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Adaptation to the Host Environment by Plant-Pathogenic Fungi

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Abstract

Many fungi can live both saprophytically and as endophyte or pathogen inside a living plant. In both environments, complex organic polymers are used as sources of nutrients. Propagation inside a living host also requires the ability to respond to immune responses of the host. We review current knowledge of how plant-pathogenic fungi do this. First, we look at how fungi change their global gene expression upon recognition of the host environment, leading to secretion of effectors, enzymes, and secondary metabolites; changes in metabolism; and defense against toxic compounds. Second, we look at what is known about the various cues that enable fungi to sense the presence of living plant cells. Finally, we review literature on transcription factors that participate in gene expression in planta or are suspected to be involved in that process because they are required for the ability to cause disease.

INTRODUCTION

Fungi are prominent among plant-pathogenic microbes, posing a persistent threat to agriculture, horticulture, and forests (38). All crops must be protected from this menace by antifungal compounds, biological control agents, agricultural practices, development of resistant cultivars, or any combination of these measures (24, 115, 195). Only a limited number of fungal species, or even strains within a fungal species, can cause disease on any crop plant. This attests to the general effectiveness of the plant immune system in limiting the growth or development of invading microorganisms. It also implies that pathogenic fungi are highly adapted to a limited set of hosts and are able to reduce the effectiveness of the defenses of only those plant species. The key to pathogenicity of fungi, therefore, lies in responding to the host environment in a way that promotes growth inside the host despite the presence of surveillance and defense mechanisms. This response involves sensing of the proper cues from the host and then adapting gene expression such that infection structures are formed, host defenses are either not induced or rendered ineffective, and all nutrients required for growth are obtained from the host.

With the emergence of transcriptomics technology, especially RNA sequencing (RNA-seq[1]), changes in fungal transcriptomes upon invasion of living plants by a variety of plant-pathogenic fungi have been documented. This has yielded a rich overview of how fungi change their gene expression upon host invasion. We summarize here what has been learned to date using transcriptomics about how plant-pathogenic fungi adapt to the host environment. The more difficult challenges are to identify the cues from the host that trigger these changes in gene expression and uncover how sensing of these cues leads to changes in gene expression. We review here the most important discoveries that contributed to answering these questions. With regard to mechanisms underlying changes in gene expression upon sensing of living plant tissue, we focus on the transcription factors (TFs) involved. Because we focus on the shift from saprophytic to invasive growth, we do not cover obligate biotrophic fungi in this review.

PLANT-PATHOGENIC FUNGI ADAPT TO THE HOST ENVIRONMENT

When attempting to discover how fungi adapt their gene expression to the living plant environment, a crucial issue is which conditions to compare. Several approaches have been taken. One basic approach is to compare fungal transcriptomes of colonized plant tissue with transcriptomes of in vitro cultures of the same fungus. In this approach, the choices of plant tissue, stage of infection and in vitro condition are crucial because these determine to a large extent which genes are identified as plant induced. More sophisticated analyses involve multiple time points of infection, combined with in vitro conditions that are known or suspected to partially mimic in planta conditions (6, 96, 103) or specific stages thereof, e.g., the plant surface (118), different tissues colonized by the same fungus (3, 47, 143), or comparisons between a wild-type and mutant strain (87, 145, 162). In addition, host-specific induction of fungal gene expression has been investigated by comparing fungal transcriptomes upon colonization of different hosts (56) or a compatible versus an incompatible interaction (71, 72, 158). An elegant approach for identification of genes that respond specifically to the living plant host is to compare gene expression between growth in living tissue and growth in the same tissue killed by freezing (7). The major transcriptomics studies we reviewed are summarized in **Supplemental Table 1**. It is important to realize that such studies do not establish causal relationships; genes induced upon colonization of the host or at certain stages of development are not necessarily required for growth under those circumstances. Nevertheless,

Supplemental Material

[1] In fact, cDNA is sequenced in most current RNA-seq methods.

these studies help to formulate hypotheses on metabolic processes, cell wall modifications, defense against host compounds, and other processes required for host colonization and also help identify candidate genes required for certain stages of pathogenicity, such as effector genes, secondary metabolite gene clusters, and genes encoding nutrient importers or secreted enzymes.

Effectors

Pathogen-secreted proteins are of primary interest in transcriptomics studies of plant-fungus interactions, as they play crucial roles in adaptation to the host environment by plant-pathogenic fungi. Two major classes of secreted proteins are commonly distinguished: enzymes and small (nonenzymatic) secreted proteins that are commonly called effectors. Effectors can be defined functionally as secreted proteins that in some way promote host colonization and/or the manifestation of disease symptoms, either by protecting the fungus from host defensive compounds such as cell wall–degrading enzymes (CWDEs) or by interfering with the host's immune system (33, 78, 122). Apart from being small (generally fewer than 300 amino acids) and commonly, but not necessarily, having high cysteine content, effectors are often fast evolving because of their participation in the molecular arms race with host plants (140, 163).

Effectors are generally associated with the biotrophic growth phase, where they play a crucial role in keeping host cells alive and the immune system suppressed. Indeed, in virtually all pathosystems investigated, expression of effector genes is induced early in host invasion and declines after a switch to necrotrophic growth. This has been observed in transcriptomics studies with *Colletotrichum higginsianum* (77, 118), *Colletotrichum orbiculare* (42), *Colletotrichum graminicola* (162), *Fusarium oxysporum* (158), *Magnaporthe oryzae* (71, 103), *Zymoseptoria tritici* (132), *Leptosphaeria maculans* (47, 54), and *Ustilago maydis* (70).

Some classes of small secreted proteins are associated with necrotrophy, e.g., members of the necrosis and ethylene-inducing peptide-1 (Nep-1)-like protein (NLP) family (50, 128, 193). In the transcriptomics studies under review here, expression of genes for such toxins, sometimes also called effectors (86), was observed for *L. maculans* (54), *C. higginsianum* (77), and *C. graminicola* (162). Interestingly, in *U. maydis*, biotrophic colonization of seedlings, adult leaves, or tassels is associated with expression of only partially overlapping sets of effector genes (143). This suggests that *U. maydis* senses not only plant tissue in general but also organ-specific cues. Nine effector genes expressed in an organ-specific manner were later shown to be required for virulence specifically in the organ in which they are expressed (137).

Secondary Metabolites

For many plant-pathogenic fungi, not only effectors but also secreted secondary metabolites (SMs) are key determinants for facilitation of plant colonization. Fungal SMs are commonly grouped based on the basic skeleton such as polyketides, synthesized by polyketide synthases (PKSs), and nonribosomal peptides, synthesized by nonribosomal peptide synthases (NRPSs). Many PKS and NRPS genes reside in gene clusters for the synthesis of a particular compound (11, 169). Well-known examples of SMs that contribute to plant diseases or food and feed contamination are trichothecenes produced by *Fusarium* (31, 98) and host-specific toxins produced by *Alternaria* (40, 126), but all filamentous fungi produce SMs and, in many cases, particular gene clusters are activated during plant invasion. Examples of fungal plant pathogens in which genes for SMs are in induced during host colonization are *Z. tritici* (72, 132), *L. maculans* (54), *Dothistroma septosporum* (10), *Botrytis cinerea* (87), and *Fusarium graminearum* (7, 56, 96, 198, 199). In most of these cases, SM gene expression is associated with necrotrophy, but in some cases expression of particular

SM gene clusters is associated with biotrophy, notably in *Colletotrichum* (42, 118, 162) and/or development of appressoria as in *M. oryzae* (145).

Secreted Enzymes

In most transcriptomics studies, a large proportion of genes whose expression is upregulated upon host invasion relative to axenic growth encode secreted enzymes, in particular, plant CWDEs (PCWDEs) and proteases. Generally, the highest number of such genes is expressed during necrotrophic growth, such as in *C. orbiculare* (42), *Colletotrichum gloeosporioides* (3), *C. graminicola* (162), *Z. tritici* (132), *D. septosporum* (10), *L. maculans* (47), and *F. graminearum* (198), signifying large-scale breakdown of plant cell walls, presumably for nutrient acquisition as fungal mass increases rapidly in this phase. In some cases, particular PCWDEs, such as pectin-degrading enzymes in *L. maculans*, are also expressed in early/biotrophic phases (54). Other fungi, such as *B. cinerea* (87) and *Penicillium expansum* (6), grow necrotrophically from the outset of infection and immediately start to express many PCWDE genes.

Despite the widespread consensus that pathogenic fungi induce expression of secreted enzymes during invasion, from most studies it cannot be concluded that this is a specific response to the living host environment. Comparisons are mostly done with axenic growth in the presence of a simple carbon source such as glucose. The absence of a sugar and the presence of complex polysaccharides already induce expression of many CWDEs (80). In an interesting approach to differentiate this type of induction with changes in gene expression that are specific to a living host environment, Boedi et al. (7) compared global gene expression in *F. graminearum* between colonization of living and cold snap–killed wheat heads. As expected, most PCWDE genes were induced in both living and dead tissue.

Nutrient Uptake

Plant colonization is commonly associated with upregulation of genes encoding membrane transporters that likely function as importers of nutrients such as sugars and amino acids. Induced expression of nutrient importers is especially prominent during the necrotrophic phase, concomitantly with secretion of hydrolases, such as in *C. graminicola* (162), *C. higginsianum* (118), *F. graminearum* (198), *F. oxysporum* (158), *D. septosporum* (10), *Z. tritici* (132), *L. maculans* (54), and *M. oryzae* (145). That this general response is not related to the presence of living plant cells is borne out by the fact that *F. graminearum* strongly induces genes for transport of ions, sugars, amino acids, and di- and tripeptides both in living and cold snap–killed wheat heads (7). For *M. oryzae* and *C. orbiculare*, induced expression of genes required for quinate uptake and utilization was noted (42, 145). Quinate is a cyclic polyol that can be used as the sole carbon source by fungi and is abundant in decaying leaf litter (59). In *C. orbiculare*, genes for quinate utilization had the highest expression during late necrotrophy (42). In *M. oryzae*, quinate permease genes are expressed at all stages of appressoria formation (145). In *F. graminearum*, members of the TPO family of polyamine transporters are highly expressed in maize, possibly to import polyamines such as spermine, putrescine, and spermidine (56), some of which induce expression of genes involved in trichothecene production (see below). Also in *F. graminearum*, genes for allantoin and allantoate transporters were observed to be enriched among genes expressed in wheat but not barley (96). A remarkable mechanism in *U. maydis*, which grows biotrophically throughout its colonization of maize, is the infection-specific expression of the sucrose-specific transporter Srt. This may help to avoid eliciting the maize immune system because it obviates the need to secrete invertase for sugar uptake. Significantly, Srt1 is required for full virulence of *U. maydis* (176).

When certain nutrients are limiting during growth in planta, fungi may also reduce the amounts needed for growth. When infecting maize stalks, *F. graminearum* shows reduced expression, compared to in vitro growth, of 36 genes predicted to be involved in the metabolism of glycerophospholipids, which are structural components of membranes (200). Further investigation revealed a connection with low phosphate availability during maize stalk colonization: Expression of the *BTA1* (betaine lipid synthase) gene, which is responsible for the synthesis of the phosphorus-free lipid DGTS (diacylglyceryl-N,N,N-trimethylhomoserine), was much higher in planta than in vitro. Expression of genes encoding putative phosphate transporters was also enhanced. The phosphate concentration in the apoplastic space was estimated to be only 0.3 mM, and deletion of *BTA1* prevented intercellular growth of *F. graminearum* in maize stalk and reduced virulence, which was restored by the addition of phosphate at the infection site (200). Enhanced expression of genes for phosphate transporters in *C. graminicola* during colonization of detached maize leaf sheaths also suggests that available phosphate levels are relatively low under those conditions (162).

Carbon Metabolism

In several plant-pathogenic fungi, the glyoxylate cycle, which is required when carbon needed for anabolism is derived from fatty acids instead of sugars, has been implicated in the ability to colonize living plant tissue, because mutants in key enzymes needed for the glyoxylate cycle, such as isocitrate lyase, are less virulent (35). In *F. graminearum*, expression of fatty acid oxidation and glyoxylate cycle genes was higher during coleoptile infection than in an in vitro culture, whereas the reverse was true for genes required for glycolysis (198). It may be that carbon is to a large extent derived from stored lipids, especially in early stages of infection. This seems to clearly be the case in *C. gloeosporioides*, which forms appressoria before penetration that accumulate transcripts for β-oxidation of fatty acids. During necrotrophic growth in mature tomato fruits, genes for glycolysis are induced instead, suggesting the use of carbon in the form of sugars derived from the plant (3). In a mutant of *C. graminicola* that cannot (fully) establish biotrophy, key enzymes of the glyoxylate cycle are expressed more highly than in the wild type, which could reflect stalling at the appressorial or early biotrophic stage (162). Also for *M. oryzae*, the formation of appressoria is associated with both β-oxidation of fatty acids and the glyoxylate cycle (145). Fatty acid generation from lipids and their β-oxidation, as well as the glyoxylate cycle, are also prominent during early infection by *Z. tritici* (132).

Detoxification and Reactive Oxygen Species Metabolism

Production of reactive oxygen species (ROS) is a universal response of plant cells to the perception of microbe-associated molecular patterns (117), and it has been shown that the ability to respond to ROS is required for the virulence of some plant-pathogenic fungi (53, 91, 111, 190) but not others (88, 113). Monitoring the expression of 206 TF genes in *M. oryzae* under various conditions, Park et al. (123) found that expression patterns during colonization of rice (78 and 150 hpi) are similar to expression patterns under oxidative stress conditions. Wheat coleoptiles accumulate more ROS at sites infected with *F. graminearum* at 16 hpi than at mock-inoculated sites (198). At that stage of infection the expression of genes for catalases and superoxide dismutases, which convert the ROS hydrogen peroxide and superoxide, respectively, has increased in *F. graminearum*. Later during coleoptile infection, at 64 hpi, expression of genes increased for extracellular ROS-producing enzymes, such as NADPH oxidases, suggesting that at that time the fungus produces ROS, coinciding with the secretion of many PCWDEs (198). Also, in *C. graminicola*, genes for proteins involved in peroxide detoxification are expressed early, during the biotrophic phase of

infection (162). Likewise, symptomless wheat leaf colonization by *Z. tritici* is associated with enrichment of expression of genes for peroxidase activity, oxidoreductase activity, and antioxidant activity, possibly to reduce ROS produced by plant cells (47, 72). The importance of protection against plant-produced compounds during infection is further suggested by increased expression of genes associated with detoxification, encoding, for example, cytochrome P450s, MDR (multi-drug resistance) proteins, and ABC transporters in *Z. tritici* (72) and *F. graminearum* (7, 199). An interesting case of interference with the plant ROS response is the effector Pep1 of *U. maydis*. This secreted protein is required for virulence and was found to inhibit peroxidases secreted by maize cells, thereby blocking the oxidative burst (63).

Siderophores

In planta upregulation of genes required for siderophores, molecules that facilitate uptake of iron, has been noted in *C. graminicola* during the transition to biotrophic growth (162) and in *C. gloeosporioides* at the appressorial stage along with the gene for the iron transport multicopper oxidase Fet3 (3). In *F. graminearum*, siderophore genes and a siderophore permease were highly expressed during maize stalk infection (199). Also in *F. graminearum*, the NRPS-encoding genes of the ferricrocin, malonichrome, and triacetylfusarinine siderophore clusters, as well as iron transporters, are specifically expressed in the living (as opposed to cold snap–killed) wheat head (7), and these gene clusters are also activated during maize stalk infection (199). Expression of the NRPS genes of the ferricrocin and triacetylfusarinine clusters was also detected early by Harris et al. (56) during colonization of wheat, barley, and maize, whereas expression of the NRPS gene of the malonichrome cluster was detected in wheat and barley but not maize. Genes for proteins related to the major facilitator MirA and a ferric reductase, which is involved in siderophore uptake, were found to be coexpressed in that study.

The Pth11 Family of G Protein–Coupled Receptors

Pth11 is a G protein–coupled receptor required for appressorium differentiation in *M. oryzae* (32). It contains an extracellular CFEM domain that is unique to filamentous ascomycetes, and it is presumed to be involved in sensing and transducing external signals that stimulate appressorium development (81, 82). In *C. graminicola*, 10 Pth11-family genes are expressed differentially in wheat: eight early and two late during infection (162). *F. graminearum* expresses six Pth11-family members at different times after infection of wheat coleoptiles (198), whereas Harris et al. (56) detected no less than 30 Pth11-family genes expressed by *F. graminearum* during colonization of wheat, barley, and/or maize, with 27 exhibiting host-preferential expression in wheat, barley, or both.

HOW DO FUNGI SENSE THE HOST?

Fungi continually sense their environment through light, chemical, and physical cues. Sensing of these cues activates signal transduction processes that induce changes in metabolism, cellular organization, and gene expression. These changes, in turn, lead to developmental processes such as sporulation, morphological changes such as redirection of growth, the ability to degrade complex organic compounds and import nutrients, and the ability to survive stressful conditions such as osmotic and oxidative environments, heat stress, and the presence of toxic compounds. Pathogenic fungi may be seen to have tuned these basic processes, present in all fungi, to sense the proximity of living plant tissue and respond in a way that promotes colonization. Beyond this, differences in

host species or different organs of the same host species can provoke different responses (56, 72, 143).

Sensing the Proximity of Plant Roots

In the soil, some fungi can sense the proximity of plant roots through compounds released by living roots and respond by growing toward the source of those compounds, a process called chemotropism (135, 167). A well-known example of a fungal response to root-released compounds is the hyphal branching response of arbuscular mycorrhizal fungi to strigolactones (2, 44). As for plant-pathogenic fungi, a remarkable recent discovery is the response of the vascular wilt pathogen *F. oxysporum* to peroxidases released by roots (168). *F. oxysporum* was found to reorient growth toward nutrients but also to active plant peroxidases, which are released by (wounded) tomato roots. The latter response requires a functional homolog of a yeast pheromone receptor, and the corresponding MAP kinase signal transduction pathway (168). Much remains to be learned about plant-released compounds that are sensed by pathogenic or symbiotic fungi and the chemotropic response that they elicit.

Sensing the Plant Surface

Pathogenic fungi that invade aboveground tissues recognize the plant surface, the cuticle, through its hydrophobicity and chemical composition. The cuticle is composed of cutin, a polymer of hydroxy fatty acids, and intracuticular and epicuticular waxes with complex compositions (14). Sensing of the rice cuticle by *M. oryzae*, which leads to the formation of appressoria, requires the membrane proteins Msb2 and Sho1. Msb2 is required for sensing hydrophobicity and cutin monomers, and Sho1 is more important for responding to primary alcohols, which are components of wax (93). The basidiomycete *U. maydis* likewise depends on Msb2 and Sho1 for the development of appressoria on the maize leaf surface, requiring both proteins for responding to a hydrophobic surface but not to the cutin monomer 16-hydroxy hexadecanoic acid (85). Next to induction of appressoria, these surface cues induce expression of effector genes associated with biotrophic growth (84). This would explain the secretion of effectors from appressorial penetration pores by *C. higginsianum* (77) and the appressorial expression of effector genes in *M. oryzae* (41, 147). Wang et al. (179) showed that in *M. oryzae*, Msb2 has overlapping functions with another mucin, Cbp1, in the formation of appressoria, and that extracellular and cytoplasmic domains of Msb2 have distinct roles in appressorium formation and invasive growth (179). *B. cinerea* requires Msb2 for the formation of appressoria or infection cushions on hard surfaces, even though *msb2* mutants of this fungus are still virulent in various plant species (87). The root-invading fungus *F. oxysporum* does not encounter a cuticle but nevertheless requires both Msb2 and Sho1 for invasive growth, root colonization, and secretion of pectinolytic activity, apparently acting through the Fmk1 MAP kinase (125). Another root pathogen, *Fusarium solani*, responds to cutin monomers by induced expression of a cutinase gene (4, 89, 90).

As mentioned above, Pth11 is required for the development of appressoria in *M. oryzae* and is suspected to be involved in host surface recognition, and expression of several Pth11 homologs is induced during colonization of wheat by *C. graminicola* and *F. graminearum* (see above). Deletion of a gene for a homolog of Pth11 in *B. cinerea* slightly reduces virulence and affects expression of genes encoding glutathione S-transferases (51). It is still unknown what signal Pth11, or any of its homologs, may sense, but there may be a connection between Pth11 and ROS homeostasis in *M. oryzae* (79).

Sensing Living Cells

As already mentioned, global gene expression in *F. graminearum* is very different between colonization of live and cold snap–killed wheat heads (7). This suggests that, apart from relatively stable chemical or physical characteristics shared between living and dead plant tissue, pathogenic fungi are able to sense living plant cells specifically. This was also suggested by studies with the effector gene *SIX1* of *F. oxysporum*, which was induced upon invasion of roots and in a plant cell culture but not in dead roots or by root exudate or root extracts (170). Possibly, living plant cells produce unstable compounds that are quickly depleted upon cell death and can be sensed by fungi. Another possibility is that living plant cells produce specific compounds only when they themselves sense the presence of microbes. An interesting example of the latter is the accumulation of polyamine putrescine, a compound that, in turn, can induce expression of genes such as *TRI5* that lead to the production of the mycotoxin deoxynivalenol (DON) in wheat heads upon infection by *F. graminearum* (45, 46). If a polyamine is indeed the key trigger for *TRI5* activation, it must be a very early host response because *TRI5* is already induced in infection cushions (8). Flavonoids such as the antimicrobial compound pisatin can induce expression of pisatin demethylase in *F. solani* (152, 153). Induced production or release of such compounds may also be a specific response of living plant cells to the presence of microbes and thereby allows fungi to sense the presence of living plant cells (58).

TRANSCRIPTION FACTORS REQUIRED FOR VIRULENCE

Signal transduction processes connect sensing of host cues to transcriptional reprogramming to adapt to the host environment. This is a vast field of research, and we mainly focus here on TFs that are required for virulence of plant-pathogenic fungi. These TFs are listed in **Supplemental Table 2**. It is important to note that some of the TFs that are required for virulence may not regulate infection-specific processes. Conversely, some TFs, such as Mzr1 and Bot6, regulating transcriptional changes that occur upon switching to an invasive lifestyle are not required for virulence (127, 201). Mzr1 from *U. maydis* is required for expression of some of the effector genes of the *MIG2* cluster but is not required for virulence. To activate *MIG* gene expression, Mzr1 requires the presence of Biz1 (201). Biz1 is a C2H2 zinc finger TF that is activated upon developmental changes (mating) preceding infection by *U. maydis* and is required for pathogenicity (39). Overexpression of *BIZ1* alone, in an *mzr1* deletion mutant, is sufficient to induce *MIG* expression (201). Bot6 is a Zn(2)Cys(6)-type TF in *B. cinerea* that is part of the *BOT* gene cluster that produces the SM botrydial, a phytotoxic sesquiterpene. Bot6 is required for the expression of the other *BOT* genes. Botrydial is, however, dispensable for pathogenicity, as is *BOT6* (127). Also, the C2H2 TF Yoh1, which regulates expression of multiple virulence-associated processes, such as phytotoxin biosynthesis, detoxification, and SM gene cluster expression, including the *BOT* cluster, is not required for virulence of *B. cinerea* (142). However, when both botrydial and botcinic acid production are disrupted, the fungus is reduced in virulence, suggesting that these two SMs are functionally redundant and contribute to virulence (28).

Supplemental Material

Morphological Changes

For many plant-pathogenic fungi, invasion of plant tissue is preceded by the production of specialized infection structures, such as appressoria or infection cushions (8, 101). However, this does not necessarily mean that these structures are required for invasive growth inside the plant. For example, when plants are directly inoculated with mycelium of an *M. oryzae HOX7* deletion

mutant, which is unable to produce appressoria, the fungus is fully able to cause disease (75). Likewise, infections of aboveground parts of plants usually start from conidia, but the ability to produce conidia is not a requirement to grow invasively into plant tissue. For example, *MoHOX2* and *FgFlbD* deletion mutants do not produce conidia but are nevertheless virulent (75, 146). Still, in a number of TF deletion mutants, reduced virulence coincides with defects in conidia production. In *M. oryzae*, many such TF genes have been identified: *CON7*, *CDTF1*, *COD1*, *COD2*, *COM1*, *COS1*, *RFX1*, *SOM1*, *TUP1*, *WOR1*, and *YCP4* are all required for full virulence as well as normal conidia production (18–20, 23, 119, 154, 184, 186, 202). Homologs of Con7 and Wor1 also function in virulence and conidiation in other fungal species (12, 68, 106–109, 121, 133, 134, 161, 164). Other TFs involved in both processes are Spt3, Spt8, Skn7, Atf1, and Swi6 in *F. graminearum*, Ste12 in *Penicillium digitatum* and *Setosphaeria turcica*, and StuA in *L. maculans* and *Stagonospora nodorum* (43, 52, 66, 67, 92, 149, 174). A reverse situation was observed for Znf1 in *M. oryzae*: Its deletion increases conidia production while reducing virulence (191).

This repeated co-occurrence of phenotypes suggests a transcriptional connection between processes required for virulence and the production of conidia. Possible connections could be a generally reduced metabolic or regulatory capacity of the deletion mutant. Indeed, some of the deletion mutants (*CON7*, *CDTF1*, *RFX1*, *SOM1*, and *TUP1*) are also impaired in vegetative growth (19, 119, 154, 184). Alternatively, it is known that Wor1 homologs and Con7 can affect cell wall composition. It is, however, unknown whether changes in cell wall composition cause the problems in conidiation (20, 119).

Secondary Metabolite Gene Clusters

Secondary metabolism in fungi is influenced by many factors [reviewed by Brakhage (11) and Tudzynski (166)], but we restrict ourselves to TFs implied in virulence. Among the clearest examples of SMs that fulfill an indispensable role in virulence are the host selective toxins (HSTs) of *Alternaria alternata*, which determine host-specific virulence (165). Although putative TF genes in several of the *A. alternata* HST gene clusters have been identified, there is currently no genetic evidence for their requirement for HST production (180). For other SMs required for (full) virulence, the effect of pathway-specific TFs, such as for *Helminthosporium carbonum* (HC) toxin, depudecin, trichothecenes, and fumonisin, has been determined (1, 13, 141, 182).

HC toxin is a cyclic tetrapeptide that is produced by *Cochliobolus carbonum* and inhibits histone deacetylases. Its production relies on the *TOX* genes at the *TOX2* locus. ToxE is the pathway-specific bZIP TF required for the expression of the other *TOX* genes (1). Depudecin, which also exhibits histone deacetylase inhibitor activity, is a polyketide produced by the *DEP* gene cluster in *Alternaria brassicicola*. *DEP6*, part of the cluster, encodes a TF that regulates expression of the five other *DEP* genes. The contribution of depudecin to virulence is, however, very small (182).

Fumonisin is a *Fusarium* mycotoxin with structural similarity to sphingolipids that interferes with sphingolipid metabolism (178). Fumonisin production is governed by the *FUM* cluster and regulated by the pathway-specific TF Fum21. Deletion of *FUM21* results in the loss of fumonisin production and loss of pathogenicity in *Fusarium fujikuroi* and *Fusarium verticillioides* (130). Production of fumonisins is also regulated on other levels. Deletion of any one of the TF genes *AreA*, *AreB*, *ART1*, *FUG1*, or *SGE1* reduces both fumonisin production and virulence (12, 74, 120, 129, 130). AreA and AreB are key TFs in nitrogen catabolite repression (166), and Art1 is required for starch hydrolysis (120). Sge1 is a homolog of Wor1, a regulator of lifestyle switching, effector gene expression, and SM production in different fungi (12).

F. graminearum produces the trichothecene DON. Trichothecenes are a group of sesquiterpenes that are potent inhibitors of protein synthesis in mammalian cells. DON production is

regulated by two pathway-specific TFs: Tri6 and Tri10. Deletion of *TRI6* or *TRI10* abolishes DON production in *F. graminearum* and severely reduces pathogenicity (141). Surprisingly, Tri10 and Tri6 control more genes than just the *TRI* cluster, and loss of DON production only partially explains the loss of pathogenicity of the deletion mutants (27, 114, 124). Tri10 affects only a few other genes, but one is a homolog of *PTH11* from *M. oryzae*, which encodes a transmembrane protein suggested to function in host sensing (see above) (124). Tri6 controls many more genes, including genes involved in lipid and nitrogen metabolism. Two of these are TFs with an NmrA domain, a domain that can act as a negative regulator of nitrogen catabolite repression (114). In a systematic study by Son and coworkers, the inability to produce DON was not coupled to a reduction in virulence (146). Deletion mutants of five uncharacterized TFs and, remarkably, Tri6 itself were still virulent, despite a complete lack of DON production. A possible explanation given is the use of a highly susceptible wheat cultivar and a growth chamber environment that favors disease. Interestingly, in all deletion mutants the production of another major SM of *F. graminearum*, zearalenone, was also abolished or reduced (146).

The production of DON is also influenced by several other factors, among which are the TFs AreA, Atf1, Art1, Fgp1 (Wor1 homolog), Myt3, Skn7, Zif1, and Swi6 (49, 68, 76, 92, 120, 172, 181). The nitrogen regulator AreA physically interacts with the Tri10 protein (64). Atf1 and Skn7 have a role in the oxidative stress response, and Myt3 affects nitrogen metabolism and conidiation (67, 76). PacC and Ap1, regulators of the pH response and oxidative stress, respectively, affect DON production, but not pathogenicity, in *F. graminearum* (105, 112). Homologs of PacC and Ap1 in other fungi are required for pathogenicity toward plants, and PacC is also required for the production of SMs in *P. expansum* (5, 53, 83, 91, 110, 111, 155, 188–190, 196). Other TFs that affect SM production are Con7, Cdtf1, and Asd4 in *M. oryzae*. Con7 and Cdtf1 are also required for conidiation, whereas Asd4 is required for nitrogen utilization (100, 119, 184). In *Verticillium dahliae* and *F. graminearum*, Mcm1, a MADS box TF, is required for normal conidiation, sclerotia formation, and SM production (183, 185).

Effector Gene Expression

Reminiscent of the pathway-specific TFs of secondary metabolism gene clusters, the TF gene *FTF1* from *F. oxysporum* is located close to small groups of *SIX* effector genes (138). Ftf1 strongly induces *SIX* effector gene expression upon overexpression, likely via direct binding to a motif found in the promoter of these effector genes (171). *FTF1* has multiple homologs in *F. oxysporum*, and a reduction of mRNA levels of the entire gene family via silencing resulted in a slight reduction of virulence (116). Expression of effector genes in *F. oxysporum* requires the presence of Sge1 (Wor1 homolog), even in strains that overexpress *FTF1* (171). As in other fungi, deletion of *SGE1* results in complete loss of pathogenicity (108). Homologs of Wor1 regulate SM production and/or effector gene expression in fungi with different infection strategies, such as *Fusarium* species, *B. cinerea*, *M. oryzae*, and *U. maydis* (12, 20, 68, 106–109, 121, 134, 161). Other regulators of effector gene expression are Vta2, the Con7 homolog of *V. dahliae*, which also affects expression of a *PTH11*-like gene, Pf2 in *A. brassicicola*, and the APSES TF StuA in *L. maculans* and *S. nodorum* (21, 66, 149, 164). Rbf1, Biz1, Mzr1, Hdp2, and Cib1 play a role in effector gene expression in *U. maydis* (see below) (39, 55, 62, 201).

Mating in *Ustilago*

In *U. maydis*, pathogenicity is intimately linked to mating. When two haploid spores of opposite mating type land on the leaf surface and fuse, subsequent signaling leads to (*a*) expression of the

PRF1 TF gene, in which the TF Tup1 is involved, and (*b*) activation of Prf1 (36, 57, 104, 194). Prf1 then incudes expression of the b mating-type loci of both genomes, which together encode the bE/bW heterodimer. bE/bW is the transcriptional regulator that initiates filamentous growth and invasion into the plant. Disruption of the mating type response by deletion of *TUP1* or *PRF1* or production of pheromones (regulated by the TF Med1) results in loss of pathogenicity in wild-type strains (17, 36, 57). If the bE/bW heterodimer is expressed in a haploid strain, said strain is able to infect without mating (70).

Downstream of bE/bW, the TF Rbf1 takes care of a large portion of the bE/bW response; downstream of Rbf1, the TFs Biz1, Hdp1, and Hdp2 are activated (62). Hdp1 is involved in filamentation, and Rbf1, Biz1, and Hdp2 are each required for pathogenicity. From the b-dependent differentially expressed genes identified, two-thirds are induced by leaf surface cues, including all TF genes of the b-cascade (bE, bW, Rbf1, Hdp1, Hdp2, and Biz1) (62, 84). Biz1 and Hdp2 are required for the expression of effector genes, and Biz1 also collaborates with Mzr1, a TF that regulates expression of some of the *MIG2* effector genes (39, 84, 201). Interestingly, two of the downstream TFs, Biz1 and Hdp2, are Sho1- and Msb2-dependently expressed, and some of the downstream responses of Biz1 and Hdp2 after exposure to surface cues from the leaf also require the proposed surface sensors Msb2 and Sho1 (84).

Activation of the bE/bW dimer also results in cell cycle arrest. This arrest is relieved after the fungus has penetrated the leaf surface, and this relief requires Clp1 (Clampless 1), which is required for pathogenicity. The *CLP1* gene is upregulated by bE/bW, but production of the protein only starts after penetration of the plant surface and may require a signal from the plant (61). Clp1 interacts with bW and Rbf, and these protein complexes inhibit bE/bW signaling and the mating response and relieve the cell cycle arrest. Clp1 also interacts with Cib1, the *U. maydis* homolog of Hac1, which is the TF regulating the unfolded protein response (UPR) that is activated upon ER stress (61). The interaction between Clp1 and Cib1 stabilizes Clp1 and is required for resistance to ER stress–inducing agents. Deletion of Cib1 also results in loss of pathogenicity. The UPR is thought to be important for the secretion of effector proteins, as their strong upregulation may cause significant stress to the ER and the secretion machinery (60, 61). Additionally, Cib1 has been shown to bind to some effector promoters directly via UPR elements and induce their expression (55). In *A. brassicicola*, the homolog of Cib1 (AbHacA) is also required for pathogenicity (69).

Nitrogen Metabolism

For the production of SMs, the form of nitrogen available is an important cue, as reviewed by Tudzynski (166). Expression of some effector genes is also influenced by nitrogen or nitrogen-responsive TFs (159). Indeed, several regulators of nitrogen metabolism are required for virulence in different fungi. Nitrogen metabolism in fungi is regulated via nitrogen catabolite repression. In the presence of a preferred nitrogen source, such as NH_4^+ or glutamine (Gln), expression of genes required for assimilation of other nitrogen sources is repressed. For these metabolic pathways to be expressed, repression needs to be relieved by removal of the preferred nitrogen source and a pathway-specific regulator needs to be activated. The latter usually occurs after perception of the compound that can be assimilated through the particular pathway. For example, the nitrate reductase gene is only expressed in the absence of NH_4^+ or Gln and the presence of nitrate. The TFs that mediate repression in the presence of a preferred nitrogen source are AreA/Nit2 and AreB. The transcriptional repressors Nmr and MeaB are part of the AreA network. Nmr can repress AreA itself, and MeaB represses some of the SM clusters (166).

The requirement of nitrogen-responsive TFs in SM gene expression and virulence raises several questions: (*a*) What is the nitrogen status in the plant? (*b*) Do nitrogen regulators play a role

in transcriptional regulation of SM and effector genes in planta? (*c*) Is a requirement for SM production and/or effector gene expression the main reason for the virulence reduction caused by the absence of nitrogen regulators?

These questions are not so easy to answer, as illustrated by the effect of nitrogen source on effector gene expression in *Cladosporium fulvum*. Expression of several effector genes is influenced by nitrogen availability in different ways. In some cases, Nrf1, the *C. fulvum* AreA homolog, is required for expression. However, during infection all effector genes but one are expressed in an Nrf1-independent manner (159). Only *AVR9* requires Nrf1 for in planta expression, and for this the AreA boxes in the *AVR9* promoter are necessary, suggesting direct binding of Nrf1 to the *AVR9* promoter (144, 159). Even in the case of *AVR9*, however, it cannot be excluded that during infection, signals other than nitrogen availability feed into the Nrf1 regulatory network, leading to nitrogen-independent recruitment of Nrf1. *AVR9* deletion has no effect on pathogenicity, whereas deletion of Nrf1 leads to reduced virulence, suggesting that Nrf1 has more in planta targets than *AVR9*.

Nitrogen availability also affects intracellular levels of Gln, and high levels of intracellular Gln activate the TOR pathway, playing a role in regulation of the cell cycle and the autophagy response, which is known to be important for appressoria formation in *M. oryzae*, and represses certain SM gene clusters (94, 99, 156). Some TFs, such as MeaB and Asd4, which are required for virulence, are also connected to TOR signaling. Asd4 affects intracellular Gln levels and, as such, affects the TOR pathway (95, 100). Among the TFs required for virulence, we find homologs of Cpc1 in *F. fujikuroi*, *Verticillium* spp., and *L. maculans* (37, 139, 160). This TF is important for responding to amino acid starvation and is upregulated in *F. fujikuroi* upon deletion of the glutamine synthetase gene, which affects TOR signaling (139).

pH

Ambient pH can be an important factor in disease development, as some fungi acidify or alkalize plant tissue as part of their infection strategy. *Penicillium* spp., *B. cinerea*, and *Sclerotinia sclerotiorum* secrete organic acids that acidify the environment. *Colletotrichum* spp., *A. alternata*, and *F. oxysporum* secrete ammonia, which alkalizes the environment (30). *F. oxysporum* also produces rapid alkalization factors (RALFs), which trigger alkalization by plant cells (102).

The pH regulator PacC is required for virulence in several fungi. PacC is activated at ambient alkaline pH and then induces expression of alkaline-activated genes and represses genes expressed under acidic conditions (30). In *PacC* deletion mutants, lytic enzymes are often misregulated. Lower cellulase, cutinase, xylanase, pectate lyase, pectin lyase, polygalacturonase, and catalase activity has been reported (83, 110, 189, 196). An increase in polygalacturonase gene expression and pectinolytic activity has also been observed in *pacc* mutants (16, 189). In *F. graminearum* and *P. expansum*, production of the SMs DON and patulin, respectively, is reduced in the absence of PacC (5, 105).

Detoxification of Host Compounds

As mentioned above, the rapid production of ROS by plant cells, the so-called oxidative burst, is one of the earliest cellular responses following pathogen recognition (15). In line with this, several different TFs required for ROS tolerance have also been found to be required for virulence. Among these are Ap1, Skn7, Vta2, Crz1, Fug1, Nuc-2, and Spt3 (29, 48, 53, 91, 111, 129, 155, 164, 173, 187, 190, 197). Some of the mutants deleted for one of these TFs also have other defects, for example, in conidiation or SM production. Ap1 is the classical example of a TF that responds

to changes in oxidative state, as it is a redox sensor and localizes to the nucleus upon oxidation (9). Indeed, in several fungi, *ap1* deletion mutants are more sensitive to oxidative stress (e.g., the *bap1* deletion mutant in *B. cinerea*) (88, 112, 157). Challenging *B. cinerea* with H_2O_2 induces expression of Bap1-dependent ROS detoxification pathways. Surprisingly, two days post inoculation, when H_2O_2 is detectable in the infected leaves, the Bap1 target genes involved in ROS detoxification are not upregulated, and the *bap1* deletion mutant is not less virulent: The fungus apparently does not suffer H_2O_2-induced oxidative stress in planta. This questions the role of the oxidative burst in the infection process and also highlights the still poorly understood differences between growth in vitro and in planta (157).

Examples of detoxification of host compounds, other than ROS, are the production by *F. solani* f. sp. *pisii* of Pda1, a demethylase that detoxifies pisatin, a compound secreted by pea plants, and requires the TF Prf1. Deletion of *PRF1* results in loss of virulence (73). A similar case is Bdtf, a TF that is essential for detoxification of brassinin and is required for virulence of *A. brassicicola* (151).

Chromatin Remodeling

An interesting feature of many plant-pathogenic fungi is their bipartite genome. Housekeeping genes reside in conserved genomic regions, often referred to as the core genome, whereas conditionally dispensable genes, such as genes required only for infection, are subtelomeric or reside in accessory chromosomes or regions. These accessory regions are often characterized by a high repeat content and a different histone code. Effector genes and SM gene clusters are often found in such regions (97, 131, 150). Compartmentalization of conditionally dispensable genes may facilitate accelerated evolution of these parts of the genome, which is necessary to stay adapted to an evolving host (25, 26).

The chromatin state of accessory genomic regions may provide another level of transcriptional control for the genes encoded there. Indeed, epigenetic regulation of SM gene clusters and of effector genes has been demonstrated (148, 175). Also, some genes required for virulence have a role in chromatin remodeling. For the nitrogen catabolite repressor AreA and for LeaA, a methyltransferase thought to have an epigenetic control function and a regulator of secondary metabolism and pathogenicity in several fungi, chromatin-modifying activity has been reported (11, 136). Finally, deletion of *SPT3* and/or *SPT8* results in strongly reduced virulence in *F. graminearum* and *B. cinerea*. Spt3 and Spt8 are components of the SAGA complex, a chromatin-acetylating transcriptional coactivator with histone acetyl transferase activity. SAGA opens up chromatin, which can allow binding of additional TFs and the transcription preinitiation complex (43, 48).

CONCLUDING REMARKS

Some of the major findings regarding adaptation to the host environment by (nonobligate) plant-pathogenic fungi are summarized in **Figure 1**. Colonization of living plant tissue requires fungi to be able to respond to a combination of developmental and environmental cues by employing sensors, signal transduction pathways, and a multitude of TFs that regulate genes required for adaptation to the host environment. Sensing cues and responding appropriately are fine-tuned processes, as borne out by the fact that all plant-pathogenic fungi are host specific, albeit with variations in broadness. However, not every gene for which expression is activated upon host invasion is required for pathogenicity, and even some TFs that are required for gene expression in planta are not required for pathogenicity. For instance, upregulation of effector genes and SM gene clusters is a hallmark of the switch from a saprotrophic to an invasive lifestyle, but individual effector genes or SM clusters are not always essential for virulence, at least not under laboratory

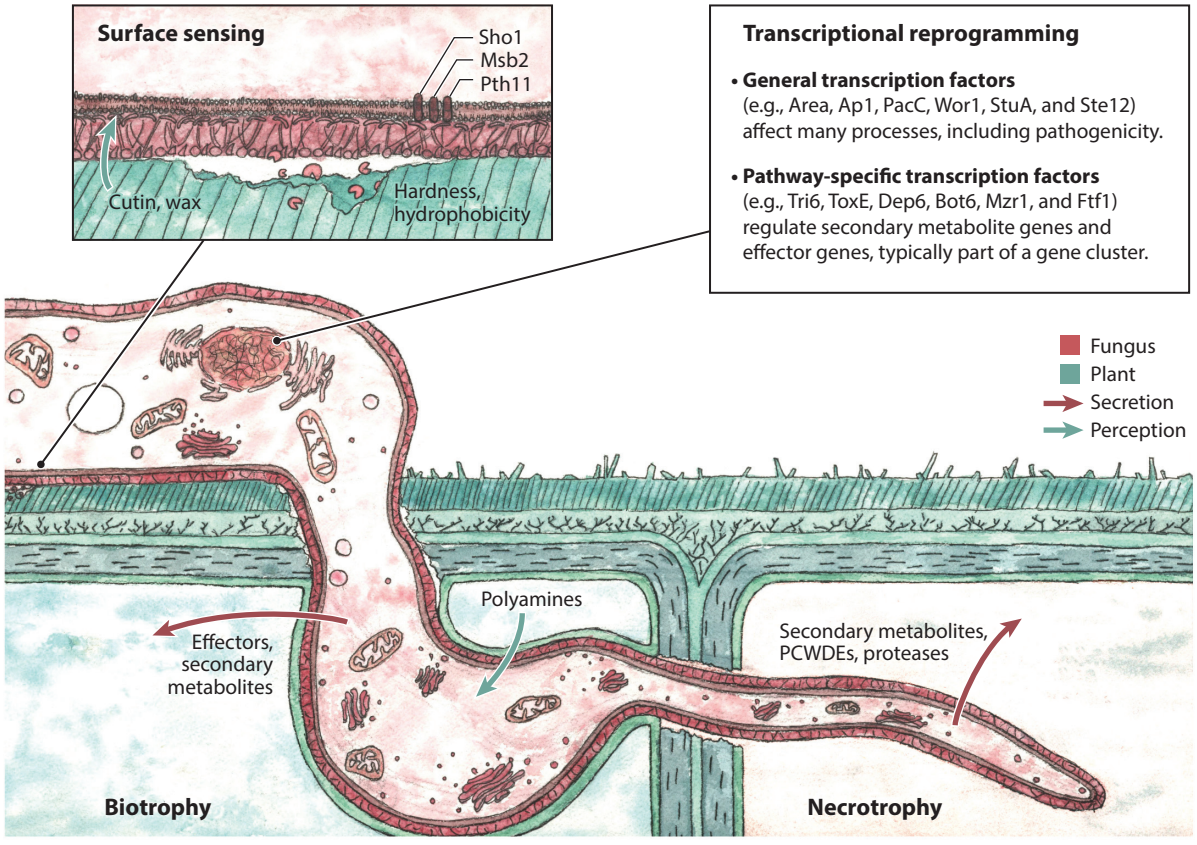

Figure 1

Adaptation to the host environment by plant-pathogenic fungi. This graphic represents a fungus that senses the plant surface, penetrates a plant cell, develops a biotrophic hypha, invades the next cell, and there switches to necrotrophic growth. Sho1, Msb2, and Pth11 are putative sensors of host signals. Abbreviation: PCWDEs, plant cell wall–degrading enzymes.

conditions. Strict assessment of the requirement of a gene for fitness, such as competition assays between mutant and wild-type strains in natural or agricultural settings, may still reveal roles for such genes.

It is also clear that there is no class of genes that can be considered to be unique for pathogenic fungi. All fungi have conserved systems to sense cues from the environment, to transduce signals, and to activate transcription, and all secrete enzymes, small proteins, and SMs. It appears that the key to pathogenicity lies in expanding the diversity of, for example, secreted proteins and by adapting conserved systems, such as a pheromone sensor (168) or a TF conserved in all ascomycetes (108), to respond to the host environment. This explains why most TFs required for virulence also have other functions. There are a few TFs that do seem to have no other important functions besides being required for pathogenicity (22, 34, 65, 116, 192). Further research is required to see whether these are really exclusively dedicated to the ability to colonize a living plant.

The study of the role of TFs required for host colonization is difficult because a mutant in such a TF cannot be studied during infection. The surprising effect of Nrf1 on effector expression in vitro and in planta and the role of Bap1 in ROS detoxification in vitro and in planta, as discussed above,

underline the importance of testing ideas in an infection situation. Conditional expression of TFs can be a solution to this problem, but the technological requirements for conditional expression are not always easy to meet and we know of no example of this in literature. Another option to study the role of TFs required for pathogenicity during infection is the use of temperature sensitive alleles, which has been used successfully to study the role of bE/bW during infectious growth of *U. maydis* in maize (177).

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