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MINIREVIEW

How the Necrotrophic Fungus *Alternaria brassicicola* Kills Plant Cells Remains an Enigma

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***Alternaria* species are mainly saprophytic fungi, but some are plant pathogens. Seven pathotypes of *Alternaria alternata* use secondary metabolites of host-specific toxins as pathogenicity factors. These toxins kill host cells prior to colonization. Genes associated with toxin synthesis reside on conditionally dispensable chromosomes, supporting the notion that pathogenicity might have been acquired several times by *A. alternata*. *Alternaria brassicicola*, however, seems to employ a different mechanism. Evidence on the use of host-specific toxins as pathogenicity factors remains tenuous, even after a diligent search aided by full-genome sequencing and efficient reverse-genetics approaches. Similarly, no individual genes encoding lipases or cell wall-degrading enzymes have been identified as strong virulence factors, although these enzymes have been considered important for fungal pathogenesis. This review describes our current understanding of toxins, lipases, and cell wall-degrading enzymes and their roles in the pathogenesis of *A. brassicicola* compared to those of other pathogenic fungi. It also describes a set of genes that affect pathogenesis in *A. brassicicola*. They are involved in various cellular functions that are likely important in most organisms and probably indirectly associated with pathogenesis. Deletion or disruption of these genes results in weakly virulent strains that appear to be sensitive to the defense mechanisms of host plants. Finally, this review discusses the implications of a recent discovery of three important transcription factors associated with pathogenesis and the putative downstream genes that they regulate.**

The bodies of plant-pathogenic fungi consist of growing hyphae and reproductive structures, although they are barely visible to the naked eye. Some fungi develop sexual spores during their life cycles, but for others, the sexual stage is unknown (1). The Dothideomycetes are a large and ecologically diverse class of fungi and contain both sexual and asexual species (2). This group includes various plant pathogens, such as *Cochliobolus*, *Pyrenophora*, *Parastagonospora*, *Leptosphaeria*, *Mycosphaerella*, and *Alternaria*. Sexual stages are not known for species of *Alternaria* (3). Some of these species are saprophytes living on dead organic materials, and others are plant parasites (4). *Alternaria brassicicola* is a destructive plant parasite that causes substantial damage to a broad range of host plants. It causes black spot disease on virtually all plant species in the Brassicaceae (5–7). These crop species include *Brassica oleracea* (vegetables), *Brassica rapa* (vegetables, oilseeds, and forages), *Brassica juncea* (vegetables and seed mustard), and *Brassica napus* (oilseeds) (8). This disease is of worldwide economic importance (5–7, 9, 10) and can result in 20 to 50% yield reductions in crops such as canola and rape (10).

Alternaria brassicicola is a necrotrophic plant pathogen that kills and absorbs nutrients from the aboveground tissues of its hosts. This mechanism is in contrast to that of biotrophic plant pathogens that feed on absolutely living host tissues. The pathogenesis mechanisms of necrotrophic fungi are simplistically described as a two-step process. The first step is the killing of host cells or inducing programmed cell death with toxins (11–17). Subsequently, the necrotrophic fungi break down the dead tissues with various carbohydrate-active enzymes (CAZys) that are commonly known as cell wall-degrading enzymes (CWDEs) (18, 19).

Several physiological and morphological characteristics of various pathogenic fungi have been demonstrated, or hypothesized, to be involved in necrotrophic pathogenesis (20). These characteristics include specialized morphology (21), secretion of secondary metabolites and toxins (22), production of lipases (23), CWDEs (18, 19), and proteases (24), and uninterrupted mycelial growth. Pathogenesis mechanisms are also affected by a pathogen's ability to cope with various environmental stresses, such as reactive oxygen species, pH fluctuation, and host defense molecules. Defects in development or in the organism's metabolism also affect pathogenesis. In this review, I describe recent progress in our understanding of the pathogenesis mechanisms of *A. brassicicola*.

RESEARCH BACKGROUND

Molecular research associated with *A. brassicicola* was focused primarily on plant responses to infection (25–31) rather than the interaction of the fungus with its host plants until its genome sequence was determined. The main reason for a lack of research on fungal genes is that conventional genetics has been difficult with *A. brassicicola*. In comparison, the biochemical aspects of pathogenesis have been actively investigated. They include the production of toxic secondary metabolites and proteins during saprophytic growth in axenic media, the parasitic colonization of host plants (32, 33), and the effects of phytoalexins on the fungus (34–36). Other research includes phenotypic characterization of naturally occurring *A. brassicicola* mutants (37) and gene expression studies to discover candidate genes associated with pathogenesis (38–40).

Molecular research on pathogenesis mechanisms used by *A. brassicicola* has been encouraged since the Lawrence group at Virginia Bioinformatics Institute and the Genome Center at Wash-

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TABLE 1 Numbers of genes encoding 12 selected proteins in 16 dothideomycete fungi[a]

Organism	Lifestyle	Order	No. of genes encoding:												
			NPS	PKS	TPS	SSP	Cut	Lip	GH	PSL	CHE	GT	PL	PE	Xylan	XE
Cochliobolus heterostrophus C4	Necro	Pleosporales	14	23	17	239	11	70	276	15	48	96	11	3	10	13
Cochliobolus heterostrophus C5	Necro	Pleosporales	14	25	17	251	13	70	292	15	51	103	11	3	10	13
Cochliobolus sativus	Hemi	Pleosporales	19	18	14	210	13	69	272	15	48	99	11	3	10	12
Setosphaeria turcica	Hemi	Pleosporales	15	27	7	201	9	60	254	14	42	94	10	3	10	9
Alternaria brassicicola	Necro	Pleosporales	7	7	8	139	9	76	249	23	41	83	19	7	9	9
Pyrenophora teres	Necro	Pleosporales	44	22	11	111	7	57	254	10	40	98	6	2	8	8
Leptosphaeria maculans	Hemi	Pleosporales	8	13	7	188	6	49	231	19	34	94	15	3	5	5
Parastagonospora nodorum	Necro	Pleosporales	10	19	7	209	7	77	273	10	50	89	6	6	14	14
Rhytidhysteron rufulum	Sapro	Pleosporales	15	34	10	205	0	73	283	17	46	89	11	4	6	26
Hysterium pulicare	Sapro	Hysteriales	10	28	5	141	2	59	271	4	38	98	2	7	7	11
Mycosphaerella fijiensis	Hemi	Hysteriales	11	8	6	143	6	28	228	5	27	97	3	3	1	8
Mycosphaerella graminicola	Hemi	Capnodiales	9	11	5	212	4	43	191	3	18	99	3	1	3	5
Mycosphaerella populicola	Hemi	Capnodiales	9	10	7	123	4	20	156	5	14	95	3	0	1	5
Mycosphaerella populorum	Hemi	Capnodiales	10	11	7	223	6	23	169	6	19	95	4	0	1	6
Dothistroma septosporum	Hemi	Capnodiales	7	6	7	133	1	28	201	4	23	110	1	3	3	4
Baudoinia compniacensis	Sapro	Capnodiales	2	2	8	67	3	36	164	0	17	85	0	1	6	3
Median			9.5	11	7	142	5	46	229.5	5.5	30.5	95	3.5	3	5.5	7
Mean			11.8	14.3	7.3	157.8	4.6	46.7	222.5	8.8	30.6	94.3	5.3	3.1	5.3	8.7

[a] Abbreviations: NPS, nonribosomal protein synthase; PKS, polyketide synthase; TPS, terpene synthase; SSP, small secretion protein; Cut, secreted cutinase; Lip, secreted lipase (excluding cutinase); GH, glycoside hydrolase; PSL, polysaccharide lyase; CHE, carbohydrate esterase; GT, glycosyltransferase; PL, pectate lyase; PE, pectin esterase; XE, xylan esterase; Sapro, saprophyte; Hemi, hemibiotroph; Necro, necrotroph.

ington University initiated their genome project. *Ab initio*, machine-annotated information from this group has been available at the Web portal of Joint Genome Initiatives (JGI; http://genome.jgi-psf.org/Altbr1/) for many years (41). Its genome was subsequently analyzed in the context of comparative genomics among 18 species of Dothideomycetes (4).

CANDIDATE GENES ASSOCIATED WITH PATHOGENESIS

There are 10,688 predicted genes in the genome of *A. brassicicola* (4). Secondary metabolites are required for the pathogenicity or virulence of several pathogenic fungi, and small secreted proteins play important roles in plant-fungus interactions. Genes encoding enzymes for secondary metabolite synthesis include those for nonribosomal peptide synthases (NPS), polyketide synthases (PKS), and terpene synthases (TPS). There are only 22 members in these three families in the genome of *A. brassicicola* compared to over 54 to 77 genes in closely related necrotrophs, such as three *Cochliobolus* species and *Pyrenophora teres* (Table 1). There are 139 genes encoding small secretion proteins in *A. brassicicola*, compared to an average of 157.8 genes in 16 Dothideomycetes. The genes that produce either unique secondary metabolites or unique small secretion proteins are putatively associated with the production of general phytotoxins or host-specific toxins.

Some CAZys abundant in the *A. brassicicola* genome might also be important for pathogenesis. There are 83 glycosyl transferases that are probably involved in the assembly of polysaccharides in fungal cell walls. The numbers are similar among other Dothideomycetes, regardless of their lifestyles. These genes may be important in the fungal life cycle and indirectly associated with pathogenesis but not specific to necrotrophy. Genes that are important for *A. brassicicola*'s necrotrophic lifestyle are speculated to be expanded. Several gene families encoding hydrolytic enzymes are increased in the genome of *A. brassicicola*. There are 76 genes encoding secreted lipases in *A. brassicicola* compared to an average of 46.7 genes among 18 Dothideomycetes. Nine of the 76 secreted lipases are cutinases in *A. brassicicola*, compared to fewer than 3 cutinases in saprophytes. Two families of CAZys are also expanded. In *A. brassicicola*, there are 249 glycosyl hydrolases that are involved in the digestion of polysaccharides. Among the glycosyl hydrolase genes, 22 and 53 genes, respectively, belong to GH61 and CBM1, which cleave cellulose. Compared with the Capnodiales, there are more hydrolases in *A. brassicicola* and other fungi in the Pleosporales. Notably, there are 19 pectate lyases and 7 pectin esterases in *A. brassicicola*, twice as many as in their homologs in other fungi. The genes whose numbers are expanded may be involved in the pathogenesis (including the initial penetration) of the host or the early colonization (by digestion) of the cuticle layer and cell wall components of host plants.

EASY REVERSE-GENETICS APPROACHES

Sexual stages are not known for this fungus, and there is no record of forward-genetics studies. In contrast to the forward-genetics approach, the reverse-genetics approach of disrupting, deleting, or labeling targeted genes with exogenous DNA fragments is simple and efficient (42, 43). It is sufficient to transfer a short linear construct into fungal protoplasts to produce disruption mutations in targeted genes. A targeted gene disruption construct can be made with a partial 250- to 600-bp-long targeted gene on one end and a selectable marker gene cassette (44) on the other end. The efficiency of targeted gene disruption is ~75% when the flanking fragment is ~1 kb long (42, 45). Linear constructs can also be used to make mutants with deletions of any targeted gene (46, 47). All constructs with either a disruption or a deletion of a target gene can be produced with just two rounds of PCR, as described for other fungal systems (48, 49). The publically available genome sequence of *A. brassicicola* (http://genome.jgi-psf.org/Altbr1/Altbr1.home.html) has been resourceful in the design of genetic constructs for the creation of mutants and subsequently in

identifying pathogenesis-associated genes and in inferring pathogenesis mechanisms.

TENUOUS EVIDENCE ON THE IMPORTANCE OF TOXINS

The importance of toxins in pathogenesis has been demonstrated for several necrotrophic fungi. For example, the toxin-producing pathogenic fungi *Cochliobolus victoriae*, *A. alternata*, and *Mycosphaerella zeae-maydis* are nonpathogenic when the toxin gene is nonfunctional (50–52). Another piece of direct evidence on the importance of toxins in pathogenesis is that toxin-deficient strains of *Pyrenophora tritici-repentis* and *Ophiostoma quercus* become pathogenic when transformed with toxin genes from the same species (53, 54). Seven *A. alternata* pathotypes produce secondary metabolites that are host-specific toxins and pathogenicity factors (51, 55–61). Gene clusters for *Alternaria* toxins are encoded on supernumerary chromosomes that are conditionally dispensable (3, 61). The apple pathotype of *A. alternata*, for example, has a toxin gene cluster on a dispensable chromosome, and the pathotype becomes nonpathogenic if the chromosome is lost (62, 63). Furthermore, the hybrid strains of two pathotypes, tomato and apple and tomato and strawberry, made by protoplast fusion produce the two toxins from the parental strains and are pathogenic to both plants affected by the parents (64, 65). These toxins are released from germinating conidia and suppress host defense reactions. Further information regarding the discovery of these toxins, the target organs of each toxin, and evolutionary implications of the toxins is comprehensively provided by Tsuge et al. (61).

Unlike with the many pathotypes of *A. alternata*, no toxins have been identified as pathogenicity factors thus far in the brassica pathogen *A. brassicicola*. Instead, several weak toxins and toxin candidates whose absence causes a slight decrease in virulence have been discovered. The histone deacetylation inhibitor depudecin is a secondary metabolite synthesized by a cluster of five genes, including a polyketide synthase (*AbPks9*) in the *A. brassicicola* genome (66). Mutation of the five genes abolishes depudecin synthesis and causes a 10% reduction in virulence compared to that of wild-type *A. brassicicola*. Diterpenoid toxins called brassicicenes are produced by this fungus and linked to gene clusters in the *A. brassicicola* genome (67). In addition, brassicicolin A recently emerged as the most selective phytotoxic metabolite produced in liquid cultures of *A. brassicicola* (32), while a weak protein toxin was previously reported (33, 68). However, the genes responsible for the production of brassicicolin A and the protein toxin have yet to be found, and their importance in pathogenesis needs to be characterized by targeted gene mutagenesis. Among 7 NPS genes, mutation of *AbNPS6* or *AbNPS2* results in a moderate-to-severe reduction in virulence, but their association with the production of phytotoxins is unknown (69). Currently, the evidence of host-specific toxins as pathogenicity factors remains tenuous. The presence and absence of host-specific toxins and the importance of general toxins in pathogenesis will be clarified as further studies progress on PKS, NPS, or TPS genes that are involved in the synthesis of secondary metabolites. In addition, 139 genes encoding small secretion proteins also warrant further studies as candidates of host-specific toxins.

MINOR ROLES OF INDIVIDUAL CUTINASES AND LIPASES

Plant pathogens encounter physical barriers presented by their host plants. Cutin is the main component of the cuticle of terrestrial plants and the first layer of defense against loss of or saturation by water. This layer also provides a defense against foliar pathogens. The role of a secreted cutin-digesting enzyme, cutinase (*Pbc1*), in fungal pathogenesis has been extensively studied and compared to those of other hydrolytic enzyme genes (70–74). The expression of cutinase genes in some fungal pathogens is regulated by contact with the host plant surface (73). The importance of the enzyme in pathogenicity has been demonstrated by using antibodies, inhibitors, or cutinase-deficient fungal mutants of both *Nectria haematococca* and *Colletotrichum* spp. (70–75). Disruption of a cutinase gene results in a loss of pathogenicity in *Pyrenopeziza brassicae* (76). The *Pbc1* homolog in *A. brassicicola* (*CutAb1* gene; gene ID AB01674.1, available at the Web portal of JGI) is expressed at high levels during saprophytic growth (77) and pathogenesis (78). However, a loss-of-function mutation of the homologous gene *CutAb1* in *A. brassicicola* as well as homologous genes in *N. haematococca*, *Magnaporthe grisea*, or *Botrytis cinerea*, does not affect the virulence of these fungi (77, 79–81). Another secreted lipase, *Fgl1* (GenBank accession number AY292529) is involved in the infection process in *Fusarium graminearum*, and disruption of the gene results in the reduction of its virulence to wheat spikes and corn kernels (23). However, deletions of four individual lipase genes, including its homologs AB10342.1 and AB09630.1, do not affect pathogenesis in *A. brassicicola* (42, 82).

The cutinase *Pbc1* in *P. brassicae* and the lipase *Fgl1* in *F. graminearum* are obviously important virulence factors, but their orthologs are dispensable for pathogenesis in *A. brassicicola*. It is possible that the functions of these genes were compensated for by other paralogs in the mutant. It is of note that there are 9 putative cutinases and 76 lipases with secretion signal sequences in the genome of *A. brassicicola* (Table 1) (4). The number of these two protein families is greater in *A. brassicicola* than the average number among other Dothideomycetes (Table 1). Although their role in pathogenesis has yet to be explored, they may be important for initial penetration and virulence. Disruption or deletion of individual genes, however, does not result in reduced virulence for reasons discussed in the following section.

CELL WALL-DEGRADING ENZYME GENES

Pectins and xylans are structural heteropolysaccharides and key components of primary and secondary cell walls of terrestrial plants. In addition, pectins are major components of the middle lamella and help bind cells together. Xylans are generally found at the interface between lignin and cellulose and are speculated to be important for fiber cohesion and plant cell wall integrity (83). Both pectins and xylans strengthen cell walls and assist in cell-to-cell adhesion in plant tissues. They also play important roles in protecting plants from pathogen invasion. These two heteropolysaccharides are rich reservoirs of sugars that are unlocked by hydrolytic enzymes produced by microbial pathogens. These sugars are used as the basic building blocks of the microbial biomass. It has been suggested that pectin-digesting enzymes play important roles in the virulence of various phytopathogenic fungi (84, 85). Subsequently, it was shown that loss-of-function mutation of genes encoding pectinolytic enzymes resulted in a reduction of virulence in *Aspergillus flavus* (86), *B. cinerea* (87), and *Claviceps purpurea* (88). In *A. brassicicola*, many genes encode pectin-digesting enzymes, which suggests their importance in a parasitic lifestyle. However, none of the pectin-degrading enzyme genes evaluated were found to be important for pathogenesis in *A. brassicicola* (42). For example, virulence was not reduced even after the

removal of the pectate lyase AB10322.1 gene (42), which was abundantly expressed during the late stage of plant infection (78). Further, disruption of a gene encoding xylanase, the AB03077.1 gene, or a regulator of a xylanase-coding gene, the AB04096.1 gene, did not affect the virulence of *A. brassicicola* (46).

Cellulose is another physical barrier to the fungus and the most abundant structural component of the primary cell wall of green plants. Cellulose is a polysaccharide consisting of a linear chain of hundreds of β-1,4-linked D-glucose units (89). Cellobiohydrolases and β-1,4-endoglucanase are specific enzymes that catalyze the hydrolysis of cellulose. A cellobiohydrolase, *CBH7*, in *Cochliobolus heterostrophus* (90) and its homolog in *A. brassicicola*, *AbCBH7* (AB06252.1), are also expressed at high levels during pathogenesis, but disruption of *AbCbh7* does not affect pathogenesis (42). In addition to *AbCBH7* (AB06252.1) in *A. brassicicola*, other hydrolytic enzyme-coding genes, such as those for chymotrypsin (AB01734.1), *N*-acetylglucosaminidase (AB02307.1), glycosyl hydrolase (AB08726.1), and serine protease (AB10439.1), are expressed at high-to-moderate levels, but mutants with disruption in these genes did not show a reduction in virulence (42). It is not unusual for mutation of an individual gene or several genes encoding hydrolases to have little or no effect on pathogenicity. This may be due to either functional redundancy (91–94) or extreme functional specialization (95–97) among gene families and individual CWDE genes. Cell wall-degrading enzymes can be grouped in families of multiple members with interconnected activities. Because of this redundancy or functional specialization, the disruption of one or several genes that encode hydrolytic enzymes can decrease their corresponding activity with no reduction in virulence (91, 92).

GENES ASSOCIATED WITH VIRULENCE AND IMPORTANT CELLULAR FUNCTIONS

Several genes whose mutation affects pathogenesis have been linked to important cellular functions in *A. brassicicola*. Mutants with these mutated genes show various phenotypes in addition to a reduction in virulence. For example, the *TmpL* gene is responsible for intracellular redox homeostasis, and its mutation causes defects in cell wall integrity, abnormal conidium development, an enhanced oxidative burst during conidiation, and hypersensitivity to oxidative stress, in addition to reduced virulence (98). Another universally important cellular function affecting pathogenesis is associated with the unfolded protein response pathway (UPRP). The UPRP is involved in a protein secretory pathway, broad aspects of cell fate, and the metabolism of proteins, amino acids, and lipids in eukaryotic cells (99). The UPRP is also an important stress-signaling pathway involved in cellular development and the adaptation of fungi to the environment. Deletion of a key regulator gene, *AbHacA*, in the UPRP causes a cell wall defect, reduced capacity for secretion of hydrolytic enzymes, slow growth on peptone-dextrose agar (PDA) or malt agar, and the complete loss of virulence (100). Cell wall integrity is affected by three other genes: *AbHog1*, *AbNPS2*, and *AbSlt2* (69, 100, 101). As in other pathogenic fungi, mutants with mutations in any of these three genes are less virulent than the wild type (102). It is reasonable to speculate that weakened cell walls make these mutants more susceptible to diverse chemicals, such as various host metabolites and reactive oxygen species. As suspected, *AbHog1* and *AbSlt2* mutants are more sensitive than the wild type to the phytoalexins brassinin and camalexin (101). The reduced virulence of these mutants is likely caused by the defective structure of their cell walls. It is also possible, however, that weakened cell walls augment the effects of uncharacterized virulence factors.

The functions of a mitogen-activated protein (MAP) kinase gene, *Amk1*, and its downstream transcription factor-coding gene, *AbSte12*, and their association with pathogenesis are mysterious. Disruption or deletion of either gene are nonpathogenic, with slow vegetative growth, a failure to form appressoria, and impaired conidium development (46, 103). In addition, *amk1* disruption mutants express putative CWDEs at lower levels than wild-type *A. brassicicola* and fail to undergo self-fusion of hyphae. The reason for the loss of pathogenicity in *amk1* mutants may be either the reduced expression of CWDE genes, slow hyphal growth, developmental defects, the inability of self-fusion, or the combined effects of all of these factors. There may be multiple cofactors or downstream genes associated with each phenotype. This hypothesis has yet to be tested. Other genes seem to have simpler functions but are still associated with pathogenesis. Mutation of the *Aso1* gene causes the loss of self-fusion and pathogenicity, although the cause-and-effect relationship between the two phenotypes is unclear (104). NPS6 is responsible for the biosynthesis of extracellular siderophores in three phytopathogens, including *A. brassicicola* (43). Mutation of this gene results in a hypersensitivity to H_2O_2 and a reduction in virulence. The proposed role of extracellular siderophores in fungal virulence is not to supply an essential nutrient, iron, to host plant tissues (43). For most genes described above and other pathogenesis-associated genes, however, the cause-and-effect relationship between their functions and the deficiencies of their mutants in pathogenesis is speculative.

REGULATOR OF CELL WALL-DEGRADING ENZYME GENES

Microbes adapt quickly and metabolize preferred carbon and energy sources, such as structurally simple glucose, rather than complex carbohydrates, such as pectin, xylan, and cellulose. This adaptation is made through the catabolite repression pathway. The repression is achieved by inhibiting synthesis of enzymes involved in catabolism of complex carbon sources other than the preferred one. This pathway is probably derepressed when complex carbons, such as pectins and celluloses, are major energy sources. Therefore, these enzymes are likely important in pathogenesis. For example, the expression levels of about half of the cellobiohydrolases and glucanases in the *Magnaporthe oryzae* genome are induced during pathogenesis (105). Knocking out a few of these genes, however, does not significantly affect virulence in *Cochliobolus carbonum* (91, 92, 106). In contrast, reducing the expression levels of many cellulases in *M. oryzae* using an RNA interference (RNAi) approach resulted in defects in penetration of the host epidermis and inhibited colonization of tissues, causing a reduction in virulence (105).

A gene encoding sucrose nonfermenting 1, *SNF1*, has a central role in carbon catabolite repression in *Saccharomyces cerevisiae* (107). Loss-of-function mutation of its homolog causes a reduced expression of multiple CWDE genes under derepressive conditions in *C. carbonum* and *Fusarium oxysporum* (106, 108). Mutants of the former exhibited an 80% decrease in virulence, while mutants of the latter show a significant delay in the development of wilt symptoms compared to their wild types. In contrast, deletion of either *SNF1*'s single-copy homolog in *A. brassicicola*, *AbSNF1*, or its downstream transcription factor *AbCre1* causes

almost no reduction in virulence (46). It is possible that the functions of *AbSNF1* and *AbCre1* are redundant to those of other unknown genes. Discovery of *AbVf19*, however, implies that the pathway is regulated by other genes in *A. brassicicola*.

The transcription factor *AbVf19* is responsible for the induction of 28 hydrolytic enzyme-coding genes during the late stage of pathogenesis (78). *AbVf19* positively regulates the expression of 15 glycoside hydrolases, 6 pectate lyases, 6 peptidases, and 1 cutinase. Only a small portion of the genes within each family encode 249 glycoside hydrolases, 19 pectate lyases, 164 peptidases, and 76 secreted lipases (4). The cutinase regulated by *AbVf19* is *CutAb1* (AB01674.1), whose expression level during plant infection was 73-fold higher in wild-type *A. brassicicola* than in the Δ*Abvf19* mutant. Other induced genes include homologous genes encoding a cellobiohydrolase, *AbCbh7* (AB06252.1, whose expression is 21-fold higher in the wild type), a peptidase (AB01734.1, whose expression is 3-fold higher in the wild type), chymotrypsin (AB01734.1, whose expression is 3-fold higher in the wild type), and pectate lyase (AB10322.1, whose expression is 3-fold higher in the wild type). Single-gene mutation of these genes did not affect the virulence of *A. brassicicola* (42, 77, 78). Most of these 28 genes are downregulated by a transcription factor, *Amr1*, which is responsible for the accumulation of melanin during conidiogenesis (45). Clearly, multiple enzyme-coding genes involved in pathogenesis are coordinately regulated by the transcription factor genes *AbVf19* and *Amr1* during the late stage of infection. It is likely that *AbVf19* induces expression of the 28 putative hydrolytic enzyme genes when the fungus needs biomolecules for energy and vegetative growth. *Amr1* suppresses these enzyme genes, however, when the fungus uses the energy to produce conidia.

PATHOGENESIS REGULATORS

There are two transcription factors that are very important for pathogenesis but probably dispensable for other cellular functions. Deletion of either gene resulted in impairment in pathogenesis without affecting other phenotypes. The transcription factor Bdtf1 is important for the detoxification of brassinin produced by *Brassica* species during pathogen infection and other stressful conditions (32, 109). Brassinin has antifungal activities against *A. brassicicola in vitro* (35). It is quickly modified and neutralized during plant infection by wild-type *A. brassicicola*, however, producing the intermediate metabolites *N*′-indolylmethanamine and *N*′-acetyl-3-indolylmethanamine (110). Genes encoding enzymes responsible for the detoxification of brassinin are yet to be identified. Studies on a transcription factor gene, the *Bdtf1* gene, using gene deletion mutants that are unable to detoxify brassinin and are ~70% less virulent than wild-type *A. brassicicola* (111), provide a path to the identification of the responsible enzymes. Comparison of gene expression profiles between the transcription factor gene mutants and wild-type *A. brassicicola* uncovered three candidate genes (112). We are in the process of characterizing their functions in pathogenesis and brassinin detoxification.

Another transcription factor, *AbPf2*, is involved in the early stage of pathogenesis. The *AbPf2* deletion mutant is nonpathogenic, but it lacks any other phenotypes in saprophytic growth, both in the presence and in the absence of stress-inducing chemicals (113). Changes in the gene expression profiles of the mutant during pathogenesis provide a clue to its loss of pathogenicity. About 1% of the genes in the genome are expressed at lower levels in the Δ*abpf2* mutant than in the wild type during the early penetration stage. Notably, the two pectate lyase AB04813.1 and AB01332.1 genes are induced by *AbPf2* as early as 4 h postinoculation. However, six other pectate lyase genes (AB05514.1, AB00904.1, AB10322, AB06838.1, AB03608, AB10575.1) regulated by *AbVf19* (78) are not induced by *AbPf2*. A total of 106 genes, including 13 putative CWDE genes, appear to be regulated by the transcription factor. Interestingly, knockout mutants of the pectate lyase AB01332.1 gene showed an ~30% reduction in virulence (Y. Cho, unpublished data). It is notable that 6 of 139 genes encoding small secretion proteins are also regulated by the transcription factor. They may act as effector proteins. The loss of pathogenicity in the Δ*abpf2* mutant may be either the cumulative effects of 106 downstream genes or caused by a few important pathogenicity factors.

PERSPECTIVES

Genes encoding cutinases, lipases, CWDEs, and proteases appear to be important for successful pathogenesis in *A. brassicicola*. A small subset of these genes is coordinately induced during the late stage and another subset during the early stage of pathogenesis by *AbVf19* and *AbPf2*, respectively (78, 113). Mutation of either regulatory gene causes a severe reduction in virulence; however, no knockout mutants of any individual genes among lipases and CWDEs has shown a significant reduction in virulence so far. These observations raise a question as to whether all lipases and CWDEs are important or whether mainly the small subset of the genes regulated by *AbVf19* and *AbPf2* are important for pathogenesis. If the second scenario is incorrect, these subsets of genes coincidentally show regulation patterns similar to those of unknown virulence-associated genes during pathogenesis. This question can be tested by knocking down their expression in *A. brassicicola* using appropriately designed RNAi constructs. In principle, the functional importance of lipases and diverse families of CWDEs in pathogenesis can be tested by knocking down a set of genes within each family beyond individual genes. Conserved regions can be targeted to reduce the expression levels of multiple genes using an RNAi approach (114). The number of targeted genes can be decided based on conserved regions of nucleotide sequence in the transcribed region among targeted genes. The extent of knockdown efficiency can be empirically determined. If the expression of most genes encoding either lipases or CWDEs is knocked down by RNAi, the corresponding mutants would use substrates less efficiently, grow more slowly, and be less virulent than the wild type when the substrates are used as major sources of nutrients. The use of RNAi in tandem with comprehensive studies on gene expression profiles would identify candidates of lipases and cutinases specialized and important for pathogenesis.

The pathogenicity of *Alternaria* species was probably acquired multiple times during their evolution. Production of similar phytotoxic secondary metabolites in several pathovars of *A. alternata* provides a clue as to how a weak pathogen becomes stronger or how a pathogen increases its host range by acquiring host-specific toxins. Unlike with *A. alternata*, however, the importance of toxic secondary metabolites in the pathogenesis of *A. brassicicola* is thus far tenuous and yet to be tested. A study of the transcription factor *AbPf2* opens another possibility that a few proteins may act as important toxins in the pathogenesis of *A. brassicicola*. It is of note that *AbPf2* regulates 6 of 139 genes encoding small secretion proteins (113), which may act as effectors. The importance of effec-

tors in the interactions between various host plants and their fungi and fungus-like oomycete pathogens has been established (115, 116). In compatible interactions, many effector proteins reengineer host gene expression, causing a suppression of the plant's defenses (117, 118). *P. tritici-repentis* ToxA and ToxB are good examples of proteinaceous toxins that manipulate host susceptibility (119). All or a few of the six effectors may have crucial roles in pathogenesis and manipulate host plants through gene-for-gene interactions between effectors and unknown plant proteins.

It is still a mystery how *A. brassicicola* kills host plant tissue before extensive colonization. A subset of CWDE genes regulated by *AbPf2* may digest cellulose and pectin in the cell wall and middle lamella, causing the eventual collapse of plant tissues. This mechanism is simple and requires only a one-sided action by the pathogen to kill the tissues of its host. It is also possible that the mechanism of pathogenesis is more complicated and that the organism may use effectors or secondary metabolites to manipulate host plants. Regardless of its mode of action, I speculate that it is most practical to study two genes that encode putative pectate lyases and six genes that encode putative effector proteins. Their explosive transient induction during early pathogenesis raises questions about how their functions in pathogenesis are associated with their mode of secretion, their final destinations in the host tissues, and interactions with host macromolecules. Gene tagging with fluorescent-protein-coding genes is a useful method to trace their movement from the moment of expression in fungal cells to their final destination in host tissues. In addition, proteins expressed in *Escherichia coli* or *Pichia* species may provide tools to clarify their functions in pathogenesis, especially the host proteins that interact with and the host responses to these proteins. Some of these proteins may turn out to be long-anticipated host-specific toxins, acting as avirulence genes or necrotrophic factors.

One reason to study molecular interactions between parasites and their host plants is to find logical methods to efficiently manage plant diseases. I suspect that key transcription factors for pathogenesis are good targets for a new class of agrochemicals. Although currently beyond our capabilities, blocking the function of *AbVf19*, *Bdtf1*, or *Abpf2* would render wild-type *A. brassicicola* nonpathogenic or weakly virulent. Further, it is technically possible to discover natural or synthetic compounds that bind and ultimately inhibit the functions of target proteins. It is time to screen chemicals targeting transcription factors in an effort to discover agrochemicals to manage plant diseases caused by *A. brassicicola*.

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