Research review

Reprogramming of plant cells by filamentous plant-colonizing microbes

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Summary

Although phylogenetically unrelated, filamentous oomycetes and fungi establish similar structures to colonize plants and they represent economically the most important microbial threat to crop production. In mutualistic interactions established by root-colonizing fungi, clear differences to pathogens can be seen, but there is mounting evidence that their infection strategies and molecular interactions have certain common features. To infect the host, fungi and oomycetes employ similar strategies to circumvent plant innate immunity. This process involves the suppression of basal defence responses which are triggered by the perception of conserved molecular patterns. To establish biotrophy, effector proteins are secreted from mutualistic and pathogenic microbes to the host tissue, where they play central roles in the modulation of host immunity and metabolic reprogramming of colonized host tissues. This review article discusses key effector mechanisms of filamentous pathogens and mutualists, how they modulate their host targets and the fundamental differences or parallels between these different interactions. The orchestration of effector actions during plant infection and the importance of their localization within host tissues are also discussed.

Introduction

Significant advances have been made in recent years in understanding fundamental mechanisms of plant innate immunity and microbial virulence. Microbial signal molecules and their cognate plant receptors have been identified in a range of interactions, and components of the plant signal transduction pathways leading to various defence responses have been characterized. Also, complete inventories of microbial pathogenicity factors can now be identified, including secondary metabolites, toxins, lytic enzymes and effector (virulence) proteins, and their expression profiles *in planta* can be documented with great precision. For biotrophic pathogens, which depend on the integrity and survival of infected host tissues, effectors have been found to be critical pathogenicity factors

involved in the suppression of the plant immune system and in metabolic reprogramming (Rafiqi *et al.*, 2012; Yi & Valent, 2013).

Pioneering research has revealed the manipulative activities of effectors injected into the host cytoplasm via the bacterial type III secretion machinery from plant-infecting bacteria such as *Pseudomonas syringae* and *Xanthomonas campestris* strains (Büttner, 2012). However, work on prokaryotic effectors is not the subject of this review. Here, we instead focus on infection strategies of filamentous plant-colonizing microbes, comprising fungi and oomycetes. We will describe the most recent findings on effector–host target interactions and highlight common and contrasting strategies of fungi and oomycetes for suppressing basal plant immunity. There is mounting evidence that effectors deployed by unrelated pathogens converge on key plant targets, although

effectors with central immune-suppressive activities are often restricted to specific pathogen species. It is also increasingly evident that mutualistic fungi that can be beneficial to their plant hosts also depend on effector proteins, although their activities lead to fundamentally different interaction outcomes.

Common fungal and oomycete strategies for modulating plant immunity

Fungi and oomycetes are very successful filamentous pathogens of plants, spanning the full spectrum of infection lifestyles from necrotrophy through hemibiotrophy to obligate biotrophy (Stassen & Van den Ackerveken, 2011; Thines, 2014). Although not related to fungi, oomycetes adopt a 'fungus-like' mode of tissue colonization, with biotrophic strains producing hyphae that grow through the extracellular milieu and project haustoria into host cells for signal exchange, nutrient acquisition and delivery of effectors to host cells (Bozkurt *et al.*, 2012; Kemen & Jones, 2012). Comparative sequencing of the genomes of different fungal and oomycete pathogens enables us to define pathogenic strain lineages and unravel the evolutionary and molecular basis of host adaptation (Tyler *et al.*, 2006; Haas *et al.*, 2009; Baxter *et al.*, 2010; Levesque *et al.*, 2010; Kemen *et al.*, 2011). Computational predictions of evolutionarily conserved and diversifying effector classes provide a platform to explore effector activities during host–pathogen coevolution, and to use effectors as probes to dissect host defence reprogramming (Stassen & Van den Ackerveken, 2011; Goritschnig *et al.*, 2012). However, the complex interplay between plant and pathogen molecules in the apoplast and across haustorial and extrahaustorial membranes into the plant cytoplasm is only superficially understood, and little is known about effector translocation mechanisms compared with phytopathogenic bacterial effectors. An important question for understanding infection biology is which host processes are targeted to dampen plant resistance – are there many or a few key sites of pathogen interference and, in the case of obligate biotrophic pathogens, how do these invasive microbes fine-tune host defences without destroying cellular homeostasis? In this regard, a better understanding of fungal and oomycete modes of host manipulation is beginning to emerge.

Interactions at the cell periphery and apoplast

A major host barrier that virulent pathogens need to overcome is resistance triggered by specialized transmembrane receptors that recognize invariant microbial structures termed pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs, hereafter referred to as MAMPs; Nürnberger *et al.*, 2004; Zipfel, 2008; Boller & Felix, 2009). Typically, MAMP perception induces a series of immune responses referred to as MAMP-triggered immunity (MTI). Insights into the molecular mechanisms underlying interference by effectors from filamentous plant pathogens suggest that, like bacteria, evasion of MAMP recognition or subversion of MAMP-triggered signalling pathways are common strategies to grow and multiply on host plants (Bozkurt *et al.*, 2012; Giraldo & Valent, 2013; Yi & Valent, 2013).

Dampening or evading MAMP recognition can occur at the plant–microbial interface through the action of apoplastic effectors, which have been identified and characterized in several biotrophs and hemibiotrophs (Figs 1, 2). For example, prevention of the binding of chitin, an N-acetyl-D-glucosamine homopolymer and major structural polysaccharide of the fungal cell wall, to lysin motif (LysM)-containing plant receptors emerges as a paradigm for effector-mediated evasion of MAMP perception. The fungal effectors Avr4 and Ecp6 from the biotrophic tomato pathogen *Cladosporium fulvum* (van den Burg *et al.*, 2004; van Esse *et al.*, 2007; de Jonge *et al.*, 2010; Sanchez-Vallet *et al.*, 2013), Slp1 from the hemibiotrophic rice pathogen *Magnaporthe oryzae* (Mentlak *et al.*, 2012), and LysMs from the wheat hemibiotrophic pathogen *Mycosphaerella graminicola* (Marshall *et al.*, 2011) act as scavengers of chitin fragments and/or protect fungal cell walls from chitinase activity. Oomycete cell walls generally do not possess chitin and it is currently unclear whether recognition of *bona fide* oomycete MAMPs, such as elicitins (Yu, 1995), Pep13-containing transglutaminases (Brunner *et al.*, 2002; Reiss *et al.*, 2011), cellulose-binding elicitor lectin (Gaulin *et al.*, 2006) or hepta-glucan (Sharp *et al.*, 1984a,b), by cognate receptors at the host cell surface is targeted for interference by effectors.

In concert with targeting MAMP perception, a central strategy of plant filamentous pathogens is to interfere with the functions of plant apoplastic enzymes which are often induced upon infection. The glucanase inhibitor GIP1 delivered into the apoplast by *Phytophthora sojae* inhibits soybean β-glucanase EgaseA, thereby blocking the release of elicitor-active glucan fragments from the pathogen cell wall (Rose *et al.*, 2002). Diverse *Phytophthora* spp. secrete a large array of effectors bearing cystatin-like protease inhibitor domains against immunity-associated host papain-like cysteine proteases (PLCPs; Tian *et al.*, 2007; Song *et al.*, 2009; Dong *et al.*, 2014). In addition, the *Phytophthora infestans* effector ARVblb2 was found to prevent secretion of the plant PLCP C14 to the apoplast in order to prevent protease-induced activation of apoplastic immune signalling (Bozkurt *et al.*, 2011; Fig. 2). The substrates of the PLCPs remain elusive and a possible function of such *Phytophthora* effectors might be to prevent the release of elicitor-active fragments from proteinaceous MAMPs.

Inhibitors of PLCPs are also found in pathogenic fungi. The first reported example was Avr2 of *C. fulvum*, targeting the tomato proteases Pip1 and Rcr3, which are also targeted by the *Phytophthora* EPIC effectors (Rooney *et al.*, 2005; Song *et al.*, 2009). The fungal biotroph *Ustilago maydis*, causing smut disease in maize, secretes another effector, Pit2, which is essential for development of disease symptoms (Doehlemann *et al.*, 2011). Pit2 is an inhibitor of apoplastic PLCPs and this function was found to be necessary for dampening maize defence (Doehlemann *et al.*, 2011; Mueller *et al.*, 2013) (Fig. 2).

Analysis of the *Ustilago* system has uncovered another class of plant enzymes that are targeted by effectors, namely the apoplastic peroxidises (Hemetsberger *et al.*, 2012). Upon host penetration, *U. maydis* secretes the effector protein Pep1, which is conserved in the barley smut *Ustilago hordei*. Deletion mutants (*U. maydis* and *U. hordei*) for *pep1* are arrested during epidermal penetration and at the same time elicit strong plant defence responses, including
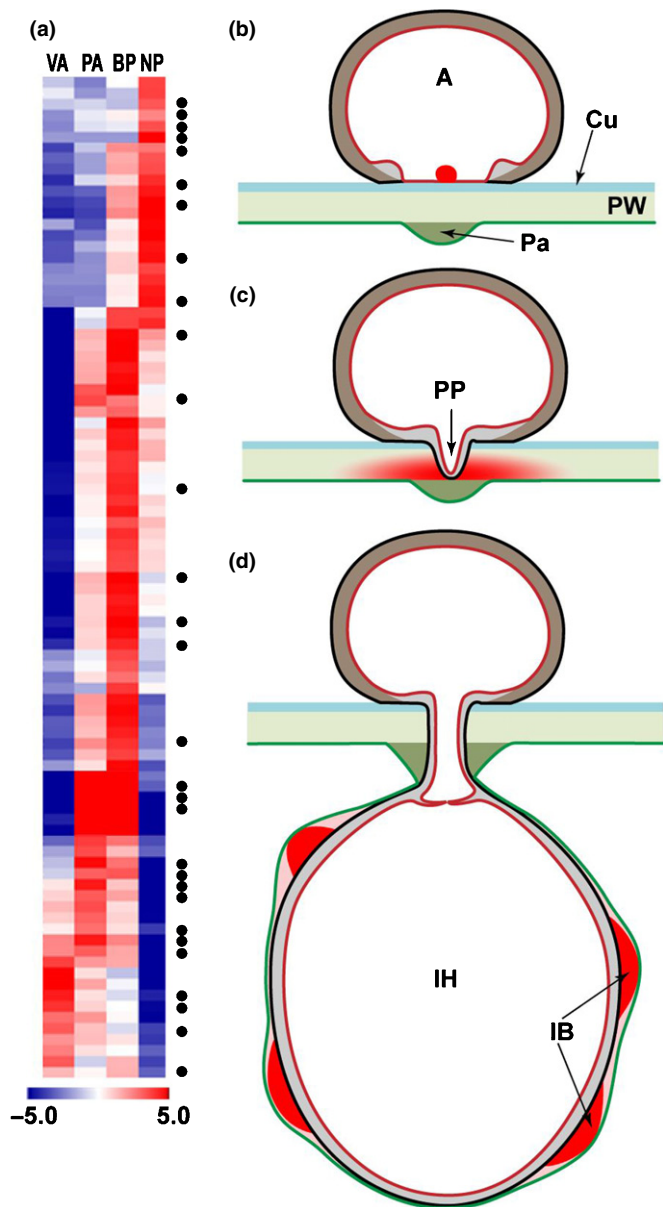
Fig. 1 Stage-specific expression and secretion of *Colletotrichum higginsianum* effectors. (a) Heat map showing waves of effector gene expression revealed by RNA-sequencing of appressoria *in vitro* (VA), appressoria *in planta* (PA), biotrophic phase (BP) and necrotrophic phase (NP). Overrepresented (red) and underrepresented transcripts (blue) are shown as $\log_2$ fold changes relative to the mean expression across all four stages. Candidate effector proteins predicted to be *N*-glycosylated (NetNGlyc) are indicated with black dots. (b–d) Focal secretion of effectors by *C. higginsianum* during and after host penetration. (b) The melanized appressorium (A) before penetration has already induced deposition of a plant papilla (Pa). The fungal plasma membrane (magenta) makes direct contact with the plant cuticle (Cu) inside the penetration pore. Effectors (red) accumulate in the pore before secretion. (c) A penetration peg (PP) emerges from the pore and penetrates the cuticle and plant wall (PW). Effectors (red) diffuse a short distance into the plant wall around the peg. (d) After penetration, a bulbous intracellular hypha (IH) develops inside a living epidermal cell. Some effectors (red) accumulate in interfacial bodies (IB) between the host plasma membrane (green) and fungal wall (grey). (Artwork by Guillaume Robin and Antonios Zampounis).

accumulation of extracellular reactive oxygen species (ROS) at sites of infection (Doehlemann *et al.*, 2009). In barley, epidermal cells attacked by *pep1*-deletion mutants show a rapid cell death response, which has hallmarks of autophagy and is distinct from the hypersensitive response (HR)-like cell death triggered in nonhost responses (Hof *et al.*, 2014). Its essential role in infection marks Pep1 as a 'core effector', suppressing PTI to allow establishment of fungal biotrophy. An intriguing question is whether other biotrophs also deploy Pep1-like effectors or have evolved alternative strategies to interfere with the extracellular, MTI-triggered oxidative burst.

Post-translational modification of apoplastic effectors is an important feature related to avoidance of immune stimulation. The described fungal LysM effectors are typically glycosylated and a recent study of *M. oryzae* showed that *N*-glycosylation by α-1,3-mannosyltransferase ALG3 is essential for the chitin-binding activity of the LysM effector Slp1 and for fungal invasive growth in rice cells (Chen *et al.*, 2014). Glycosylation of another apoplastic effector, BAS4, is reduced in the *M. oryzae* Δ*alg3* mutants. Moreover, *N*-glycosylation is critical for pathogenesis in *U. maydis* (Fernandez-Alvarez *et al.*, 2013), in which at least two apoplastic effectors, Pep1 and Pit2, are glycosylated. Similarly, in *C. higginisanum*, 31% of candidate effectors carry at least one predicted *N*-glycosylation site (Fig. 1a). Glycosylation therefore probably impacts effector function by modifying protein size, stability, conformation, hydrophobicity and/or resistance to host proteases.

Intracellular targeting and manipulation of postinfection host defences

Microbe-associated molecular pattern perception drives the activation of host intracellular signal transduction pathways, leading to the production of antimicrobial metabolites and hydrolases (Macho & Zipfel, 2014). Hence, successful establishment of infection relies on pathogen delivery of effectors inside host cells to dampen MTI signalling. One starting point for identifying candidate fungal and oomycete effectors that are delivered to the host apoplast or cytoplasm has been to catalogue pathogen-derived sequences expressed during infection and then classify effector types based on the possession of a predicted signal peptide and, in the case of oomycetes, host translocation motifs (Baxter *et al.*, 2010; Cabral *et al.*, 2011; Fabro *et al.*, 2011; Kemen *et al.*, 2011; Stassen & Van den Ackerveken, 2011). In oomycetes, computational prediction of one important family, the RXLR/EER effectors, was aided by the presence of conserved motifs in known 'avirulence' proteins that are recognized by Resistance proteins in ETI (Stassen & Van den Ackerveken, 2011). Data suggest that the RXLR and/or 'EER' motifs, positioned after the signal peptide and preceding variable C-terminal domains, contribute to effector delivery from haustoria to host cytoplasmic compartments (Whisson *et al.*, 2007; Kale *et al.*, 2010). Analysis of the genome effector complement in the oomycete biotroph *Albugo laibachii* revealed, besides the RXLRs, a novel class of translocated 'CHXC' effector (Kemen *et al.*, 2011). In fungi, no clear translocation motif has been identified, suggesting different mechanisms for delivery to host cells. In effector candidates of powdery mildews, a YxC motif was
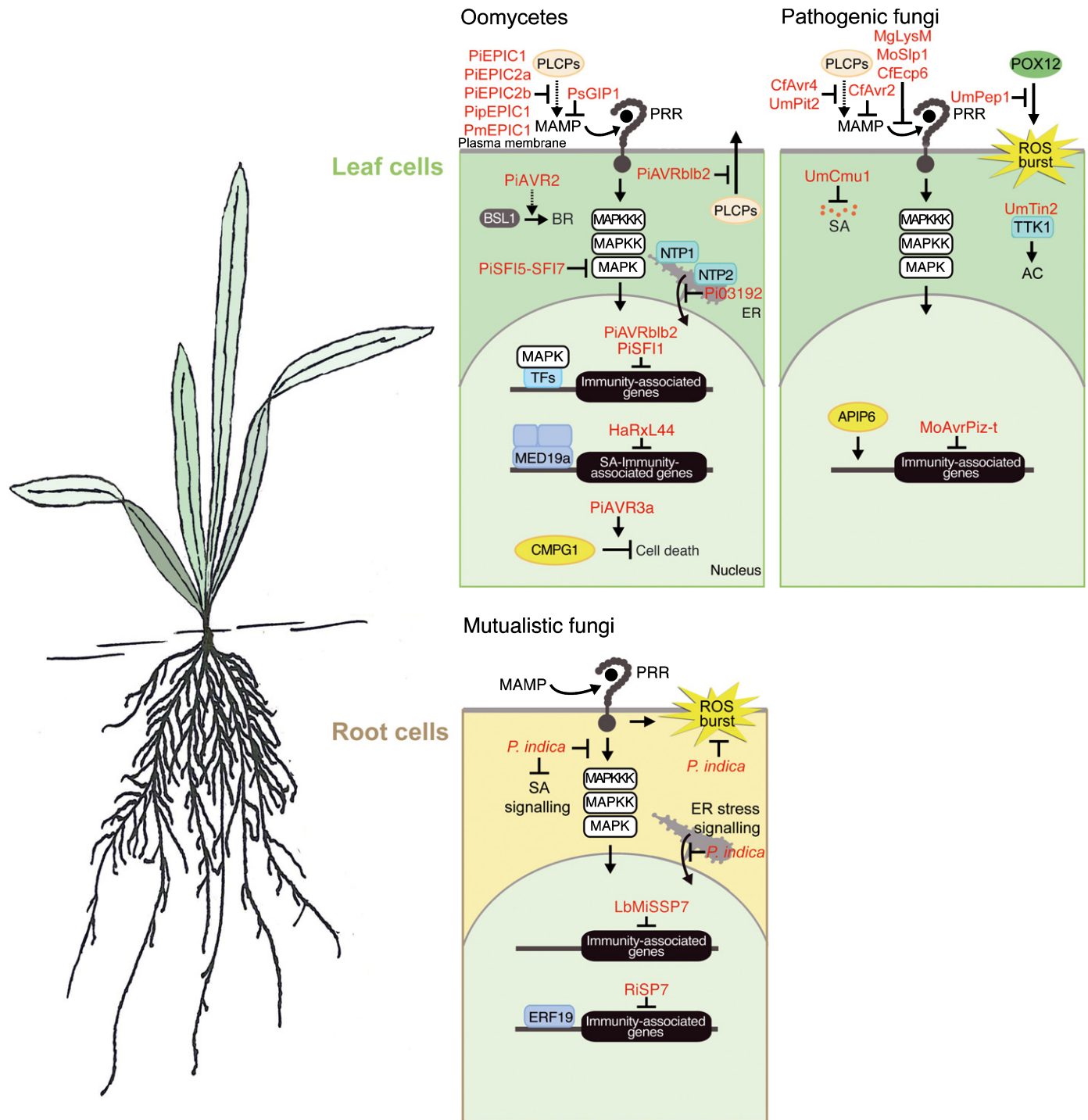
Fig. 2 Overview of the microbe-associated molecular pattern (MAMP)-suppressing function of apoplastic and intracellular effectors secreted by filamentous fungi/oomycetes. Perception of MAMPs by plant pattern recognition receptors (PRRs) initiates a MAMP-triggered immune programme (MTI) that is evolutionarily conserved in all plants. Both pathogenic fungi and oomycetes have coevolved effectors to compromise MTI. *Pirifomospora indica* exemplifies the need of mutualists to suppress root MTI and host signalling at different levels. Components of basic plant defence and interfering pathogen effectors (in red) are depicted. Solid line, demonstrated function; dashed line, hypothetical function. See main text for additional details. MAPK, mitogen-activated protein kinases; MAPKK, mitogen-activated protein kinase kinases; MAPKKK, mitogen-activated protein kinase kinase kinases; PLCPs, Papain-Like Cysteine Proteases; POX12, peroxidase POX12; TTK1, maize transcription factor bound by effector Tin2; AC, anthocyanin; SA, salicylic acid; ROS, reactive oxygen species; BR, brassinosteroid signaling, BSL1, BSU1-like ser/thr phosphatase; ERF19, Ethylene-Responsive Factor 19; ER, endoplasmic reticulum; NTP1-2, NAC targeted by *Phytophthora* 1-2; MED19a, Mediator subunit 19a; CMPG1, ubiquitin-protein ligase CMPG1; APIP6, AvrPiz-z Interacting Protein 6.

found to be enriched, but there is no evidence that this motif confers translocation ability (Godfrey *et al.*, 2010). Therefore, to define fungal effector candidates, small peptides with secretion signals remain the chief selection criteria (Petre & Kamoun, 2014).

Functional characterization of large families of candidate RXLR effectors derived from pathogenic oomycetes has been aided by high-throughput assays for suppression of immune responses induced by the elicitin INF1 (*Phytophthora*) or flg22 (bacterial MAMP) using *in planta* or cell culture systems (Oh *et al.*, 2009; Fabro *et al.*, 2011; Zheng *et al.*, 2014). Some important leads to the host targeting of effectors were provided by matrix-wide yeast two-hybrid analyses, as illustrated by the identification in Arabidopsis of interaction 'hubs' for *Hyaloperonospora arabidopsidis* (*Hpa*) effector proteins (Mukhtar *et al.*, 2011). Importantly, this study also revealed that Hpa effectors share several potential common targets with the bacterial pathogen *P. syringae* (Mukhtar *et al.*, 2011). One further screening strategy for candidate expressed RXLRs utilizes the type III secretion machinery (TTSS) of *P. syringae*, an Arabidopsis-infecting bacterial pathogen, to deliver C-terminal RXLR domains fused to the signal peptide of *P. syringae* effector AvrRps4 (Sohn *et al.*, 2007). This bacterial 'effector detection vector' (EDV) system was employed to search for candidate effectors dampening bacterial MAMP-triggered defences and enhancing bacterial growth and to prioritize RXLRs for deeper analysis (Cabral *et al.*, 2011; Fabro *et al.*, 2011). Also, transient expression in protoplasts provides a pathogen-free system to test MAMP signalling suppression activity of RXLR effectors (Zheng *et al.*, 2014). Although relatively high-throughput, these heterologous assays might miss certain maturation steps or post-translational modifications associated with haustorial delivery and have the potential to mislocalize effectors inside host cells (Fabro *et al.*, 2011). Transgenic hemibiotrophic oomycete effector delivery systems might provide a more faithful proxy for host translocation and cellular interference (Schornack *et al.*, 2010; Kemen *et al.*, 2011; Anderson *et al.*, 2012), although interpretation of such assays might be complicated by the action of endogenous effectors. Nevertheless, such heterologous assays revealed that RXLR effectors suppress different steps of MAMP-induced signalling. The finding that RXLR effectors SFI5-SFI7 of the hemibiotrophic potato pathogen *P. infestans* suppress early MTI signalling events – that is, those occurring within minutes of MAMP recognition, such as post-translational mitogen-activated protein kinase (MAPK) activation or a ROS burst – coupled with their localization at the host plasma membrane, suggests that these effectors target a MAMP receptor complex (Zheng *et al.*, 2014). Numerous RXLR effectors from the biotrophic Arabidopsis pathogen *Hpa* were also found to suppress a MAMP-elicited ROS burst (Fabro *et al.*, 2011).

Following early MTI events, nuclear transcriptional reprogramming of immunity-associated genes takes place. Notably, the *P. infestans* RXLR effector PITG_03192 was found to interact with the NAC transcription factors NTP1 and NTP2 at the host endoplasmic reticulum, thus preventing their relocalization into the nucleus following MAMP elicitation (McLellan *et al.*, 2013). Other studies have shown that a number of RXLR effectors from *H. arabidopsidis* and *Phytophthora* spp. target distinct subnuclear compartments where they modify host immune signalling, in some

cases through interaction with components of the plant transcriptional/post-transcriptional- or ubiquitin-proteasome degradation machinery (Caillaud *et al.*, 2012a,b, 2013; Zheng *et al.*, 2014). Nuclear localization of *P. infestans* SFI1 is also required for suppression of MAMP-induced gene expression (Zheng *et al.*, 2014). Moreover, *P. infestans* AVR3a interacts and stabilizes the host U-box E3 ubiquitin ligase CMPG1, causing suppression of INF1-induced host cell death (Bos *et al.*, 2010; Gilroy *et al.*, 2011). Interestingly, a similar mode of MTI interference was displayed by *M. oryzae* AvrPiz-t, which interacts with and destabilizes the RING E3 ubiquitin ligase APIP6 during infection (Park *et al.*, 2012).

Hyaloperonospora arabidopsidis HaRxL44 interacts with and destabilizes mediator subunit 19a (MED19a), a positive transcriptional regulator of salicylic acid (SA)-induced gene expression (Caillaud *et al.*, 2013). SA is an important MAMP-induced plant stress hormone in resistance against biotrophic pathogens (Vlot *et al.*, 2009). Thus, HaRXL44 manipulation of the host transcriptional machinery provokes a finely tuned shift in the balance of stress hormone signalling to favour the parasite. Manipulation of SA signalling by biotrophic fungi was also reported, as illustrated by Cmu1 from *U. maydis*, which attenuates the synthesis of SA by converting its precursor chorismate into the aromatic acid precursor perphenate (Djamei *et al.*, 2011). Hormonal changes and intricate crosstalk between different hormone signalling pathways are important for the execution and control of MTI and effector-triggered immunity (ETI) programmes (Tsuda *et al.*, 2009). A tradeoff was recently reported between MAMP-triggered immunity and brassinosteroid-controlled plant growth and development (Albrecht *et al.*, 2012; Belkhadir *et al.*, 2012; Lozano-Duran *et al.*, 2013). Therefore, the interaction established between *P. infestans* RXLR effector AVR2 with BSU1-Like ser-phospha-tase-1 (Saunders *et al.*, 2012), a positive regulator of brassinosteroid signalling in Arabidopsis, provides a useful molecular probe for testing the antagonism between MTI and BR signalling pathways.

Orchestration of effectors during plant colonization

Stage- and tissue-specific action of effectors

Many filamentous pathogens deploy their effector repertoires with great precision in a stage-specific manner. For example, among RXLR effector genes of the hemibiotrophic oomycete *Phytophthora sojae*, Wang *et al.* (2011) recognized three different expression patterns corresponding to induction at the early onset of infection, haustorium formation (biotrophy) and necrotrophic stage. Notably, while the early effectors suppressed ETI, haustorial stage effectors preferentially suppressed PTI (Wang *et al.*, 2011).

Similarly, during infection of Arabidopsis by the hemibiotrophic anthracnose fungus *Colletotrichum higginsianum*, effector genes are transcribed in a series of waves, suggesting that different subsets of proteins are required during appressorial penetration, biotrophic growth inside living host cells and the transition from biotrophy to necrotrophy (Fig. 1a; Kleemann *et al.*, 2012; O'Connell *et al.*, 2012). Also the barley powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* expresses distinct waves of effector candidates during early infection stages (Hacquard *et al.*, 2013). In the

U. maydis system, effector functions at specific stages of infection became evident from fungal gene knockout approaches. For example, the *U. maydis* Pep1 effector described earlier is required for initial host penetration, while deletion mutants for *pit2* are not impaired in epidermal penetration but fail to maintain biotrophy (Doehlemann *et al.*, 2009, 2011). Moreover, mutants for the Tin-effectors (Tin1-Tin5) that are encoded by the largest *U. maydis* effector cluster (Cluster 19a) show more specific effects at later stages of infection (Brefort *et al.*, 2014). Of particular interest is the effector Tin2, whose deletion results in a reduced size of *Ustilago*-induced tumours, and, most obviously, a complete loss of anthocyanin production in the infected maize tissue, which is a typical feature in wildtype infections (Brefort *et al.*, 2014). A set of elegant experiments revealed that Tin2 prevents degradation of the maize ZmTTK1 kinase, which regulates anthocyanin formation. By stabilizing ZmTTK1, Tin2 channels the host metabolism towards anthocyanin accumulation and away from pathogen-induced lignin formation. This, in turn, facilitates fungal prolif-eration towards the vascular tissue and expansion of tumours (Tanaka *et al.*, 2014; Fig. 2).

Another level of complexity in effector-orchestrated manipula-tion of host cells comes from the finding that *U. maydis* effectors not only act in a stage-dependent manner but are also activated depending on the infected host organ. A transcriptomic approach found that the pathogen expresses distinct sets of effector genes when colonizing leaves or inflorescences (Skibbe *et al.*, 2010). This organ specificity could be functionally verified by a knockout screen of effector-candidate genes (Schilling *et al.*, 2014). Strikingly, a set of nine *U. maydis* effectors was found to be required for tumour formation in a strictly organ-specific manner – that is, the respective deletion mutants were impaired in symptom formation only in leaves, while staying fully virulent in flower infections or vice versa (Schilling *et al.*, 2014).

Establishing that effectors are deployed in a tissue-specific manner raises the question of how a pathogen senses its specific host environment to tailor expression of its infection weaponry. Information on mechanisms regulating effector gene expression is still scarce, but in many pathogens these genes are activated specifically during growth *in planta* and not in infection structures formed *in vitro*, suggesting that they depend on plant-derived cues. In *C. higginsianum*, comparison of the transcriptomes of appress-oria formed on polystyrene and on Arabidopsis leaves revealed that > 1500 genes were induced by host contact, including numerous effector genes and 12 secondary metabolism gene clusters (O'Connell *et al.*, 2012). In this instance, host recognition was mediated by the mature, melanized appressoria of the fungus before penetration. Given that the cell walls of *Colletotrichum* appressoria, like those of *Magnaporthe*, are highly impermeable, the 200-nm-diameter penetration pore at the base of the cell, where the fungal plasma membrane makes direct contact with the plant cuticle, may provide a nanoscale window for the fungus to perceive host signals (Fig. 1b). Thus, in addition to their well-established roles in adhesion to plant surfaces and penetration, appressoria appear to function as sensing organs.

Very few transcriptional regulators of effector genes have been identified to date. Examples are SGE1 in *Fusarium oxysporum*

(Michielse *et al.*, 2009) and FOX1 in *U. maydis* (Zahiri *et al.*, 2010). However, a recent study of *Leptosphaeria maculans* (a *Brassica*-infecting ascomycete pathogen) suggests that the coordi-nated expression of effector genes during infection of oilseed rape is at least partially controlled through epigenetic mechanisms (Soyer *et al.*, 2014). Thus, RNAi silencing of two key heterochromatin regulators, *HP1* and *DIM5*, resulted in chromatin decondensation and the derepression of numerous effector genes during growth *in vitro* (Soyer *et al.*, 2014).

Targeting of effectors to the biotrophic interface

Two *C. higginsianum* effectors, ChEC6 and ChEC36, accumulate inside the appressorial pore before the penetration peg breaks through the plant cuticle, as revealed by confocal imaging and immunogold labelling of fluorescent protein-tagged effectors (Kleemann *et al.*, 2012; Fig. 1b,c). During penetration, these effectors are then secreted into a small region of the plant cell wall directly beneath the appressorium. Thus, the appressorial pore also provides a portal for the focal secretion of effectors at the penetration site. In the oomycete *Phytophthora parasitica*, a subset of RxLR effectors are presumed to be secreted by appressoria during penetration of host roots (Evangelisti *et al.*, 2013). Among these, PSE1 was found to perturb auxin physiology, raising the possibility that this effector locally modulates auxin concentrations at the penetration site. However, most effectors of filamentous plant pathogens have been identified from haustoria or intracellular hyphae formed after penetration into host cells. These infection structures are typically enveloped by a specialized host-derived membrane termed the extrahaustorial (or perihyphal) membrane, across which effectors must be translocated to reach the plant cytoplasm. Beautiful work has shown haustorial secretion and uptake by the plant cell for the flax rust (*Melampsora lini*) effectors AvrL567 and AvrM (Rafiqi *et al.*, 2010). Recently, the crystal structure of AvrM was solved, identifying functionally important effector surface domains for host cell entry and, in certain flax genotypes, detection by the plant immune system (Ve *et al.*, 2013).

Analysis of the rice blast fungus *M. oryzae* suggests the existence of two distinct secretion pathways to target effectors to this interfacial zone. Effectors destined for translocation into host cells (cytoplas-mic effectors) preferentially accumulate in a novel compartment called the biotrophic interfacial complex (BIC), which forms at the tips of primary hyphae soon after host cell entry and is enriched with plant membrane material (Giraldo & Valent, 2013; Giraldo *et al.*, 2013). By contrast, apoplastic effectors do not enter host cells and accumulate more uniformly over the entire fungal cell surface. Using a combination of pharmacological and genetic approaches, Giraldo *et al.* (2013) showed that while apoplastic effectors are secreted via the classical endoplasmic reticulum (ER)–Golgi route, secretion to the BIC engages an unconventional pathway that involves exocyst components Exo70 and Sec5 and the t-SNARE Sso1.

Structures resembling BICs, termed interfacial bodies, are also present on the intracellular hyphae of *C. higginsianum*, located between the fungal wall and host plasma membrane, where they similarly act as foci for the accumulation of a subset of effector proteins (Kleemann *et al.*, 2012; Fig. 1d). However, in contrast to

BICs, interfacial bodies are not enriched with plant membranes, and they are also smaller, more numerous and randomly distributed over the fungal cell surface. Whether *Colletotrichum* effectors arrive at interfacial bodies via an alternative secretion pathway is currently unknown. BIC-like structures have not yet been found in haustorium-forming pathogens, but in some rust fungi, long, tubular extensions of the extrahaustorial membrane protrude far into the host cytoplasm. In a recent study of the bean rust pathogen *Uromyces fabae*, it was found that the effector RTP1 accumulates within these protuberances before being translocated into the host cytoplasm (Kemen *et al.*, 2013). Remarkably, RTP1 can self-assemble into filaments *in vitro* and *in planta*, through a process of β-aggregation, similar to amyloid proteins, but the function of RTP1 in pathogenesis remains unclear. In the case of haustoria of the powdery mildew *Golovinomyces orontii* infecting Arabidopsis, the extrahaustorial membrane is much thicker than the normal plant plasma membrane and becomes highly convoluted, with elaborately branched evaginations protruding into the extrahaustorial matrix (Micali *et al.*, 2011). An important challenge for future research will be to determine whether any of these v interface compartments serve as sites for the localized transfer of effectors into host cells (Kemen *et al.*, 2013).

Host colonization by mutualists vs pathogens – same hurdles, different outcomes

Colonization by beneficial microbes (mutualists) provides various benefits to plants. These range from an improved nutrition (e.g. mycorrhizas, N_2-fixing rhizobia) and plant development to enhanced plant stress adaptation (e.g. sebacinoid endophytes; Parniske, 2008; Bonfante & Requena, 2011; Qiang *et al.*, 2012a; Oldroyd, 2013). Mutualistic symbioses provide adaptive flexibility and competitiveness for plants to conquer new ecological niches and habitats (Redman *et al.*, 2002; Weiss *et al.*, 2011). In order to benefit the plant, mutualists need to colonise the host root system using a biotrophic or hemibiotrophic strategy. During the interaction, the plant plasma membrane is invaginated to establish nutrient exchange organs (e.g. arbuscules), which, although morphologically and functionally resembling pathogenic feeding structures such as haustoria (Parniske, 2000), reflect fundamentally different interaction outcomes. While pathogenic haustoria aid nutrient acquisition by the invading fungus, mutualistic organs like arbuscules mediate the bidirectional exchange of nutrients between host and fungus. The establishment of such interfaces is as demanding for mutualists as it is for pathogens and requires a high degree of host adaptation and communication (Parniske, 2008; Spanu, 2012; Oldroyd, 2013). Plants have developed various chemical strategies to attract mutualists, which in the case of arbuscular mycorrhizal fungi (AMF) have evolved to a sophisticated molecular dialogue (Bonfante & Requena, 2011; Schmitz & Harrison, 2014). Plants release strigolactones to induce hyphal branching and root-directed fungal growth (Akiyama *et al.*, 2005), and AMF produce a cocktail of molecules, known as Myc factors, including lipochitooligosaccharides (Myc-LCOs), short chitooligosaccharides (COs) and possibly other as yet uncharacterized substances, to prepare root cells for colonization (Bonfante &

Requena, 2011; Maillet *et al.*, 2011; Genre *et al.*, 2013; Nadal & Paszkowski, 2013). Some of these Myc factors are supposed to be specifically recognized by the extracellular LysM motif of plasma membrane-localized receptor like kinases to activate a signalling cascade that is required for root mycorrhization.

Establishment of a beneficial symbiosis, irrespective of whether it is accompanied by precolonization communication, does not prevent the activation of immunity against the attracted mutualists. Root cells are equipped with an acutely sensitive immune system that does not necessarily distinguish between mutualists and pathogens (Jacobs *et al.*, 2011; Kloppholz *et al.*, 2011). Thus, mutualists also exhibit an array of immunoactive MAMPs (e.g. chitin) and crude extracts from their hyphae or spores are as potent as pathogen-derived MAMPs in MTI (Jacobs *et al.*, 2011; Kloppholz *et al.*, 2011). If not suppressed, plant defence responses to mutualist-derived MAMPs can even abort the interaction, underlining the exquisite fine-tuning of root immunity required to control mutualist colonization (Jacobs *et al.*, 2011; Kloppholz *et al.*, 2011). The extent to which the mycorrhization pathway interferes with immune signalling is still unclear.

Nodulation (Nod) factors used by N_2-fixing rhizobia for nodulation in legumes are chemically related to Myc-LCOs and have immunosuppressive activities in both soybean and the AMF nonhost Arabidopsis, indicating that this response might be independent of Myc-factor receptors (Liang *et al.*, 2013). Interestingly, heterologous coexpression of *Medicago truncatula* Nod-factor receptors, MtLYK3 and MtNFP, induced immunity in *Nicotiana benthamiana* leaves (Pietraszewska-Bogiel *et al.*, 2013), while Medicago *nfp* mutants lacking MtNFP were more susceptible to the root pathogens *Colletotrichum trifolii* and *Aphanomyces euteiches* (Rey *et al.*, 2013), indicating the immune-activating capacities of these receptors. Hence, 'symbiotic' LysM receptors might recognize structurally related MAMPs (e.g. chitin oligomers) in addition to Nod factors (and perhaps Myc factors) (Gust *et al.*, 2012). These studies indicate that plant perception of mutualistic microbes, in particular AMF, is a complex process in which the perceived fungal signals need to be integrated by distinct receptors and signalling pathways to produce the desired output. Therefore, AMFs have developed different strategies to inhibit root immunity. In addition to the delivery of short-chain COs and Myc-LCOs to elicit the symbiotic pathway (Maillet *et al.*, 2011; Genre *et al.*, 2013), AMFs deliver effector proteins to counteract defence pathways (Kloppholz *et al.*, 2011) (for details, see the following section). Considering the potential signal 'overload' experienced by roots at the rhizosphere (Bakker *et al.*, 2013; Bulgarelli *et al.*, 2013) as well as the ability of plant parasites (e.g. *Striga* sp.) and eventually even pathogens to hijack plant-mutualist communication and signalling to locate and colonize roots (Cook *et al.*, 1966), plants might ultimately rely on an immune system that cannot discriminate between pathogens and mutualists.

Effectors in mutualistic interactions – trailblazers or triggers of mutualism?

The effectiveness of MTI relies on three steps: MAMP perception, rapid activation of signalling cascades and translation of signalling

into MTI (e.g. by the ER). The mutualistic fungus *Pirifomospora indica* blocks immune signalling at a point immediately downstream of MAMP recognition. While the fungus does not apparently interfere with MAMP recognition itself, it stalls the MAMP-triggered oxidative burst, MAPK phosphorylation and defence gene expression (Jacobs *et al.*, 2011; P. Schäfer, unpublished). In addition, *P. indica* reduces the execution of immune responses by disturbing ER-triggered stress signalling and thereby synthesis of antimicrobial proteins (Qiang *et al.*, 2012b; Fig. 2). Effector candidates have been identified in the *P. indica* genome (Zuccaro *et al.*, 2011; Lahrmann *et al.*, 2013) and the fungal capacity for specifically blocking MTI and ER signalling suggests employment of effectors by *P. indica* in a similar manner as pathogens (Fabro *et al.*, 2011; Saunders *et al.*, 2012; Zheng *et al.*, 2014).

A recently described AMF effector is the protein SP7, which is delivered by *Glomus intraradices* to plant cell nuclei where it interacts with the transcription factor ERF19, a member of the AP2-EREB family participating in *Medicago* immunity (Kloppholz *et al.*, 2011). Perception of AMF MAMPs induces expression of ERF19, which is sufficient to suppress mycorrhizal colonization. The *Glomus* effector SP7 blocks expression of ERF19 and promotes root colonization by the AMF *Rhizophagus irregularis* (Kloppholz *et al.*, 2011). Similarly, the ectomycorrhizal fungus *Laccaria bicolor* uses the effector MiSSP7 to modify the transcriptome of *Populus trichocarpa* root cells (Plett *et al.*, 2014). MiSSP7 interacts with the transcription factors JAZ5 and JAZ6 from *P. trichocarpa* to modulate host jasmonate-related developmental pathways and promote the Hartig net formation; a hyphal network that surrounds root cells to establish bidirectional nutrient exchange (Plett *et al.*, 2014). These studies show that mutualistic symbionts are able to interfere with MTI and host signalling and rely on manipulation of host defences for successful root colonization. The finding that mutualists use effectors to reprogram host immunity immediately raises the question of why these effector activities are not detected by the root immune system, as mobilization of ETI has not been observed in these symbioses. It is unlikely that mutualistic effectors alter host signalling in a way that is not detectable by R proteins. One possibility to explain the absence of ETI in mycorrhizal infections is that AMF have opted to reduce their effector arsenal to avoid stoking an arms race, similar to their limited repertoire of cell wall-degrading enzymes (Tisserant *et al.*, 2013). Two recent releases of the long-awaited genome of the first AMF, *Rhizophagus irregularis*, tend to support this idea (Tisserant *et al.*, 2013; Lin *et al.*, 2014). Thus, Lin *et al.* (2014) showed that the secretome of *R. irregularis* is significantly depleted (between 1 and 2% of the proteome) compared with pathogenic fungi. However, annotation of the *R. irregularis* genome is not yet complete and preliminary data suggest that several effector genes require reannotation before concluding absence (R. Betz & N. Requena, pers. comm.). Alternatively, plants might have evolved organ-specific differences in effector detection as suggested by a study in *Arabidopsis* in which the accession Ws-0 carrying the RPP1 R gene cluster mediates ETI against a recognized strain of the oomycete pathogen *H. arabidopsidis* in leaves but not in roots (Hermanns *et al.*, 2003). Mutualists might have evolved additional

strategies to avoid recognition or be tolerated by plant root cells. Obligate biotrophs are dependent on host photosynthates (e.g. carbohydrates) for reproduction. It is also well established that plant cells monitor their energy status and activate stress signalling under nutrient starvation (Baena-Gonzalez & Sheen, 2008). Moreover, energy deprivation triggers an immune response in mammals that is independent of Toll and immune deficiency (IMD) pathways but requires the nuclear activity of FOXO, a key transcription factor of immune signalling upon nutrient deficiency (Becker *et al.*, 2010). The delivery of nutrients by mutualists (e.g. phosphate by AMF), together with the status of roots as a sink tissue for photosynthates and other primary metabolites, might help to avoid activating immunity, and mutualists might specifically use effectors to control the nutrient status or hormone pathways associated with energy metabolism in colonized root cells (Eveland & Jackson, 2012). The ability of mutualists to produce hormones (e.g. auxin by *P. indica*) or modify hormone signalling (Jacobs *et al.*, 2011; Hilbert *et al.*, 2012; Floss *et al.*, 2013) might therefore represent a strategy to maintain symbioses in addition to overcoming immunity at early interaction stages.

Conclusions and challenges for the future

Detailed molecular and cytological studies combined with genome and expression datasets produced by next-generation sequencing technologies have massively enhanced our understanding of plant host–microbe interactions over the last decade. The known microbial effector repertoires are helping to determine host range and adaptation strategies (Spanu *et al.*, 2010; de Wit *et al.*, 2012; Lahrmann *et al.*, 2013). Accumulating data support an evolutionary concept which connects nonhost resistance, pathogen host range and host specialization. This view on plant–microbe compatibility concludes that changes in pathogen host range are driven (and reflected) by effector variation (Schulze-Lefert & Panstruga, 2011), as was nicely shown for the *Phytophthora* EPIC protease inhibitors (Dong *et al.*, 2014). Future functional studies will be able to address epistatic relationships between particular effectors in and between cell compartments and the apoplast during disease progression. Also, novel effector classes, particularly those with noncanonical secretion and uptake mechanisms, will probably be discovered by exploiting comparative genome evolution and collecting more effector crystal structure data to understand more completely the molecular basis of effector uptake, action and evolution (Chou *et al.*, 2011; Leonelli *et al.*, 2011; Ve *et al.*, 2013).

Mutualists and pathogens secrete effectors to redirect plant host signalling and metabolism for colonization (Kloppholz *et al.*, 2011; Plett *et al.*, 2014). Effectors of mutualists and pathogens probably target an overlapping set of host processes, because both microbial groups are committed to overcoming immunity (Zamioudis & Pieterse, 2012) and modulating plant metabolism for accommodation (Fig. 2). In clear contrast to mutualistic effectors, the concerted effector actions of pathogens result in disease. Are there fundamental distinctions between mutualistic and pathogen effectors, and, if so, what ultimately determines these two very different host–microbial outcomes? Answers to these questions will provide clues to understanding mutualism to the same extent as

pathogenic interactions. Future approaches will probably take advantage of synthetic biology solutions to elucidate differences between pathogenic and mutualistic outcomes. Particularly for mutualistic interactions, we expect microbe–microbe communication within the complex root microbiome to be of fundamental relevance. Investigating this as yet largely unexplored territory should lead to a more comprehensive view of plant–microbe biology.

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