Form and Function of Fungal and Oomycete Effectors

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Abstract

Plants are able to recognize conserved features of potential microbial invaders and mount an active defense in most cases. Over the course of evolution, a number of these microbes including plant pathogenic fungi and oomycetes have evolved means through the secretion of small molecules (effectors) to block these defenses and promote virulence. In recent years, research has uncovered a wealth of knowledge regarding how effectors function within the plant cell to promote disease. Function of effectors ranges from altering plant cellular metabolic pathways and signaling cascades, RNA silencing, anti-microbial inhibition, and interfering with recognition machinery. The importance of understanding effector function has given rise to a new area of research termed effectoromics, which in this review refers to high-throughput studies to elucidate the function of a large number of candidate effector genes. Effectoromics research has led to the identification of a number of effectors with redundant function, indicating that pathogenic fungi and oomycetes contain effectors that are individually dispensable but functionally redundant that act synergistically to promote disease.

Keywords

Effector; Reactive Oxygen Species (ROS); Systemic Acquired Resistance (SAR); Effectoromics; MAMP-triggered immunity (MTI)

Introduction

The interactions of fungi and oomycetes with plant hosts play a significant role in many ecosystems. In some situations, interaction with organisms such as mycorrhizal fungi confer beneficial properties in the plant rhizosphere (Haverkort et al., 2008; OERKE, 2005; Soka and Ritchie, 2014). On the other hand, although plants do not typically succumb to invading microorganisms, fungi and oomycetes have evolved to become pathogens and cause millions of dollars of loss in annual agricultural revenue in a number of instances. In recent years much light has been shed on the molecular basis of these interactions (Lo Presti et al., 2015; Ortíz-Castro et al., 2009). During initial infection stages, plant cell surface-localized pattern recognition receptors (PRRs) are able to recognize conserved elements of the invading microbe, which are referred to as microbe-associated molecular patterns (MAMPs). Molecular communication between PRRs and MAMPS triggers a defense response called MAMP-triggered immunity (MTI), which is sufficient to deter many would-be pathogens. However, pathogens evolved molecules, often small, secreted proteins that not only block MTI but also alter specific plant processes for the benefit of the invading pathogen, which are known as effectors (Dodds and Rathjen, 2010; Lo Presti et al., 2015; Rovenich et al., 2014; Thomma et al., 2011). During the course of the molecular arms race between pathogens and their hosts, plants evolved mechanisms to recognize these effectors, collectively known as resistance (R) proteins. This recognition allows the plant to mount a defense

response often involving the hypersensitive response (HR) to kill the invading pathogen, thus causing effector triggered immunity (ETI) (Martin et al., 2003). The continued interplay between the invading pathogen and host plant is encapsulated in the “zig-zag” model, which elegantly describes the evolutionary warfare between the invading pathogen and host plant (Jones and Dangl, 2006).

Effectors have been shown to be essential for the virulence of numerous pathogenic fungi and oomycetes. Data is now beginning to emerge that reveal the remarkable array of plant processes that are targeted by pathogen effectors (Bozkurt et al., 2012; de Jonge et al., 2011; Lo Presti et al., 2015; Rovenich et al., 2014; J. Win et al., 2012). In some instances, understanding of how effectors work has led to extraordinary scientific breakthroughs. For example, knowledge of TAL effectors from the plant pathogenic *Xanthomonas* species enabled the development of a powerful genome editing tool called TALENS (Bogdanove and Voytas, 2011; Bogdanove et al., 2010). This tool has been recently used as a novel cancer treatment (Reardon, 2015). This and other applications would not have been possible had it not been for understanding how effectors from plant pathogenic bacteria affect host plant biology and underscores the powerful knowledge that can be gained from understanding how effectors function within the host.

Although TAL effectors from bacterial plant pathogens are well characterized, how a majority of effectors from filamentous microbes interact with and modify their host targets is still largely unknown. Characterizing how effectors function within plant cells is essential for understanding how pathogens overcome their plant hosts and has become a driving force in molecular plant pathology (Hogenhout et al., 2009). This review uses specific examples to describe the current knowledge of how effectors from fungi and oomycetes manipulate the host plant for their own benefit. In addition, progress in developing systematic high throughput strategies to identify unknown effectors and functionally define their role in virulence, hereby defined as effectoromics is discussed.

Cellular Effectors

The location of fungal or oomycete effectors within the plant provides clues to how they function to affect virulence of the invading pathogen. Effectors from fungi and oomycetes have been shown to operate at two main locations: in the host cell and in the apoplastic space between adjacent cells (Giraldo and Valent, 2013). Within the host cell, effectors from filamentous organisms interact with numerous plant components to alter plant metabolism, signaling pathways, and gene transcription to manipulate the plant defense response.

One of the primary mechanisms through which effectors block defense responses is through the alterations to the plant metabolic pathways (Djamei et al., 2011; Liu et al., 2014; Plett et al., 2014). Salicylic acid (SA) and jasmonic acid (JA) are two essential phytohormones that act antagonistically in response to pathogen infection (Niki et al., 1998). SA acts to mediate MTI, to regulate host cell death, and is a key player for systemic acquired resistance (SAR), which is a broad spectrum response to pathogen invasion that prepares a plant and its progeny for pathogen attack (Dempsey et al., 2011; Fu and Dong, 2013; Lo Presti et al., 2015). JA is also involved in plant defense responses as well as cell

development and vegetative growth (McDowell and Dangl, 2000; Thomma et al., 2001). Cmu1, a chorismate mutase from the maize pathogen *Ustilago maydis*, is secreted into the host cell during infection to reduce SA levels (Djamei et al., 2011). Similarly, effectors Pslsc1 from *Phytophthora sojae* and Vdlscl from *Verticillium dahliae* are required for full virulence of both pathogens and reduced the amount of SA when expressed within plant cells (Liu et al., 2014). Like Cmu1, these effectors act as isochorismatases that hydrolyze isochorismate, a precursor to SA, and downregulate SA mediated defense responses (Liu et al., 2014). Plant pathogenic nematodes have also shown to secrete chorismate mutases into plant cells during pathogenesis, indicating that manipulating SA levels is a common target of many plant pathogenic organisms (Bekal et al., 2003; Doyle and Lambert, 2003). Effectors have also been shown to affect JA signaling. The ectomycorrhizal fungus *Laccaria bicolor* secretes the effector MiSSP7 during plant colonization that is targeted to the plant nucleus and is essential for the establishment of mutualism between the fungus and the plant (Plett et al., 2011). To promote mutualism, MiSSP7 interacts with and prevents the degradation of the transcriptional repressor protein PtJAZ6 to prevent JA-induced changes in gene transcription (Plett et al., 2014).

Beyond alterations of SA and JA, effectors from filamentous plant pathogens have been shown to alter the levels of other hormones and metabolites in order to gain entry into the host plant (Evangelisti et al., 2013; Tanaka et al., 2014). Tin2 from *U. maydis* interacts with and prevents the degradation of the maize protein kinase ZmTTK1, which is responsible for the activation of genes involved in anthocyanin biosynthesis. Maize infected with Tin2 knockout strains of *U. maydis* show strong lignification at the sites of infection, indicating Tin2 redirects metabolite production into anthocyanins and away from other metabolites that would otherwise be beneficial for plant defense (Tanaka et al., 2014). Finally, PSE1 from *Phytophthora parasitica* alters the levels of auxin through the redistribution of the auxin efflux carriers PIN4 and PIN7 (Evangelisti et al., 2013).

RNA silencing serves as a major defense mechanism against RNA viruses in plants, where viral RNA is recognized as a DAMP and induces small interfering RNAs (siRNAs) to guide the cleavage of viral RNAs (Ding, 2010). In response, viruses have developed suppressors of RNA silencing that interfere with the RNA silencing machinery and allow for the virus to proliferate within the host (Vance and Vaucheret, 2001). Viruses, however, are not the only organisms to block RNA silencing in order to benefit infection. Two effectors from *Phytophthora sojae*, PSR1 and PSR2, suppress RNA silencing and enhance susceptibility to both *P. sojae* as well as viral infection (Qiao et al., 2013). PSR1 was shown to bind with an evolutionarily conserved nuclear protein known as the PSR1-interacting protein 1 (PINP1), which likely is involved in small RNA processing. Silencing this protein in *Arabidopsis* leads to not only developmental defects but also hypersusceptibility to *Phytophthora* infections, which is similar to transgenic plants expressing the PSR1 protein (Qiao et al., 2015). A PSR2-like effector from the related species *P. infestans* can also suppress RNA silencing, indicating that a conserved class of effectors that suppress RNA silencing exists within the genus *Phytophthora* (Xiong et al., 2014). These examples show that RNA silencing suppression is a key strategy for infection not only in viruses, but also in oomycetes as well (Qiao et al., 2013).

Components of plant signaling pathways are also targets of effectors from filamentous pathogens. MAPK cascades are important for both MTI and ETI and thus effectors have evolved to block these

pathways in order to stop the immune response (Boller and Felix, 2009; Martin et al., 2003). PexRD2 from *P. infestans* is one such effector that interacts with the kinase domain of MAPKKKε and disrupts the signaling pathway. MAPKKKε is a positive regulator of immunity-related cell death and the overexpression of PexRD2 or the silencing of MAPKKKε within *Nicotiana benthamiana* promoted the growth of *P. infestans*, indicating that this effector has evolved to interact with MAPKKKε to interrupt plant immunity-related signaling (King et al., 2014).

Effectors from filamentous pathogens have also been shown to directly affect plant transcription in order to downregulate genes involved in defense responses. SP7 from the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* and HaRxL44 from the oomycete pathogen *Hyaloperonospora arabidopsidis* are two examples of effectors that directly affect plant gene transcription (Caillaud et al., 2013; Kloppeholz et al., 2011). SP7 was shown to localize to the plant nucleus and interact with the transcription factor ERF19 to block the plant immune system (Kloppeholz et al., 2011). Furthermore, SP7 increased the duration of the biotrophic phase of infection when expressed within the rice blast fungus *Magnaporthe oryzae*, indicating that this effector plays an important role in suppressing defense responses for the establishment of fungal growth within the plant (Kloppeholz et al., 2011). HaRxL44 from *H. arabidopsidis* is another nuclear localized effector that interacts with and degrades Mediator subunit 19a (MED19A) which is a positive regulator of immunity against *H. arabidopsidis* and responsible for transcription changes within *Arabidopsis* (Caillaud et al., 2013). Transgenic *Arabidopsis* plants expressing HaRxL44 saw an induction of transcription associated with JA signaling, which is similar to what is observed in *Arabidopsis* plants infected with *H. arabidopsidis*. Therefore, HaRxL44 seems to affect the balance between JA and SA signaling through the degradation of MED19A, thus affecting defense-related transcriptional changes (Caillaud et al., 2013).

Reactive oxygen species (ROS) play an essential role in MTI and have a direct role in cell wall protein cross-linking, phytoalexin production, callose deposition, and SAR (O'Brien et al., 2012). Crinklers (CRN for CRinkling and Necrosis) are a well characterized family of effectors from plant pathogenic oomycetes that have been shown to affect ROS levels in plants (Rajput et al., 2014; Zhang et al., 2015). PsCRN63 and PsCRN115 from *P. sojae* interact directly with plant catalases, modulating their activity for the induction or suppression of H₂O₂ homeostasis and plant cell death (Zhang et al., 2015). Plant catalases are localized to the peroxisomes and convert H₂O₂ into water and oxygen, thus contributing to the proper ROS balance within the plant (Du et al., 2008). PsCRN63 promotes plant cell death through interacting with and destabilizing plant catalases whilst PsCRN115 counteracts this process to maintain the proper H₂O₂ levels and block plant cell death (Zhang et al., 2015). Another well characterized suppressor from *P. sojae*, PsCRN70, suppresses the accumulation of H₂O₂ (Rajput et al., 2014). While the exact mechanism for how PsCRN70 modulates plant ROS levels is unknown, it is clear that controlling the level of ROS during disease development is critical for virulence.

The plant ubiquitination system has emerged as a common target for numerous effectors. AvrPiz-t from *M. oryzae* interacts with the rice RING E3 ubiquitin ligase APIP6 and suppresses its ubiquitin ligase activity. Silencing APIP6 in rice leads to a significant reduction of ROS generation, defense-related gene expression, and increases the susceptibility of rice to *M. oryzae*, indicating that AvrPiz-t blocks MTI through the inactivation of APIP6 (Park et al., 2012). Avr3a is a well characterized effector from *P.*

infestans and exists in two allelic forms: AVR3a^{KI} and AVR3a^{EM} (Bos et al., 2006). Avr3a^{KI} was shown to strongly interact with and stabilize the host ubiquitin E3 ligase CPMG1 to suppress HR during the biotrophic phase of infection, whereas Avr3a^{EM} and a mutated form of Avr3a^{KI} with a deleted C terminal tyrosine, Avr3a^{KI-Y147del}, had weak to no interaction with CPMG1 (Bos et al., 2010; González-Lamothe et al., 2006). New research indicates, however, that all forms of Avr3a suppresses defense responses triggered by the PAMP receptor FLS2 and that Avr3a associates with a plant GTPase Dynamin-Related Protein 2 (DRP2) which is involved in receptor-mediated endocytosis (Chaparro-Garcia et al., 2015). Therefore, this effector can associate with multiple targets within the host to block plant cell death.

The ability of effectors to suppress MTI not only applies to inhibiting the signaling of defense but also to physically block or alter the necessary components of defense from reaching their intended target. Vesicle trafficking for exocytosis within plants has been implicated in the secretion of defense proteins at the site of infection and thus has been hypothesized as being targets for effectors (Zhao et al., 2015). Avr-Pii from *M. oryzae* was found to interact with OsExo70-F2 and OsExo70-F3, two rice proteins presumably involved in exocytosis (Fujisaki et al., 2015). Although overexpressing Avr-Pii in *M. oryzae* or knocking down of both OsExo70-F2 and -F3 did not have an effect on the level of immunity within rice, Avr-Pii interacting with OsExo70-F3 plays a crucial role in the resistance mediated by the rice R protein Pii (Fujisaki et al., 2015). The powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*) secretes the effector BEC4 during the early stages of infection and has been shown to interact with ADP ribosylation factor-GTPase-activating proteins (ARF-GAP), which is a key player of membrane trafficking in eukaryotic cells (Nielsen et al., 2008; Schmidt et al., 2014). How BEC4 interacts with and modulates the activity of the ARF-GAP protein as well as affect the virulence of *Bgh*, however, remains to be fully understood. The effector Pi03192 from the tomato late blight pathogen *Phytophthora infestans* interacts with the transcription factors NTP1 and NTP2 at the ER in tomato cells and prevents their localization to the nucleus (McLellan et al., 2013). Finally, the effector PexRD54 from *P. infestans* has been shown to stimulate autophagosome formation through binding to the autophagy protein ATG8CL. Interestingly, PexRD54 also interacts with the host cargo receptor Joka2 to deplete it out of ATG8CL autophagosomes to promote disease susceptibility. Although it is currently unknown how Joka2 contributes to immunity, this example shows that *P. infestans* alters autophagosomes for their own benefit (Dagdas et al., 2016). These examples highlight how effectors prevent the activation of defense through the mis-localization or alteration of plant cellular components during infection.

Apoplastic Effectors

The apoplast is a pivotal battleground between plants and many invading filamentous pathogens. It is a hostile environment due to the production of various glucanases and chitinases by host plants that aim to disrupt the pathogen cell wall and stop the pathogen from advancing. These enzymes also release MAMPs that are recognized by plant PRRs and signal the production of phytoalexins, PR proteins, ROS, and lignins for the strengthening of the plant cell wall (Sanchez-Vallet et al., 2014). Effectors from filamentous pathogens that function in the apoplast have been shown to play a central role in blocking these plant responses in a variety of ways. Additionally, some effectors aid in the breakdown of plant cell walls and inhibit plant derived enzymes, adding to the complexity of functions that apoplastic effectors possess.

A number of cell wall degrading enzymes (CWDEs) that break down plant cell wall components during infection are considered effectors. CWDEs are thought to aid in the invasion of plants by hydrolyzing plant cell wall components such as pectin and cellulose. However, a high level of redundancy within fungal genomes and only few reports indicating a direct contribution of these enzymes to virulence throws their importance into question (Doehlemann and Hemetsberger, 2013; Walton, 1994). In *U. maydis*, simultaneous deletion of its three pectinase genes did not impair virulence (Doehlemann et al., 2008). However, knockdown of glycoside hydrolase family 6 and 7 genes as well as the silencing of the *M. oryzae* xylanase genes resulted in weakened infection from *M. oryzae* in rice (Nguyen et al., 2011; Van Vu et al., 2012). How and to what extent CWDEs aid in virulence is an area that needs further exploration.

To protect the invading hyphae from plant-produced hydrolytic enzymes, fungi secrete LysM effectors into the apoplast that bind chitin to prevent the degradation of the fungal cell wall and block the triggering of MTI (Chen et al., 2014; de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Mesarich et al., 2015; Sanchez-Vallet et al., 2014; van den Burg et al., 2006; van Esse et al., 2007). Mg1LysM and Mg3LysM are two LysM effectors from *Mycosphaerella graminicola*, the causal agent of Septoria tritici blotch disease, that bind to chitin at the fungal cell wall to protect against plant derived hydrolytic enzymes (Marshall et al., 2011). Avr4 from *Cladosporium fulvum* is a well-studied LysM effector that also binds chitin at the fungal cell wall to prevent chitinase enzymes from degrading the fungal cell wall (van den Burg et al., 2006). Expression of this gene in *Arabidopsis* enhances susceptibility to a variety of fungal pathogens, but the virulence of bacterial and oomycete pathogens remains unchanged. Furthermore, expression of Avr4 in *Fusarium oxysporum* f. sp. *lycopersici* increases its virulence (van Esse et al., 2007). Orthologues of this gene in other closely related Dothideomycete fungi all bind chitin, illustrating the importance of this gene family across closely related fungi (Mesarich et al., 2015).

Chitin is a well-characterized MAMP that triggers MTI when recognized by PRR proteins present in the plant cell wall (Lo Presti et al., 2015). In addition to protecting the fungal cells from degradation by plant chitinases, LysM effectors also sequester free-floating chitin present within the apoplast as a means to avoid MTI. Slp1 from *M. oryzae* does this by binding to and sequestering chitin that is present within the apoplast and prevents the triggering of MTI (Chen et al., 2014; Mentlak et al., 2012). Ecp6 from *C. fulvum* is also secreted into the apoplast during infection and sequesters chitin to prevent the elicitation of MTI (de Jonge et al., 2010). These effectors compete for chitin binding with the chitin-elicitor binding protein (CEBiP) and receptor chitin elicitor receptor kinase 1 (CERK1), two well characterized PRR proteins that mediate MTI through the recognition of chitin during filamentous pathogen invasion (Kaku et al., 2006; Miya et al., 2007). Structural analysis of both these proteins indicate that in the presence of chitin oligosaccharides, both CERK1 and CEBiP form dimers and it is hypothesized that upon dimerization, CERK1 and CEBiP trigger MTI (Hayafune et al., 2014; Liu et al., 2012). Structural analysis of Ecp6 indicates that two of the three LysM domains bind chitin in a high affinity manner, but the third LysM domain does not bind chitin as strongly as the other two but can still block chitin-triggered immunity (Sánchez-Vallet et al., 2013). Therefore, this LysM domain is hypothesized to block the receptor dimerization to further contribute to blocking MTI (Sánchez-Vallet et

al., 2013). More work is needed to confirm this hypothesis, but if true this would be the first report of a single fungal effector showing multiple virulence functions.

Beyond the capacity to bind to and isolate chitin, effectors have been shown to inhibit glucanase enzymes produced by the plant in order to block MTI and any anti-microbial activity these enzymes might have (Rose et al., 2002; Sánchez-Rangel et al., 2012). A class of effectors from *P. sojae* termed glucanase inhibitor proteins (GIPs) are secreted during disease development and specifically interact with and prevent endoglucanase enzymes that are secreted by the plant (Rose et al., 2002). GIP1 from *P. sojae* is one such effector that interacts with the soybean endoglucanase EgaseA, thus preventing the release of oligosaccharides and the triggering of MTI (Rose et al., 2002). Fumonisin B1 (FB1) has been shown to help mediate the virulence of *F. verticillioides* by targeting plant derived β -1,3-glucanases and modulating them during infection (Sánchez-Rangel et al., 2012). These examples point to effectors directly interacting with plant derived enzymes and blocking their action.

An important group of plant enzymes involved in the immune response are the papain-like cysteine proteases (PLCPs) that are secreted from the plant into the apoplast during infection. Although it is well documented that these enzymes are activated by the presence of SA, are able induce PR-gene expression, and trigger the host cell death response, the exact mechanism through which these enzymes prevent pathogen invasion is still unknown (van der Hoorn and Jones, 2004). Their importance in plant defense, however, is highlighted by the large number of effectors from filamentous pathogens that inhibit the activity of numerous PLCPs. EPI1 and EPI10 from *P. infestans*, for example, are Kazal-like serine protease inhibitors (EPI's) that both target and inhibit the serine protease P69B, indicating that two distinct and structurally divergent protease inhibitors can target the same protease and underlies the importance of this strategy during infection (Tian et al., 2005, 2004). Another effector from *P. infestans* is AVRblb2, which localizes around haustoria during infection and interacts with the PLCP C14, preventing its secretion from the plant into the apoplast (Bozkurt et al., 2011). Knockdown of C14 via RNAi-silencing leads to the plants becoming more susceptible to *P. infestans*, indicating the essential role AVRblb2 plays in virulence (Bozkurt et al., 2011). In addition, *P. infestans* secretes cystatin-like effector proteins (EPICs) EPIC1 and EPIC2B and, like AVRblb2, have been shown to target the protease C14 (Kaschani et al., 2010). EPIC1, EPIC2B, and AvrBlb2 from *P. infestans* are very different proteins yet all target C14, indicating the importance of blocking this enzyme through convergent evolution of multiple effectors. The *C. fulvum* effector Avr2 has been shown to interact with multiple tomato Cys proteases, such as Rcr3 and Pip1, and acts as a virulence factor by inhibiting these proteases (van Esse et al., 2008). Interestingly, EPIC1 has also been shown to interact with and inhibit RCR3 while EPIC2B inhibits both RCR3 and PIP1 (Song et al., 2009; Tian et al., 2007). Avr2 was also shown to weakly inhibit C14 (van Esse et al., 2008). Multiple variant residues among the C14 amino acid structure indicate that this enzyme is under diversifying selection and the multiple effectors that inhibit C14 most likely interact at different locations on the enzyme surface (Kaschani and Van der Hoorn, 2011).

Protease inhibitors secreted into the apoplast have been shown to not only be produced by filamentous pathogens, but also by the host plant. Pit2 from *U. maydis* is an essential effector for the

establishment of biotrophy during the early stages of infection as well as the induction of tumor formation (Mueller et al., 2013). Yeast-2-hybrid and tandem mass spectrometry analysis indicated that Pit2 interacts with the maize cysteine proteases CP1A, CP2, and XCP2 via a novel inhibitor domain present on the Pit2 protein (Mueller et al., 2013). In addition, *U. maydis* infection into maize induces the transcription of CC9, which is a maize-encoded cystatin that is secreted into the apoplast during infection and is required for full virulence of *U. maydis* (van der Linde et al., 2012). CC9 was found to interact with CP1A, CP2, and XCP2 proteases in addition to CP1B and the cathepsin B-like protease as well (van der Linde et al., 2012). These proteases have been linked directly to SA-mediated defense responses, indicating that both Pit2 and CC9 function to block protease function for the downregulation of defense responses related to SA signaling (Doehlemann and Hemetsberger, 2013; Mueller et al., 2013; van der Linde et al., 2012). These examples and the ones listed in the aforementioned paragraph illustrate that although the precise mechanism through which host-derived proteases function in defense is currently unknown, their inhibition by effectors secreted from filamentous pathogens or from the host itself is crucial for successful invasion.

Reactive oxygen species (ROS) and their rapid accumulation within the plant during infection of filamentous pathogens comprises an essential component to plant defenses. ROS has been linked to not only direct antimicrobial roles, but also to cellular signaling for defense gene expression, HR, cell wall protein cross-linking, phytoalexin production, callose deposition, and SAR, just to name a few (O'Brien et al., 2012). The *U. maydis* effector Pep1 was originally identified as a virulence factor that is essential for pathogenicity and localized to the apoplast during disease development (Doehlemann et al., 2009). Pep1 was shown to interact with and inhibit the maize peroxidase POX12 *in vivo* through fluorescence complementation assays, thus suppressing the ROS-generating machinery and early plant defense responses (Hemetsberger et al., 2012). To date this is the first example of a fungal effector that directly interferes with the apoplastic ROS-generating system (Doehlemann and Hemetsberger, 2013).

The effectors presented thus far have been shown to operate at discrete locations within the host plant cell or apoplast to affect virulence. There are other effectors, however, whose location within the plant cell is ambiguous or unknown yet still play a major role in pathogenicity in filamentous pathogens. CfTom1 from *C. fulvum* is a glycosyl hydrolase (GH10) that interacts with and detoxifies the steroidal glycoalkaloid α -tomatine from tomato, which is an antimicrobial compound produced in the tomato leaves and green fruits that present a constitutive chemical barrier against a wide range of fungal and bacterial pathogens (Ökmen et al., 2013; Osbourn, 1996). Although α -tomatine was shown to be present in both the plant cellular vacuole and within the apoplast during *C. fulvum* infection, the exact location where CfTom1 operates to detoxify α -tomatine is currently unknown (Ökmen et al., 2013). These and the other examples presented in this review underscore the importance on understanding the location of effector function in order to elucidate how the effector functions to affect the virulence of the invading filamentous pathogen.

3D Structures of Filamentous Pathogen Effectors

In recent years the pace of discovery and assignment of functional roles that effectors play in mediating the virulence of numerous filamentous pathogens has increased due to advancements in

genomics, bioinformatics, proteomics, biochemistry, and cell biology. In addition, technologies for biophysical characterization and crystallization of effectors from plant pathogenic microorganisms has led to a deeper understanding of how effector and target interact (Wirthmueller et al., 2013). Research into the structure of effectors from filamentous pathogens has largely focused on how R proteins interact with effectors to mediate ETI, but understanding the tertiary structure of these effectors has also led to a deeper understanding of how these effectors operate within the plant cell.

The AVR-Pik allele, AVR-PikD, from *M. oryzae* interacts with the rice R protein Pikp-1 and is thought to be the oldest pair of interacting partners in co-evolutionary time due to the high level of polymorphisms within both the Avr and R gene (Kanzaki et al., 2012). Yeast two-hybrid and *in planta* co-immunoprecipitation assays revealed that AVR-Pik physically binds to the N-terminal coiled-coil (CC) domain located at the N-terminal of Pik (Kanzaki et al., 2012). Crystal structure analysis of this interaction revealed that AVR-PikD binds as a dimer to Pikp-1 at the Heavy-Metal Associated (HMA) domain located between the CC and the nucleotide binding (NB-ARC) domain with high affinity (Maqbool et al., 2015). Both AVR-Pik and the HMA region of Pik exhibit high levels of nucleotide polymorphisms that result in amino acid changes, indicating co-evolutionary warfare occurring at this region (Kanzaki et al., 2012). These studies establish the structural basis of interaction between an Avr and R protein that interact directly and opens the door for engineering rice plants for resistance to *M. oryzae*. In addition, the crystal structure of AvrPiz-t from *M. oryzae* was also determined, which was shown to form a six-strand β -sandwich fold and revealed that cysteine bonds are important for recognition by the rice R protein Piz-t (Zhang et al., 2013). As mentioned previously, it has been shown that AvrPiz-t interacts with the rice E3 ubiquitin ligase APIP6 (Park et al., 2012). Like AVR-Pik, determining the structure of AvrPiz-t will illuminate how it interacts with APIP6 and thus unlocks the possibility for engineering rice plants for resistance.

The power of determining the physical structure of effectors from filamentous pathogens includes not only determining what motifs are important for their function, but also predicting how they function to promote virulence (Sperschneider et al., 2015). AvrL567 from *M. lini* revealed a β -sandwich fold and no close structural homologues, but evidence of DNA binding *in vitro* points to a potential virulence function of this effector (Wang et al., 2007). Interestingly, AvrL567 shares a high degree of similarity to the ToxA toxin produced by the tan spot fungus *Pyrenophora tritici-repentis* insofar as both proteins share the same β -sandwich fold (Sarma et al., 2005). It was shown that ToxA localizes to the cytoplasm and chloroplasts where it exerts its toxic effect, but how it affects virulence is still currently unknown (Manning et al., 2008; Sarma et al., 2005). In addition to ToxA and AvrL567 sharing a common motif, several characterized effectors from various oomycete pathogens share the same WY-domain fold (Joe Win et al., 2012). The presence of similar physical structures on effectors from different pathogens could indicate a common virulence function or evolutionary origin.

Effectoromic Screens

To date, relatively few effectors have been identified and assigned a functional role in virulence (Sperschneider et al., 2015). Effectoromics screens aims to expedite this process via high-throughput approaches that functionally characterize a large number of effector candidates (Figure 1) (Petre et al.,

2015). These effectoromics experiments usually include approaches to first define the most likely set of candidates, often considered to be small cysteine-rich secreted proteins in the case of fungal pathogens or contain an RXLR motif in oomycete pathogens (Guyon et al., 2014; Win et al., 2007). Due to large numbers, particularly for fungal pathogens, other criteria are used to refine the list of candidate effectors. For example, comparative genomics studies can be used to identify genes undergoing diversifying selection. In the two-speed genome hypothesis, effectors are generally considered to be undergoing more rapid evolution to escape host detection (S. Dong et al., 2015). Such studies often conclude that secreted proteins are evolving more rapidly than other genes, although this is not universally true and other genes lacking a so-called signal peptide show evidence of diversifying selection. Many, but not all pathogen genomes are rife with repetitive elements and a number of studies have shown that known effectors are associated with such elements (Raffaele and Kamoun, 2012). Their close proximity is consistent with the hypothesis that regions rich in repetitive elements are prone to mutation and recombination, which facilitates more rapid evolution of associated genes. One such mechanism, Repeat Induced Point (RIP) mutation, is well documented in both pathogenic and non-pathogenic fungi and can result in mutations extending well beyond duplicated (repetitive) DNA sequences (Hood et al., 2005). In addition to signatures of diversifying selection and proximity to repetitive sequences, examining their expression levels and cellular location in infected plants can help further refine candidate effectors. Differential expression early during infection is typically taken as a requirement for an effector (Mosquera et al., 2009).

Once candidate effectors are identified, the critical step is to ascertain function. In many but not all instances, simply knocking out (or down) a single effector has little or no observable effect on virulence (Mosquera et al., 2009). It is hypothesized that in the molecular arms race between pathogens and their hosts, pathogens have assembled a vast army of virulence proteins and consequently loss of a single combatant will have little effect on the outcome of the battle, in general. Effectoromics screens for inducers and suppressors of plant cell death have become a popular method to link function to candidate effectors due to the relative ease of screening a number of effector proteins from pathogenic oomycetes and fungi. Effectors that induce the host cell death response potentially act as inducers of cell death for necrotrophic and hemibiotrophic pathogens. Screening candidate effector genes from *M. oryzae* expressed during disease development revealed five genes that were able to induce host cell death when expressed within rice protoplasts (Chen et al., 2013). Interestingly, four of these five also induced plant cell death when expressed within the model plant *N. benthamiana*, indicating common mechanisms of cell death induction between the two plant species (Chen et al., 2013). Conversely, numerous effectors have been identified that suppress the host cell death response, potentially acting to suppress HR during infection for biotrophic and hemibiotrophic pathogens. Five candidate effectors identified from *M. oryzae* were able to suppress plant cell death induced by the BAX gene, the characterized Six6 effector from *F. oxysporum* suppressed I-2-mediated cell death, and a homologue of CgDN3 from *C. gloeosporioides* that is present in *C. orbiculare* suppressed plant cell death induced by the NIS1 gene, which is also from *C. orbiculare* (Y. Dong et al., 2015; Gawehns et al., 2014; Yoshino et al., 2012). Interestingly, seven effectors identified from the apple canker fungus *Valsa mali*, a necrotroph, suppressed BAX-induced plant cell death (Li et al., 2015). Knocking out of one of the seven identified

suppressors, VmEP1, caused a significant reduction in virulence, alluding to the possibility that suppression of ETI-related HR is important for necrotrophic pathogens (Li et al., 2015).

Effectors that either suppress or induce plant cell death have been found to be expressed at certain times during infection for hemibiotrophs, corresponding to either the biotrophic or necrotrophic phase of infection. Transcriptome analysis of effector genes from *C. higginsianum* expressed during disease development showed that effectors that suppressed host cell death were expressed during the early stages of disease development, whilst effectors able to induce cell death were expressed later (Kleemann et al., 2012). This coordinated expression of effector delivery suggests effectors play a significant role in mediating the infection strategy of *C. higginsianum* and potentially other fungi.

Effectoromics screens from oomycete pathogens are aided by the RXLR motif, making the selection criteria for effectors to test for plant cell death inducing or suppressing activity much easier than in fungi. Thus, many more effectors from oomycetes have been characterized as suppressors or inducers of cell death. Of 169 candidate effectors from *P. sojae* that contain an RXLR sequence, 107 could suppress plant cell death triggered by BAX and/or the INF1 gene when expressed within *N. benthamiana* while 11 were found to be able to induce cell death (Wang et al., 2011). Two different classes of effectors were identified based upon their ability to suppress plant cell death induced by different genes, indicating the possibility that different effectors block different pathways of the cell death triggering machinery (Wang et al., 2011). Analysis of the effector protein Avr1b established that the conserved WY motif is essential for suppressing BAX-mediated cell death not only within *N. benthamiana*, but also within yeast and soybean, indicating its importance for virulence within the plant (Dou et al., 2008). In addition to RXLR effectors, there are other oomycete effectors that have been shown through effectoromics screens to be able to induce cell death. Four necrosis and ethylene-inducing-like protein (NLP) genes from *P. capsici* and eight from *P. sojae* were found to induce cell death when expressed within the leaves of dicots (Dong et al., 2012; Feng et al., 2014). Beyond screens that test for suppression or induction of plant cell death, effectors from the oomycete pathogen *Hyaloperonospora arabidopsidis* have been shown to suppress callose deposition, another hallmark of MAMP triggered immunity (Fabro et al., 2011). PcCRN4 from *P. capsici* was shown to not only induce plant cell death when expressed within the leaves of *N. benthamiana*, *N. tabacum*, and *Solanum lycopersicum*, but knockout strains caused an increase of callose deposition at the site of infection, indicating the role that this effectors play in virulence (Mafurah et al., 2015). These experiments point to the possibility that while some effectors are essential for the full virulence of filamentous pathogens, a vast majority of effectors have small, quantitative effects that are individually dispensable. When acting together at specific time points, however, these effectors have a significant effect on virulence.

Future Directions

The functional identification of effectors from filamentous pathogens that can induce or suppress plant cell death is a key discovery in unraveling the complex nature of host-pathogen interactions. However, this is just the first step in the quest: the means through which these effectors affect plant defenses arguably remains the Holy Grail, the mythical chalice that amongst other things will provide food in infinite abundance. Common plant targets of effectors have emerged from bacteria,

oomycetes, and fungi, indicating conserved hubs that are important for disease development of multiple pathogens (Mukhtar et al., 2011; Weßling et al., 2014). Manipulating ROS and the ubiquitination system, for example, have been shown to be common targets of effectors from filamentous and bacterial pathogens, indicating their importance for disease development (Banfield, 2015). Understanding how effectors manipulate common targets and how they work together to down-regulate the defense responses of plants is not only essential for understanding and managing disease development, but also may lead to unexpected discoveries and technologies.

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Table 1. Characterized effectors from fungi and oomycetes			
Effector	Organism	Function	Reference
BEC4	<i>Blumeria graminis f. sp. hordei</i>	Interacts with ADP ribosylation factor-GTPase-activating proteins (ARF-GAP)	Nielsen et al. 2008; Schmidt et al. 2014
Avr4	<i>Cladosporium fulvum</i>	Binds chitin at the fungal cell wall to protect against plant derived chitinases	van den Burg et al. 2006
Ecp6	<i>Cladosporium fulvum</i>	Sequesters free floating chitin present in the apoplast to prevent MTI and potentially interferes with recetor protein dimerization	de Jonge et al. 2010;(Sánchez-Vallet et al. 2013
Avr2	<i>Cladosporium fulvum</i>	Targets and inhibits multiple cysteine proteases	van Esse et al. 2008
CfTom1	<i>Cladosporium fulvum</i>	Detoxifies α -tomatine from tomato, which is an antimicrobial compound constitutively produced by tomatos	Ökmen et al. 2013; Osbourn 1996
FB1	<i>Fusarium verticillioides</i>	Targets and inhibits plant derived β -1,3-glucanases	Sánchez-Rangel et al. 2012
SP7	<i>Glomus intraradices</i>	Interacts with the transcription factor ERF19 to block the plant immune system	Kloppholz et al. 2011
HaRxL44	<i>Hyaloperonospora arabidopsidis</i>	Induces the degradation of MED19A and affects the balance of JA and SA, which affects defense-related transcriptional changes	Caillaud et al. 2013
MiSSP7	<i>Laccaria bicolor</i>	Interacts with the PtJAZ6 protein in the plant nucleus to prevent its degradation and block JA-induced changes in gene transcription	Plett et al. 2011
AvrL567	<i>Melampsora lini</i>	Binds to DNA <i>in vitro</i> , but virulence function is currently unknown	Wang et al. 2007
AvrPiz-t	<i>Magnaporthe oryzae</i>	Interacts with APIP6, a RING E3 ubiquitin ligase, to suppress MTI	Park et al. 2012
Avr-Pii	<i>Magnaporthe oryzae</i>	Interacts with OsExo70-F2 and OsExo70-F3, which are two proteins involved in exocytosis. Avr-Pii interacting with OxExo70-F3 is crucial for Pii mediated resistance	Fujisaki et al. 2015
Slp1	<i>Magnaporthe oryzae</i>	Sequesters free floating chitin present in the apoplast to prevent MTI	Mentlak et al. 2012; Chen et al. 2014
Mg1LysM and Mg3LysM	<i>Mycosphaerella graminicola</i>	Binds chitin at the fungal cell wall to protect against plant derived hydrolytic enzymes	Marshall et al. 2011
PexRD2	<i>Phytophthora infestans</i>	Interacts with the kinase domain of MAPKKK ϵ to interrupt plant defense signalling	King et al. 2014

Avr3a	<i>Phytophthora infestans</i>	Stabilize the E3 ligase CMPG1 in order to suppress HR during the biotrophic phase of development. Also associates with DRP2, which is involved in receptor-mediated endocytosis	Bos et al. 2010; González-Lamothe et al. 2006; Chaparro-Garcia et al. 2015
Pi03192	<i>Phytophthora infestans</i>	Prevents the transcription factors NTP1 and NTP2 from reaching the nucleus, which is required for full virulence of <i>P. infestans</i>	McLellan et al. 2013
PexRD54	<i>Phytophthora infestans</i>	Binds to the autophagy protein ATG8CL to alter autophagosomes	Dagdas et al. 2016
EPI1 and EPI10	<i>Phytophthora infestans</i>	Both inhibit the serine protease P69B	Tian et al. 2004; Tian et al. 2005
AVRblb2	<i>Phytophthora infestans</i>	Prevents the secretion of the cysteine protease C14 into the apoplast	Bozkurt et al. 2011
EPIC1 and EPIC2B	<i>Phytophthora infestans</i>	Targets and inhibits multiple cysteine proteases	Kaschani et al. 2010; Tian et al. 2007; Song et al. 2009
PSE1	<i>Phytophthora parasitica</i>	Redistributes the auxin efflux carriers PIN4 and PIN7 to alter auxin concentrations during disease development	Evangelisti et al. 2013
PsIscl1	<i>Phytophthora sojae</i>	Hydrolyzes isochlorismate, which is a precursor to SA to downregulate SA mediated defense responses	Liu et al. 2014
PSR1 and PSR2	<i>Phytophthora sojae</i>	Suppress RNA silencing in plants, which makes plants more susceptible to fungal and viral infection. PSR1 interacts with PINP1, a nuclear protein involved in small RNA processing	Qiao et al. 2013; Qiao et al. 2015
PsCRN63	<i>Phytophthora sojae</i>	Promotes cell death through the destabilization of plant catalases	Zhang et al. 2015
PsCRN115	<i>Phytophthora sojae</i>	Maintains proper hydrogen peroxide levels to suppress plant cell death	Zhang et al. 2015
PsCRN70	<i>Phytophthora sojae</i>	Suppresses the accumulation of hydrogen peroxide to make <i>N. benthamiana</i> more susceptible to <i>P. sojae</i>	Rajput et al. 2014
GIP1	<i>Phytophthora sojae</i>	Interacts with and inhibits the soybean endoglucanase protein EgaseA to prevent MTI	Rose et al. 2002
Cmu1	<i>Ustilago maydis</i>	Acts as a chorismate mutase to reduce SA levels during infection	Djamei et al. 2011
Tin2	<i>Ustilago maydis</i>	Interacts with ZmTTK1 to redirect metabolite production away from defense-related metabolites during	Tanaka et al. 2014

		infection	
Pit2	<i>Ustilago maydis</i>	Interacts with multiple maize cysteine proteases	Mueller et al. 2013
Pep1	<i>Ustilago maydis</i>	Interacts with the maize peroxidase POX12 to suppress ROS signaling	Hemetsberger et al. 2012;Doehlemann & Hemetsberger 2013
Vdlscl	<i>Verticillium dahliae</i>	Hydrolyzes isochorismate, which is a precursor to SA to downregulate SA mediated defense responses	Liu et al. 2014

Figure Legend

Figure 1. An effectoromics workflow begins with selecting candidate genes that fulfill some or all of the following criteria. Are upregulated during infection based on transcriptomics data generated from certain stages of disease development, contain a motif that is characteristic of other known effectors such as an RXLR or WY motif, and or contain an N-terminal signal peptide indicating that they are secreted. Additionally, effectors are often selected based on whether they show evidence of being under various selective forces, as well as their proximity to repetitive sequences within the genome. Finally, effectors are selected based on size, having been shown to be typically small proteins. Upon selection, recombinational cloning techniques such as Gateway or Golden Gate are typically employed for high-throughput cloning of candidates. Subsequently, candidates are mobilized into a number of different vectors for functional assays. Transient expression in planta is used to assay for induction of cell death, whereas the ability to suppress cell death when challenged with known plant cell death inducers is evidence for suppressor function. Tagging effectors with fluorescent proteins is commonly used to observe subcellular location. Identifying plant interacting partners is usually done through *in vitro* and *in vivo* techniques, such as yeast-two hybrid or co-immunoprecipitation followed by mass spectrometry. Resistance (R) proteins that recognize the effector can be identified by expressing the candidate effector in the leaves of isogenic plant varieties containing single R proteins. A hypersensitive response (HR) indicates that a particular R protein can recognize the effector and triggers a resistant response. Finally, measuring plant defense responses (such as the ROS burst or defense gene expression) in the presence of the effector provides insight as to how the effector modulates plant defenses for the benefit of the pathogen

Figure 1

1.) Candidate Effector Selection

2.) Recombinational Cloning

3.) Functional Characterization