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Cell Communication and Fungal Virulence: A Review

Tinashe J Runyanga*, Yubin Chen and Fei Sun

Department of Plant Pathology, South China Agricultural University, Guangzhou, 51064, China

Review Article

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***For Correspondence**

Runyanga TJ, Department of Plant Pathology, South China Agricultural University, Guangzhou 510642, China, Tel: +8620-38604779, +861 3246839824; Fax: +862 0-85280292.

E-mail: tina123run@yahoo.co.uk

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ABSTRACT

Effective message conveyance is a fundamental aspect for all living organisms. Fungal microbes utilise multifaceted signal transduction systems to perceive and respond to a broad diversity of environmental cues. Well-studied signalling pathways include the protein kinase A/ cyclic AMP (cAMP), protein kinase C (PKC)/mitogen-activated protein kinase (MAPK), cascades, and the calcium–calcineurin signalling pathway. Fungal microbes also rely heavily on effector protein secretion upon coming into contact with the appropriate host cell surface. Intra or inter-cellular communications do exist during sex in the true fungi (Ascomycota and Basidiomycota). Sex pheromones are implicated to mediate mating partner recognition. Unravelling the mechanisms of fungal communication and response to environmental stimuli could open up new technological innovations on how to manage fungal contamination and infestations within biological ecosystems. However, due to the complexity of microbial cellular systems and their species diversity, much of the information and knowledge gained over years on fungal communication is still scattered, a scenario which could hinder research initiatives that can reduce fungal virulence among agricultural systems. Therefore, this review discusses cellular communication pathways that directly or indirectly lead to fungal virulence in order to provide research insights towards virulence reduction. Mechanisms discussed include, but are not limited to, signal transduction systems, cell surface receptors, effector proteins and sex pheromones.

INTRODUCTION

In host-fungi systems, cellular dialogue can be friendly or hostile. Hostility is typically confined to pathogenic fungi when interacting with their preferred hosts, whereas, friendly relations exist among symbiotic strains. A plethora of research output information on how pathogenic fungi convey messages to establish successful host infection is available [1-5]. In the infection process, penetration is facilitated by reception and transduction of external signals, which trigger developmental and morphogenetic processes preceding host penetration [6-9]. A diversity of extracellular effector molecules and morphogenic proteins plays key roles in signal transduction, morphogenesis and manipulation of the host [10]. Communication by extracellular signals usually involves six steps; 1) synthesis of signal molecules, 2) release of signal molecules, 3) transport of signals to target cells, 4) detection of signal by specific receptor proteins, 5) change in cellular processes triggered by receptor-signal complex and 6) removal of signals which often eliminate cell response [10,11]. Effectors are secreted by the pathogens' secretion system. So far cell secretion systems (Type I-IV) in addition to the recently discovered Type VI have been identified. Among them T3SS (Type III secretion system) and T4SS (Type IV secretion system) deliver effectors directly into the host cell without going through extracellular matrix. Apoplastic effectors are secreted into extracellular spaces where they affect extracellular targets and surface receptors, whereas cytoplasmic effectors are translocated inside the host cell, usually through specialized structures like haustoria [12]. Such molecules are secreted into the intercellular interface between the pathogen and the host or are delivered inside the host cell [6]. Extracellular effector proteins associated with virulence among pathogenic fungi include hydrolytic enzymes, hydrophobins, metallothioneins and tetraspanins [13].

Fungal mating processes across sexually reproducing species involve complex pathways required to trigger partner recognition, hyphal fusion (anastomosis) and subsequent nuclear fusion (karyogamy) between partners [14]. The biochemical processes that control the dialog between potential fungal mating partners (intra-specific mating) among different fungal groups are very diverse. Yeast (*Saccharomyces cerevisiae)* uses mitogen-activated protein kinase (MAP) pathways to respond to spectra

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of environmental stimuli to control cellular processes like morphogenesis and stress adaptation [15]. The structural organisation of such MAP signal transduction pathways is conserved from yeast to mammalian cellular systems. In spite of interesting research findings available in the fungal research community regarding MAP signalling and pathogenesis, fungal microbes are continuously causing havoc in agriculture. The ultimate objective of a plant disease mycologist is to come up with a better therapeutic method for a disease.

Cellular communication pathways are major targets for attempting to design novel antifungal drugs and agro-chemicals. The plasma membrane is presumed to be the major site for information reception in eukaryotes and is always the target of most antifungals including fungicides. Concisely summarising and discussing what is currently known with respect to cell communication and virulence from well-studied fungal systems will provide insights and research guides to researchers working on non-model fungal species such as the sugarcane smut fungi (*Sporisorium scitamineum)*, whose genome sequence has been recently published [16]. Therefore, this review will provide an overview of cell signalling with much emphasis being given to phytopathogenic fungi. Potential practical implications of understanding cell-cell communication are provided.

FUNGI MATING SYSTEMS AND CELL COMMUNICATION

Mating systems in fungi model organisms give strong evidence of the importance of cellular communication. Homothallism, and heterothallism are two mating systems common in fungi **(Figure 1)**. Homothallism is when compatible mating occurs between mitotic descendants of the same haploid genotype. It is achieved through several mechanisms including: a) presence of both mating type alleles in a haploid genome b) replacement of the active cassette in the mating type locus by a copy of the alternative transcription factor gene(s) that is present elsewhere in the genome, such as what has been discovered in ascomycete yeasts [17]. c) endoduplication that restores the diploid condition or fusion of two haploid nuclei carrying the same mating type allele [18-20].

However, pseudo-homothallism is exhibited in some fungi where post-meiotic haploid nuclei of opposite mating types are packaged into spores for dispersal so that restitution of the diploid state can always be made possible within the same cell. Hence, there is no requirement for scavenging for a compatible partner for conjugation [21-22]. Heterothallism is when mating can only occur between individuals of different mating types **(Figures 1A and 1B)**. Two forms do exist namely bipolar heterothallism and tetrapolar heterothallism. Bipolar mating is where the mating type is governed by single genetic loci that harbour only two alleles and thus yield only two compatible mating types [22]. Most basidiomycete fungi exhibit a tetrapolar mating system that is governed by two loci, with mating only possible between haploid cells carrying different alleles at both loci. In these species, tightly linked genes resident on the first locus encode pheromones and pheromone receptors involved in regulation of cell fusion. The second locus encodes homeodomain proteins that function in non-self-recognition, controlling post-fusion compatibility [23]. Independent segregation of the two mating type loci in tetrapolar systems is associated with high levels of allelic polymorphism, yielding potentially thousands of mating types, hence the need for tight cell communication and signalling for proper signal transmission in order to give a desired output at various levels of growth and developmental processes.

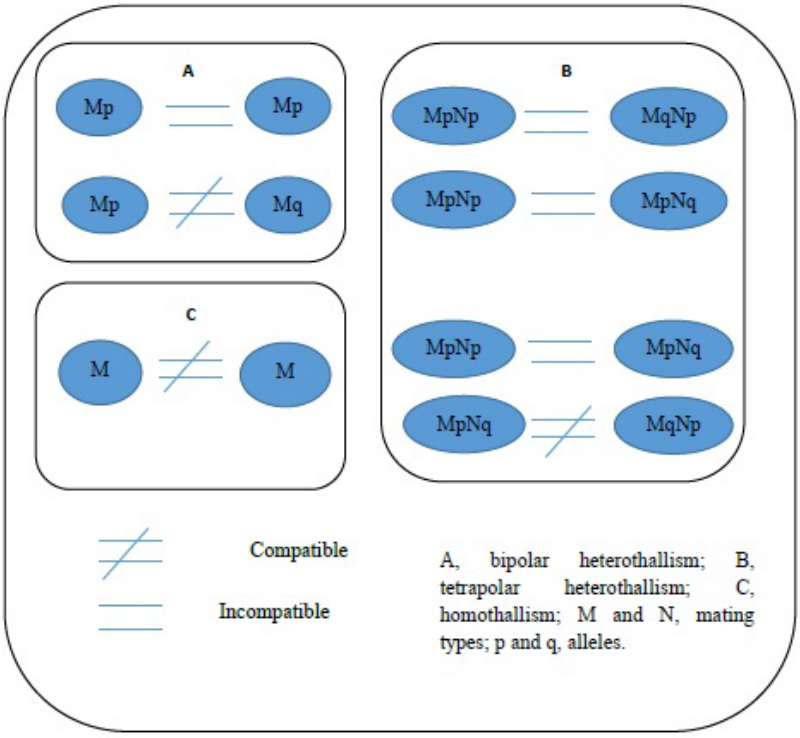

Figure 1. Mating systems known among fungal microbes.

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MAP KINASE SIGNALLING PATHWAYS

MAPKs are highly conserved protein modules in eukaryotic cells that are used for sensing and responding to changes in the outside environment [24,25]. MAPK pathways are co-ordinated cascades of phosphorylation reactions that convey signals from the cell surface to the nucleus. The core of MAPK pathways consists of three kinases. The downstream MAPK is activated by a MAP kinase kinase (MAPKK), which in turn is activated by a MAP kinase kinase kinase (MAPKKK) [26]. The mechanisms of sensing and response to external signals vary and determine the specificity of each pathway. Specialized scaffold proteins also participate in MAPK pathways to facilitate signalling fidelity. Sensors are heterotrimeric G-protein-coupled receptors or other membrane-bound receptors, whose responses typically trigger transcriptional activation/repression of appropriate genes [27].

In bakers' yeast there are at least five MAPK pathways, which mediate the response to pheromones during mating, trigger filamentous growth during nitrogen starvation, respond to osmotic shock and other stresses, cope with cell wall stress or initiate sporulation [28]. Among human pathogenic fungi, APK pathways have been well characterized in *C. albicans* (Monge). This is partly because the MAPK pathway mediates the dimorphic yeast to initiate hypha transition, a morphological change essential for infection with *Candida*. Similar to other pathogenic fungi, MAPK pathways in *C. neoformans* are crucial for its survival in the host [29]. In the corn smut disease fungi *Ustilago maydis,* mating and pathogenic development is regulated by a MAPK cascade involving the three known major kinases-MAPKKK, MAPKK and MAPK [30]. In terms of their role in information transfer, MAPKs cascades have dual roles in some key fungal cellular life stages. MAP kinases are involved in mating response, filamentation and invasive growth in *S. cerevisiae* [31]. Generally, a common feature of signalling pathways is that they do not operate in isolation but rather form a fine-tuned network consisting of cross-talks, feedback loops and compensatory mechanisms. Deciphering novel pathogenesis-related signalling processes associated with the MAP kinase cascade should be expected to yield sound therapeutic measures in fungal pathogens of crop plants.

Cyclic AMP (cAMP) Signalling Pathway

Many pathogenic fungi utilize the cAMP signalling pathway to trigger diverse cellular responses, including virulence. Membrane-bound adenylyl cyclase (Cdc35) converts ATP into cAMP, which serves as a second messenger. cAMP then activates protein kinase A (PKA), which in the resting state is a heteromeric tetramer with coupled regulatory (Bcy1) and catalytic subunits (Tpk1, Tpk2 and Tpk3) [32,33]. In the absence of cAMP, the regulatory subunit is bound to the catalytic subunit and inhibits kinase activity. Binding of cAMP to the regulatory subunits dissociates the catalytic and regulatory subunits activating protein kinase A (PKA) [34]. Subsequent phosphorylation of downstream targets by PKA induces cellular responses, including nutrient sensing, growth regulation, stress responses and pseudohyphal growth in *S. cerevisiae* [35]. In the human basidiomycetes fungal pathogen, *Cryptococcus neoformans,* the cAMP- mediated signalling pathway also contributes to cellular responses that initiates virulence traits such as melanin production, capsule formation and invasive hyphal growth.

The Cryptococcus capsule is one of the pre-eminent virulence factors and is considered to protect the pathogen from the host defence [36]. Capsule production is induced in response to carbon dioxide (CO_2) levels present in the host [37]. The cAMP–PKA pathway is involved in a signalling relay through both a CO_2 sensing system and a G-protein-coupled receptor system to produce and properly locate virulence factors [38]. Thus, the cAMP pathway is a central regulatory conduit governing virulence of *C. neoformans*. Taken together, the cAMP–PKA signalling pathway could be a potential target for developing therapeutic fungicides.

Ca^{2+}/Calcineurin Signalling Pathway

Calcineurin (CN) is a highly conserved Ca^{2+}/calmodulin regulated type 2B protein phosphatase that is crucial for mediating cellular stress responses. Stress-induced transient increases of intracellular Ca^{2+}/ concentrations from either intracellular stores or extracellular sources are sensed by calmodulin (CaM), a small protein containing four E-helix and F-helix (EF-hand motifs) for binding Ca^{2+} ions. The Ca^{2+} -CaM complex then activates various target proteins including protein kinases and the phosphatase CN [39,40].

Functional CN consists, in its inactivated form, of two subunits, a 60 kDa catalytic (CNA) and a 19 kDa regulatory subunit (CNB) [41]. The calcium/calcineurin pathways are highly conserved in yeast, filamentous fungi and mammals. Many studies associate this pathway with the control of fundamental aspects of fungal growth, development, reproduction and virulence [42]. A well-known CN substrate is the conserved zinc finger transcription factor Crz1 which is translocated to the nucleus to regulate expression of over 160 target genes, including those involved in cell wall integrity and ion homeostasis [43]. In the human pathogens *Candida albicans*, *Candida dubliniensis* and *Aspergillus fumigatus*, the CN-dependent cascade controls cell wall integrity, stress resistance and response, morphogenesis, serum survival and virulence [44]. For *C. albicans,* it has also been shown that CN is a key mediator of Hsp90 dependent azole resistance [45]. In plant pathogenic fungi such as *Aspergillus oryzae*, *Magnaporthe oryzae*, *Ustilago maydis* and *Ustilago hordei*, a role for CN was not only suggested in hyphal growth, but also in environmental stress adaptation, for instance, under alkaline pH or high NaCl concentrations, during formation of conidia and appressoria, during sexual development, maintenance of cell wall integrity, and virulence [46]. Furthermore, in *S. cerevisiae* and *U. maydis* CN was shown to be a putative antagonist of the protein kinase A (PKA) demonstrating a connection between the CN and the cAMP dependent cascades [47]. A common feature of most, if not all of the above highlighted cell-cell communication pathways is that all

messages are destined to the nucleus for the purpose of decoding appropriate genomic information to give the desired response. The cell-cell communication networks are presumed highly complex but tightly regulated at the biochemical and molecular level. Identification of key nodes of the regulatory circuitries associated with virulence among fungal pathogens of plants should open new doors for development of sound control measures. However, cautious and innovative approaches are needed since it is well-known that genomes of pathogenic microbes are not static but rather dynamic due to the frequent occurrence of mutations and other structural changes associated with transposable genetic elements and jumping genes; and genetic recombination during sexual reproduction.

CELL SURFACE RECEPTORS

Cell surface receptors play an essential role in the biological systems of single- and multi-cellular organisms. Receptor proteins bound to the cell's plasma membrane provide specific sites to which water-soluble signalling molecules bind [48]. Transmembrane receptors are able to transmit information from outside the cell to the inside because they change conformation when a specific ligand binds to it. Some cell surface receptors dimerise or undergo oxidative or reductive electron transfers instead of conformational changes during cell signalling. Various external stimuli, either friend or foe, are perceived through virtually conserved cell surface receptors followed by intracellular information signal transduction events used to trigger transcription of appropriate target genes in order to give the desired output. It is not surprising that cell surface receptors are major players of the pathogenesis process, since nearly 50% of all drugs and fungicides in clinical use target cell surface receptors. These proteins and their ligands remain extremely important targets for structure- based drug design and novel fungicide development [49]. Major types of cell surface receptors explored so far and their roles in cell signalling are summarised in **Table 1**.

Table 1. Cell surface receptors and their roles in cell signaling.

Cell surface receptors	Role/function	References
Fas receptors (FasR)	Recognition of death factors such as (FasL and Tnf), thereby initiating the apoptotic pathway.	Ibáñez and Simi [33] Kaufmann et al. [54] Chiang et al. [78]
Receptor tyrosine kinase(RTK)	Recognition of survival and growth factors such as insulin-like growth factor 1 (IGF1), transforming growth factor α (TGFα) and epidermal growth factor (EGF).	Moloney [9]
G-protein coupled receptors (GPCRs)	Relay signals to downstream intracellular effectors and exhibit complex signalling cascades; pharmacological, internalisation and recycling properties. Examples are chemokines, hormones, neurotransmitters and serotonin.	Fujita et al. [62] Maurice et al. [71] Grimmelikhuijzen and Hauser [37]
Integrins	Participate in signaling pathways directed to actin cytoskeletal organisation, regulation of cell behavior including survival, differentiation and growth.	Cabodi et al. [75] Carduner et al. [76]
Cytokine receptors	Signalling pathway that activates induction of expression of antimicrobial peptides thus are key players in host defence against microbes or antigens.	Chang and Dong [77]
Smoothened (SMO) and patched (PTC) receptors	Key signal transducers of the hedgehog (Hh) pathway.	Xie et al. [20]

Successful pathogens are those that have developed cell-cell communication mechanisms to evade host immune defence responses. Cell surface receptors are therefore primary sites for stimuli perception. Once such primary sites are disrupted, virulence could be compromised and the host is unaffected. Understanding of the role of these receptors in cell signalling among a broad spectrum of fungal pathogens has facilitated the development of antifungals specifically targeting these receptors [50-52]. Cell surface receptors are mostly proteins and researchers now argue that drug design against proteins is non-specific because of polypeptide variability. Scientists are making efforts to design antifungals with a gene target mode of action [53]. However, developing gene targeting drugs would be very difficult because such molecules have to overcome several barriers (cell wall, cell membrane and cytosol) before entering the nucleus or nucleolus.

THE PHEROMONES AS CHEMICAL MESSENGERS

Pheromones are substances that mediate communication between individuals of the same species [54]. Behavioural and physiological responses are often changed when an individual senses a pheromone. Known pheromones commonly expressed in fungi are sex pheromones [55,56]. Sex pheromones have gained much attention in the study of fungi pathogenesis. Fungi, like many vertebrates and insects, use pheromones to attract their mates. In most fungal host pathosystems successful mating has been consistently associated with virulence [57]. Mating-type-specific pheromones are secreted from cells and are sensed by cells of the opposite mating-type. In ascomycetes and basidiomycetes, the pheromones are detected by pheromone receptors, which are cell surface seven transmembrane G-protein coupled receptors (7-TM GPCRs) [58]. These receptors are activated upon pheromone

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binding and trigger the downstream-signalling pathways that lead to successful mating. **Table 2** shows known fungal mating pheromones and their functions. Research on genetic or physical ways of disrupting the biosynthesis or sensing mechanisms of these pheromones will open avenues of reducing fungal multiplication and virulence.

Table 2. Mating pheromones from various fungi.

Pheromone	Fungal species	Behavioural response	Pheromone size in terms of amino acids (aa) residues
a-factor	*S. cerevisiae*	Mediate mating partner recognition and cell fusion	13
Rhodototrucine A	*R. toruloides*	Mediate dikaryotic transition from unicellular yeast stage to dikaryotic mycelial phase.	11
M-factor	*S. pombe*	Cell fusion of compatible mating partners	9
Tremerogen A- 9291-I	*T. brasiliensis*	Necessary for successful cellular conjugation	14
Tremerogen A-10	*T. mesenterica*	Cell conjugation	12
Tremerogen A-13	*T. mesenterica*	Cell conjugation	13
a1	*U. maydis*	Mating partner recognition and cell fusion	13
a2	*U. maydis*	Mating partner recognition and cell fusion	9
MATα	*C. neoformans*	Dimorphic transition from yeast to filamentous form	12

To appreciate how pheromones participate in fungal cell signalling, description of extensively studied model ascomycete yeast *S. cerevisiae* pheromone response signalling pathway is presented. The pheromone receptor Ste2 activates the Gα subunit Gpa1 in yeast and promotes the release of the Gβ Ste4 and Gγ Ste18 subunits from the heterotrimeric G protein complex, which in turn activates the PAK kinase Ste20 and downstream MAPK cascade, Ste11, Ste7, and Fus3 [59].The scaffold protein Ste5, the PAK kinase Ste20, and the Cdc24/Far1 complex are three main downstream targets of the Gβγ complex. When bound to Ste5, the Ste4/Ste18 complex facilitates its membrane recruitment and places the scaffold proteins, mitogen-activated protein kinase (MAPK) module and Ste20 into close proximity to enable signalling circuit activation [60,61]. The MAPK module is a three-tiered phosphorelay system composed of Ste11 (MAPKKK), Ste7 (MAPKK) and Fus3 (MAPK). Upon signal activation, the phosphoactivated Fus3 releases the downstream transcription factor Ste12 from inhibition by Dig1/Dig2, which induces the expression of several mating-specific genes [62,63]. When cells sense a pheromone and activate the mating pathway, their transcriptional profile changes, cell cycle arrest occurs, and they exhibit a chemotactic response toward the mating partner or host cell [64].

Components of the pheromone-signalling pathway, from the upstream receptor–G protein complex to the downstream transcription factor, are required for these mating responses [65]. The activation of transcription factor Ste12 promotes mating. In the extensively studied maize smut phytopathogen, *U. maydis,* mating success has been shown to be the major important first step of the pathogenesis process. Thus, the pheromone dialogue governs cell-cell recognition and fusion, which results in cellular morphological transitions, for example from yeast form to filamentous form. Extensive research is underway to elucidate pheromone signalling, however, the polygenic nature of such vital signalling pathways could be slowing the progress. Besides, with the ongoing genome sequencing projects, vast amounts of genomic information is being generated, which, however, could be of limited usefulness due to the existing gap in bio-informatics. Despite this, one interesting aspect of cell-cell communication is that it is evolutionarily conserved among eukaryotes. Therefore, closing the few but complex knowledge gaps with respect to cell-cell communication and pathway cross-talks among model eukaryotic pathogens could be a better strategy to dissect current and newly emerging pathogens leading to better control.

EFFECTOR PROTEINS

Effectors are generally defined as “molecules secreted by plant-associated organisms that alter host-cell structure and function” [66]. Current knowledge of the role of fungal effectors during plant infection is still limited. The secretomes play crucial roles during plant-pathogen interactions. Cellular information exchange between pathogen and host through effector protein secretion, often termed hetero-communication, is a vital component of pathogen virulence. Notably, most effectors of fungi and oomycetes are small secreted proteins (especially small, secreted, cysteine-rich proteins), with the exception of some non-proteinaceous toxins and secondary metabolites [67]. Many effectors in host- specific necrotrophic fungi, hemibiotrophic fungi, biotrophic fungi and oomycetes have been well documented [68-70]. Carbohydrate-active enzymes (CAZymes) are major components of the secretome of phytopathogenic fungi and their hosts. CAZymes are responsible for the breakdown, biosynthesis or modification of glycoconjugates and oligo- and polysaccharides by degrading, modifying, or creating glycosidic bonds. They participate in a spectra of important biological processes, including cell signaling, cell wall synthesis and energy production [71,72]. Plant cell wall degrading CAZymes such as pectinases and xylanases have been identified as important pathogenicity or virulence factors [73].

Successful breaking through the plant cell wall is an essential step for the colonization of host tissues for most phytopathogens. However, most of the secreted proteins lack specificity, typical of necrotrophic fungi with a remarkably broad host range, such

as *S. sclerotiorum*, is poorly understood. The fungal-plant interplay depends on mutual recognition, signalling, and expression of pathogenicity and virulence factors, from the fungal side. It is further influenced by the existence of passive, preformed, or inducible defence mechanisms in the plant, resulting in compatible (susceptibility) or incompatible (non-host, basal or host specific resistance) interactions. From a genetic point of view, and according to the gene-for-gene interaction hypothesis, resistance results from the combination of a dominant avirulence (*Avr*) gene in the pathogen and a cognate resistance (*R*) gene in the host; the interaction of both gene products leads to the activation of host defence responses such as the hypersensitive response, that arrests the growth of fungi [74-88]. This hypothesis has been experimentally demonstrated for a number of pathosystems, mainly involving biotrophic fungi, with a number of avirulent genes identified [66,68]. Regardless of encoding for plant cell wall degrading enzymes, many genomes of phytopathogenic fungi also encode for fungal cell wall degrading enzymes. However, the expression patterns and the exact roles of these fungal cell wall-degrading CAZymes during fungal infection and development are still unknown. Probably these fungal cell wall- degrading enzymes participate in partial degradation of the wall to eliminate rigidity and promote flexibility during endogenous host tissue invasion. Experimental evidence is still lacking to either verify or falsify the hypothesis. However, it could be assumed that most answers to these mysteries could only be realised if extensive dissection of cell-cell communication pathways using diverse approaches is done.

CONCLUSION AND FUTURE DIRECTION

Development of a multicellular organism not only involves endogenous programming of individual cells but also cellular communication and coordination. Synthetic (anabolic) cellular processes always surpass degradative (catabolic) processes in actively growing cells; therefore strict cellular communication does exist to maintain cellular homeostasis. A more virulent pathogen is one with an inherent ability to aggressively multiply within the host tissue. Development of a microbial phytopathogen such as *U. maydis* involves both endogenous programming by fungal regulatory proteins and cell signalling. In this case, signalling occurs between both the fungus and host because *U. maydis* is a biotrophic pathogen. Any form of external molecules that might target and negatively interfere with cell signalling components discussed above could lead to the development of better therapeutic methods. In most if not all pathosystems studied so far, the plasma membrane is always the major playground for information perception among pathogens and hosts. Thus far, most antifungals on the market target the plasma membrane through attacking the ergosterol biosynthesis pathway or specifically interact with ergosterol leading to loss of membrane integrity and cell death. Many cases of antifungal resistance are emerging as a result of continuous use of antifungals of the same mode of action. It is therefore now time to look beyond the plasma membrane in order to come up with effective control measures. In this current functional genomic era, dissection of the cell-cell communication circuitries among economically important phytopathogens should be embraced. Further, effector proteins could be exploited in development of biomarkers for the enhancement of knowledge on phytopathogenic-host interactions and development of sound diagnostic methods.

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