions, interfering with plant proteases and peroxidases. For example, the Avr2 effector from the biotrophic fungal pathogen *Cladosporium fulvum* suppresses basal defense through inhibition of specific host proteases.<sup>15–17</sup> On the other side, cytoplasmic and nuclear effectors affect host defense mechanisms by

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targeting proteins involved in plant immune signaling cascades. Moreover, they also manipulate various plant processes, further predisposing the host cellular machinery to act in a pathogen-conducive manner.<sup>18,19</sup> As their names suggest, cytoplasmic effectors target cytosolic components or are redirected to other organelles, while nuclear effectors transit via the cytosol but have a different purpose than the other two effector types (described in subsequent sections). The biology of infection of obligate biotrophic pathogens is rather unique due to the establishment of haustoria. The different strategies deployed by intracellular biotrophic hyphae produced by various pathogens to secrete their effectors are beautifully illustrated by Giraldo and Valent.<sup>13</sup> In this mini-review, we offer a retrospective of the molecular interactions between obligate biotrophic pathogens and their hosts, speculating on this rather intimate relationship at the molecular level and focusing on cellular components representing potential effector targets.

### **Effector Terminology: Virulence/Avirulence Factors vs. Effectors**

It is pertinent to demystify the terminological ambiguity around effectors since, until recently, their nomenclature was contingent upon host reactions. When a molecule from a particular pathogen modulates the host's defensive cover to increase the pathogen's fitness, it is called a virulence factor. However, when the same molecule is recognized by host immunoreceptors, thereby failing to augment pathogenicity and instead triggering a defense response, it is referred to as an avirulence factor. This variation in pathogenicity is a commonly-occurring phenomenon. A particular effector may be a virulence factor on one host and an avirulence factor on another, a situation observed even within a single plant species where interactions are race-specific. Because of this inconsistency, terms such as virulence and avirulence have their limitations, since they are dependent on the specific host system in which they have been observed. The above discussed terminology in plant pathology is thus rather different from that employed in the medical field. In plant immunity, the terms virulence and avirulence are mainly related to the plant's ability to resist or succumb to the pathogen, thus depending on plant genotype.<sup>9</sup> In the medical field, avirulence refers to the loss of a virulence component belonging to the pathogen. Consequently, an inclusive and neutral term such as "effector" is preferred,<sup>20</sup> as it accounts for all the molecules secreted by a pathogen during infection that alter host cell structure or function.<sup>21</sup>

As mentioned earlier, Flor's work was instrumental in establishing the gene-for-gene concept.<sup>9,10</sup> Flor was quite foresighted when he noted that, for each gene conditioning a reaction in the host, there is a corresponding gene that conditions pathogenicity in the pathogen.<sup>9</sup> His deduction came from studies on the inheritance of pathogenicity in flax rust (*Melampsora lini*) and on the inheritance of resistance in flax (*Linum usitatissimum*).<sup>10</sup> Many years later, the flax/flax rust pathosystem remains instrumental in our understanding of the molecular aspects of gene-for-gene interactions. This pathosystem enabled inroads to be

made in the molecular interaction between R- and Avr-protein, mainly through studies of L and M resistance genes and their corresponding Avr loci. Flax rust *AvrL567* genes, whose products are recognized by the L5, L6, and L7 R-proteins of flax, are highly diverse and under diversifying selection pressure, with 12 sequence variants identified from six rust strains.<sup>22</sup> Ravensdale et al.<sup>23</sup> studied direct molecular interactions between L5 and L6 (two alleles of L) and their avirulence targets in detail. Site-directed mutagenesis in *AvrL567* and the construction of chimeric L-proteins revealed that the recognition specificities of L5 and L6 are conditioned by their leucine-rich repeat regions. Their study indicated that mutations in the TIR or NB-ARC domain also affect recognition, which prompted the authors to suggest that interaction with the Avr ligand directly competes with intramolecular interactions, causing R-protein activation.<sup>23</sup> The AvrM effector from flax rust also interacts directly with the flax R-protein M, and this interaction can also be observed in yeast two-hybrid assays. Catanzariti et al. showed that the C-terminal domain of AvrM is required for M-dependent cell-death, consistent with the fact that it interacts with M-protein in yeast.<sup>24</sup> Furthermore, these authors demonstrated that C-terminal 34 amino acids formed a structured domain (unlike the N-terminal part of the protein), and gel filtration revealed that AvrM-A can dimerize.<sup>22</sup> Recently Ve et al. resolved the structure of AvrM and AvrM-A and showed that both possess an L-shaped fold and form a dimer with an unusual nonglobular shape.<sup>25</sup>

The avirulence properties of AvrM and AvrL have been described, but yield no clues with regard to their targets and their potential virulence functions. Few rust effectors have been shown to be expressed during infection and translocated to host cells. One of these effectors is rust-transferred protein 1 (RTP1), which belongs to a family of effector proteins specific to the order Pucciniales.<sup>26</sup> RTP1 from *Uromyces fabae* was the first rust effector demonstrated to localize in host cells, and it was also observed that the transfer of the protein was dependent on the developmental stage of haustoria.<sup>27</sup> RTP1 translocates from the extra-haustorial matrix, where it first accumulates, transits through the cytoplasm, then further moves to the nucleus.<sup>27</sup> Unlike most localization studies cited herein, which are mainly based on green fluorescent protein (GFP) fusion and transient expression, RTP1 localization was assessed by immunolocalization during *Uromyces fabae* infection using four independently-raised polyclonal antibodies.<sup>27</sup> RTP1 sequence analyses indicated that the C-terminal domain exhibited similarities to cysteine protease inhibitors, and RTP1 was indeed shown to inhibit proteolytic activity.<sup>26</sup>

### **Effector Type, Localization, and Function**

When dealing with a subject as broad as effectors, it is worthwhile to classify them to the extent that current knowledge in this domain will allow. Therefore, in an attempt to draw clear lines, they can be largely divided into three major groups based on their localization and site of activity: apoplastic, cytoplasmic and nuclear/nucleolar effectors.

As the name suggests, apoplastic effectors are localized to plant extracellular spaces. This class of effectors includes, but is not restricted to, small and cysteine-rich proteins which function primarily by inhibiting host proteases, hydrolases, glucanases, and other lytic enzymes.<sup>13</sup> Recent models suggest that these could be the first effectors to potentially activate the plant defense response (PTI).<sup>13</sup> The architecture of these effectors, often having a signal peptide and a cysteine-rich C-terminus, is highly reminiscent of plant small signaling peptides,<sup>28</sup> which may reflect the prototypic structure that a protein must harbor to survive its passage in apoplastic space. However, apoplastic effectors may have a much more refined mechanism and could exert a long-lasting action in protection of the pathogen cell wall or in chelating/neutralizing antimicrobial compounds being secreted by the host.

On the other hand, cytoplasmic effectors have the duty of dealing with host cells at a much more intricate level. Cytoplasmic effectors are active once they reach the plant cytoplasm and tend to target plant defense signaling components. Effectors from *P. syringae* have been shown to target anti-pathogenic vesicle trafficking and kinase-based recognition activity of the host, a prime defense component.<sup>29</sup> Some effectors may also transit through the cytoplasm to reach their final destination (e.g., organelles).

Nuclear effectors are seemingly ultimate weapons in the inventory of pathogens, since they are thought to suppress the immune response from upstream. Nuclear effectors could potentially shut off master switches of the immune machinery or reprogram host transcription to the benefit of pathogens. A recent investigation of 49 putative effectors from *H. arabidopsidis* revealed that 33% localized strictly to the nucleus, and an additional 33% were nucleo-cytoplasmic.<sup>30</sup> Since several effectors tend to migrate toward the nucleus, it would be logical to assume that some R-proteins act in the nucleus. Indeed, several R-proteins, such as SNC1, N and RPS4, were found to localize to the nucleus.<sup>31-34</sup> Tobacco TIR-NB-LRR R-protein N localizes to the nucleus in the absence of its elicitor, the *Tobacco mosaic virus* p50 helicase fragment,<sup>32</sup> lending support to a default presence of R-proteins in the nucleus to monitor their corresponding effectors rather than being relocalized upon effector binding. However, SNC1 and N nuclear accumulation is reduced at elevated temperatures, making their mode of action temperature-dependent.<sup>35</sup> It was demonstrated recently that ETI is more active at low temperatures (10–23 °C), while PTI takes over at higher temperatures (23–32 °C).<sup>36</sup> It has also been shown that bacterial pathogens strive and multiply at higher temperatures but secrete their effectors more actively at lower temperatures.<sup>37,38</sup> These observations suggest that the immune system of plants is adapted to pathogen physiology. However, some pathogens prefer more temperate environments (around 18 °C) for optimal growth.<sup>39,40</sup>

### Nucleolar-Localized Effectors

Computer software, such as NOD, PSORT II, and WoLF PSORT, can predict the subcellular localization of various proteins, but that of very few candidate effectors has been verified

experimentally<sup>41-43</sup> relative to the wealth of those from all plant pathogens. A number of plant pathogen-secreted effector proteins have been reported to localize in the nucleus, but most localization studies have been conducted with GFP-tagged assays. It should be noted that GFP fusion may abrogate proper effector localization, either by hiding a sorting signal or by inducing change in the 3D structure of native effectors which could prevent interaction with a protein involved in true effector localization. In addition, most of these experiments are transient assays and do not examine localization during infection. Therefore, although GFP represents a very powerful tool at our disposal to identify subcellular effector localization, care should be taken when analyzing the results. However, since GFP does not diffuse to the nucleolus, it is safe to assume that nucleolar localization is effector-driven. RXLR effectors, such as HaRxLL3b, HaAtr13 Emoy2 and HaRxL44 from *Hyaloperonospora arabidopsidis*, localize to the nucleolus of plant cells.<sup>30</sup> In *Phytophthora capsici*, CRN effectors all localize to the nucleus, and at least two have been found to accumulate in the nucleolus, suggesting that there might be subnuclear localization domains.<sup>44</sup>

The nucleolus is a multifunctional subcellular organelle critically involved in ribosome biogenesis and protein synthesis.<sup>45</sup> Several DNA viruses and retroviruses are known to target the nucleolus. Umbravirus ORF3, potato leafroll virus capsid protein and influenza virus nucleoprotein are some examples of viral proteins localizing to the nucleolus.<sup>46-49</sup> Given that viruses are entirely dependent on the host machinery to translate their genome into proteins, they are expected to target the nucleolus. However, one can wonder why biotrophic filamentous pathogens would target this subnuclear compartment. The effector HaRxL44 from the obligate biotrophic pathogen *H. arabidopsidis* was recently shown to target nucleolar (and nuclear) Mediator subunit 19a (MED19a). This interaction results in MED19a degradation in a proteasome-dependent manner. MED19a degradation appears to shift transcription from salicylic acid-responsive defense to jasmonic acid and ethylene-responsive transcription, thereby conning the host to enhance its susceptibility.<sup>50</sup>

### Haustral Accommodation: Cellular Rearrangements through Reprogramming

What happens once a pathogen gets access to its host? How does the host respond to the pathogen's demands? And what are the overall cellular dynamics in play? Answering such questions becomes a lot more imperative when dealing with obligate biotrophs, because of their intimate relationship with the host and since they can only survive in living cells. Obligate biotrophic pathogens thus have to be subtle when dealing with their host after invasion. First of all, they have to keep host immunity in check at all times by suppressing PTI. Second, they have to continuously feed from plant cells. Finally, they need to steadily propagate and multiply.

Fungal spores grow on plant surfaces upon germination. It has been shown that the rust fungus *Uromyces appendiculatus* uses topographical cues for orientation and the formation of

infection structures.<sup>51</sup> Once *U. appendiculatus* detects a 0.5- $\mu\text{m}$  ridge, which it interprets as the presence of the stomatal lip (its entry point into tissue), it starts producing its infection structure.<sup>51</sup> When the pathogen has forced its way into plant tissue, nutrient acquisition and defense suppression occur primarily through haustoria, although effectors are also released from growing hyphae. Support for such a mechanism is lent by deep sequencing of the biotrophic growth phase of *Colletotrichum higginsianum* during *A. thaliana* infection.<sup>52</sup> In this pathosystem, effector genes are expressed in consecutive waves associated with pathogenic transition, and some are expressed before host invasion at the appressorial stage.<sup>52</sup> In fact, multi-stage transcriptome analysis of *Melampsora larici-populina*, the causative agent of the poplar leaf rust (obligate biotroph), revealed that a number of small-secreted proteins were even expressed in resting urediniospores.<sup>53</sup> Therefore, we can infer that suppression of plant immunity starts prior to the formation of haustorial structures in host tissue. While our understanding of molecular partners at play is progressing, we have made few inroads into the establishment of plant–haustoria interactions and post-invasion events. Dynamic interplay could be mainly driven by the invader, and as we progress in this review, we will examine some important phenomena that may hold clues to these questions.

It should not be difficult to conceptualize massive host cellular reprogramming occurring in response to the development of haustoria. Haustoria are found to be surrounded by endoplasmic reticulum, actin cytoskeleton and cytoplasm, along with the accumulation of Golgi bodies and mitochondria.<sup>54</sup> It has also been observed that a significant amount of tonoplast is present around these complexes.<sup>54</sup> To host such critical appendages, cells have to expand their plasma membrane tremendously. Haustoria are separated from the host cytoplasm by an extra-haustorial matrix (EHM). The EHM has been speculated to be mostly of host origin, sealed from haustoria by a haustorial neck band.<sup>55,56</sup> However, it differs from the plasma membrane in both cytological and biochemical properties.<sup>55,57</sup> The EHM also appears to vary in composition over time.<sup>58,59</sup> Recently, Lu et al.<sup>60</sup> reported that some plasma membrane resident proteins relocalize to the extra-haustorial membrane during infection. For example, the aquaporin PIP1;4 and the calcium ATPase ACA8 remained at the plasma membrane during infection with either *H. arabidopsidis* or *Phytophthora infestans* while the syntaxin PEN1 (penetration deficient 1), the synaptotagmin SYT1 and the remorin StREM1.3 were present in the extra-haustorial membrane around *P. infestans* haustoria. Interestingly, this relocalization appears to be pathogen-dependent since PRR FLS2 localized in the EHM of *P. infestans* but remained at the plasma membrane and was excluded from the EHM in *H. arabidopsidis*. However, the most remarkable feature of this cellular rearrangement is the position of the nucleus. Studies have shown that the *Arabidopsis* nucleus stays close to *H. arabidopsidis* haustoria,<sup>30</sup> and this is presumably driven by the actin cytoskeleton.<sup>61,62</sup> It is possible that proximity of haustoria to the nucleus enables pathogens to deliver their effectors more quickly to the nucleus for cell reprogramming. Proximity of the nucleus to the intruder would thus be driven by the pathogen per se, but one cannot exclude that host

plants could steer this process autonomously to respond quickly to pathogen attack.

## Vesicular Trafficking as a Possible Pathogen Target

Pathogens are known to target host vesicular trafficking, a key element of plant defense.<sup>30</sup> In *H. arabidopsidis*, 26% of examined effectors have been found to localize to membranes, the majority of them (18%) associating with the endoplasmic reticulum.<sup>63</sup> *Arabidopsis* cells hosting *H. arabidopsidis* haustoria develop bulging vesicular structures compared with non-infected cells,<sup>30</sup> the occurrence of such vesicles being attributed to presence of the pathogen. It is possible that the formation of these vesicles is driven by a particular effector or effectors to upset vesicular movement and disrupt any organized defense response. They may also be pathogen-driven and provide the extra-phospholipid bilayer required at the plasma membrane to accommodate fast-expanding haustoria. Regardless, support for the fact that these are vacuolar structures comes from the observations of very similar structures in cotyledons of transgenic *Arabidopsis*  $\gamma$ -TIP-GFP plants.<sup>64</sup> Other types of membrane structures have been shown to differentially localize around haustoria formed by *H. arabidopsidis* and *P. infestans*.<sup>60</sup>

HaRxL17 localizes to the EHM during infection by *H. arabidopsidis*. However, in the absence of the pathogen, it localizes to the tonoplast where its ability to enhance plant susceptibility is possibly linked with a task in plant cell membrane trafficking.<sup>30</sup> Since tonoplast is located close to the EHM along with the effector HaRxL17 in the event of infection, the effector may be interfering with plant cell membrane trafficking, and interestingly, this also suggests a role for tonoplast in EHM formation. However, no single effector has been reported to cause the bulb-like vesicular structures observed in the presence of growing pathogens,<sup>29</sup> and it is not clear whether it is a plant defense response or an effector-driven process. Surprisingly, our understanding of the detailed mechanism of vacuolar biogenesis is still limited, justifying the need to push the investigation further into such peculiar vesicular structures. It is difficult to elucidate possible pathways being targeted by pathogens to hinder vesicular trafficking and eventually give rise to these bulb-like structures. In *A. thaliana*, a point mutation in the deubiquitinating enzyme AMSH3 renders cells incapable of forming central lytic vacuoles. In addition, *amsb3* mutant cells accumulate autophagosomes and incorrectly sort their vacuolar protein cargo.<sup>65</sup> Vacuoles are important in various plant defense mechanisms, and two vacuole-mediated mechanisms have been postulated to affect programmed cell death.<sup>66</sup> In one of them, vacuolar-processing enzymes mediate vacuolar membrane disruption, thus releasing vacuolar content into the cell cytoplasm (demonstrated for viral infection).<sup>67</sup> In the second proposed mechanism, vacuole fusion with the plasma membrane enables the extracellular release of vacuolar content (demonstrated in bacterial infection).<sup>68</sup> Interestingly and coincidentally, phenotypic similarity between vesicular structures from *amsb3* mutants and cells hosting haustoria can be noticed.<sup>60,65</sup> This concurring vesicular signature suggests that pathogens

could be targeting AMSH3 (or similar components) to alter the vesicular pathway.

Octomeric–exocyst complexes could also be targeted by pathogens, given that the exocyst architecture plays an important role in vesicular tethering and redefining cell polarity, which are integral to plant defense responses.<sup>69</sup> Targeted exocytosis occurs during infection, and freshly-synthesized, defense-related compounds are delivered to infection foci, which eventually leads to asymmetrical plasma membrane development. Small GTPases from the Rab and Rho families are known to be essential in this process which involves delivery, anchoring, and integration of secretory vesicles to the plasma membrane,<sup>70,71</sup> whereas the exocyst complex works as a scaffold in tethering operations.<sup>72,73</sup> The final process of attachment is mediated by the integral membrane proteins v-SNARE and t-SNARE, where plasma membrane and vesicle bilayers are fused together to complete the process.<sup>74,75</sup> It has already been demonstrated that upon mutating, two exocyst subunits—Exo70B and Exo70H1 from *Arabidopsis* plants—are more susceptible to infection, validating their importance in plant immunity.<sup>69</sup>

PEN1 is a classic example of proteins preventing penetration by pathogens. PEN1 encodes a syntaxin known to interact with the SNARE proteins SNAP33 and VAMP72<sup>76</sup> and regulates papillae formation in cells under attack.<sup>77</sup> Papillae are bell-shaped cell wall appositions deposited in epidermal cells. Within papillae, various secondary antimicrobial metabolites accumulate along with lytic enzymes and reactive oxygen species, which stops the pathogen penetration peg. In *Arabidopsis*, PEN1 is found in significant amounts when the non-host fungus *Blumeria graminis* f. sp. *hordei* endeavors an unsuccessful invasion. However, when the host fungus *Erysiphe cichoracearum* successfully penetrates *Arabidopsis* cells, PEN1 is then downregulated.<sup>77</sup> The *pen1* single mutant allows increased penetration of the non-host fungus *B. graminis* f. sp. *hordei*, thereby showing that PEN1 helps in procuring an effective penetration barrier.<sup>77</sup> Thus, PEN1 could participate actively in polarizing secretion events that lead to papillae formation.<sup>77</sup>

## Conclusions

Obligate biotrophic phytopathogens have evolved a robust and elaborate offensive strategy to invade their host by deploying numerous effector proteins. It appears that the effectors inventory of pathogens is organized around different types of molecules, which have unique capabilities and functions. Therefore,

most so-called effectors should be considered candidate effectors. A crude way to envision effector deployment is to see apoplastic effectors at the onset of attack, performing all the bullwork and setting the stage for more sophisticated weaponry. True cytoplasmic effectors could act at the intermediate stage by deactivating local surveillance, paving the way for nuclear effectors to enter the nucleus, taking over the entire defensive network and stalling the complete immune set-up. Nucleolar effectors from various pathogens are increasingly being reported,<sup>44,78,79</sup> and it is likely that they have an important function in pathogenesis. Many cellular processes, including plant defenses, depend on the formation of new proteins. Thus, further study needs to be undertaken to understand the task of nucleolar effectors. Some effectors are also involved in disrupting vesicle trafficking and as such, they may be compromising vacuolar integrity, which is believed to play a significant role in plant defense. Plant cells hosting haustoria experience unique cellular rearrangements that are likely influenced by haustoria themselves and driven by secreted effectors.

As genome-sequencing costs are falling, the full sequences of many more genomes are becoming available. Despite the dazzling speed at which effector catalogs can be assembled, functional study of effectors remains a relatively slow and strenuous process. In obligate biotrophs, functional studies of effectors by virulence assays are hindered by the lack of molecular genetic approaches. As a result, alternative tactics with heterologous systems are increasingly being adopted. Given the very large repertoire of effectors observed in obligate biotrophic fungi, such as rusts that encode over 1000 small secreted proteins,<sup>80,81</sup> one could propose that the outcome of each effector may be a lot more subtle than the bacterial effectors of *Pseudomonas syringae* that have roughly 30 or so effectors,<sup>82</sup> and a direct, quantifiable impact on virulence may prove difficult to observe since the cumulative result of many effectors may be required. Alternatively, redundancy could explain the huge number of effectors in filamentous pathogens. In either case, deciphering the interactions of these effectors will likely reveal many unknown components of various plant processes. With these issues in mind, localization remains one of the first aspects to consider when assessing effector functions. In addition, combination of genetic evidence and protein–protein interaction approaches, either yeast two-hybrid assay, co-immunoprecipitation, or bi-molecular fluorescence complexes, may prove to be the best ways of investigating effectors from biotrophic pathogens.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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