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Review

At Death's Door: Alternaria Pathogenicity Mechanisms

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The fungal genus *Alternaria* is comprised of many saprophytic and endophytic species, but is most well known as containing many notoriously destructive plant pathogens. There are over 4,000 *Alternaria*/host associations recorded in the USDA Fungal Host Index ranking the genus 10th among nearly 2,000 fungal genera based on the total number of host records. While few *Alternaria* species appear to have a sexual stage to their life cycles, the majority lack sexuality altogether. Many pathogenic species of *Alternaria* are prolific toxin producers, which facilitates their necrotrophic lifestyle. Necrotrophs must kill host cells prior to colonization, and thus these toxins are secreted to facilitate host cell death often by triggering genetically programmed apoptotic pathways or by directly causing cell damage resulting in necrosis. While many species of *Alternaria* produce toxins with rather broad host ranges, a closely-related group of agronomically important *Alternaria* species produce selective toxins with a very narrow range often to the cultivar level. Genes that code for and direct the biosynthesis of these host-specific toxins for the *Alternaria alternata sensu lato* lineages are often contained on small, mostly conditionally dispensable, chromosomes. Besides the role of toxins in *Alternaria* pathogenesis, relatively few genes and/or gene products have been identified that contribute to or are required for pathogenicity. Recently, the completion of the *A. brassicicola* genome sequencing project has facilitated the examination of a substantial subset of genes for their role in pathogenicity. In this review, we will highlight the role of toxins in *Alternaria* pathogenesis and the use of *A. brassicicola* as a model representative for basic virulence studies for the genus as a whole. The current status of these research efforts will be discussed.

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Alternaria toxins and small chromosomes

With the exception of studies regarding pathogen-derived host-specific phytotoxins, the physiological and molecular mechanisms underlying the interactions between necrotrophic phytopathogenic fungi and their respective host plants are largely unexplored. Several different types of genes have been shown to be involved in fungal pathogenesis for necrotrophs. These range from genes that encode proteins such as cell wall degrading enzymes, toxins, and transporter proteins, to those involved in signal transduction cascades such as mitogen activated protein (MAP) kinases. Many unanswered questions regarding fungal pathogenicity, especially pertaining to *Alternaria* species, still remain. All of the plant pathogenic *Alternaria* species to date have been reported to produce host-specific toxins (HSTs) and/or non-host specific toxic substances, both having very diverse biochemical structures (Rotem, 1994; Thomma, 2003). For many of the plant pathogenic *Alternaria* species, toxin production has been clearly demonstrated to be essential in enabling disease development on a particular host(s). The species *A. alternate* is ubiquitous in nature and mainly has a saprophytic lifestyle. Their transition from a nonspecific and nonpathogenic life to that of a host-specific pathogen involved the acquisition/evolution of host specific toxins, hereafter referred to as HSTs (Rotem 1994). For example, there are at least 7 known host-parasite interactions in which HSTs produced by *A. alternate* pathotypes are responsible for disease (Akamatsu et al., 1997; Otani et al., 1995) making them a relatively unique system for addressing the evolution of virulence and host specialization. Toxins produced by *A. alternate* pathotypes are mainly low molecular weight secondary metabolites. Some toxins, such as the AAL-toxin, produced by *A. alternate* f. sp. *lycopersici* (synonym the tomato pathotype of *A. alternate*, synonym *A. arborescens*), has been shown to be a sphingolipid-like molecule structurally similar to fumonisins (Gilchrist, 1997; Wang et al., 1996). Other toxins of diverse structure include

cyclic despipeptide-based molecules such as the AM-toxin from the apple infecting *A. alternate* pathotype, *A. alternate* f.sp. *mali* (Johnson et al., 2000).

Most fungal host-specific toxins are metabolites, but in the case of the wheat pathogen, *Pyrenophora tritici-repentis*, a toxic peptide has been shown to be a major virulence factor (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995). Similarly, AB-toxin produced by *A. brassicicola* is proteinaceous, and interestingly only produced when on host plants (Otani et al., 1998). *Alternaria* species also produce suits of toxins that are non-host specific. In addition to AB-toxin, *A. brassicicola* produces other toxic substances including despipeptides and fucicoccin-like compounds (Cooke et al., 1997; MacKinnon et al., 1999; Mckenzie et al., 1988). *A. solani* has been reported to produce non-HSTs such as alternaric acid and zinniol, in addition to a HST (Maiero et al., 1991). Both HSTs and non-HSTs may be involved in any or all stages of infection by *Alternaria* species. It is reasonable to assume even that non-HSTs may not be the primary determinant of disease but may serve as important virulence factors (Rotem, 1994).

Although *Alternaria* species synthesize structurally diverse suits of toxic substances, some *A. alternate* pathotypes share common toxin biosynthetic building blocks. For example, AF-toxin of the strawberry pathotype, AK-toxin of the Japanese pear pathotype, and ACT-toxin of the tangerine pathotype have a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structural moiety (Feng et al., 1990; Kohmoto et al., 1993; Nakashima et al., 1985; Nakatsuka et al., 1986, 1990). Most interestingly, the strawberry and tangerine pathotypes are also pathogenic to Japanese pear cultivars susceptible to the Japanese pear pathotype (Kohmoto et al., 1993). Indeed, the tangerine and pear pathotypes actually produce two closely related but distinct toxins (ACT-toxin I and II; AK-toxin I and II, respectively), while the strawberry pathotype produces three (AF-toxins I, II, and III), all sharing the common moiety and differing within a pathotype by a simple side group. While both AK-toxin I and II are toxic only to susceptible Japanese pear cultivars, AF-toxin I of the strawberry pathotype is toxic to susceptible cultivars of both strawberry and pear, and AF-toxin II is toxic only to pear (Kohmoto et al., 1993). ACT-toxin I of the tangerine pathotype is toxic to susceptible cultivars of both citrus and pear, while ACT-toxin II is more toxic to pear, but also elicits symptoms on citrus (Kohmoto et al., 1993). Concordant with the proliferation of HST production in this clade of *Alternaria* pathogens, it was found that the genomes of these related pathogens contained small additional chromosomes when compared to related strains that are strictly saprophytic in nature (Akamatsu et al., 1997). As the DNA sequences corresponding to the toxin biosynthesis genes became available, two characteristics became evident: 1) these genes were part of larger gene clusters responsible for toxin production; 2) these toxin biosynthetic clusters were localized to the small chromosomes noted previously (Akamatsu et al., 1997). These clusters, however, can occur in different numbers on these small chromosomes or on small chromosomes of differing sizes depending on the individual isolate assayed. For example, genes in strawberry responsible for AF-toxin production are each present in multiple copies, all on a single 1.05-Mb chromosome (Hatta et al., 2002). When additional strains of this pathotype were evaluated, five had these sequences on the 1.05-Mb chromosome, and one strain, T-32, had an additional chromosome of 1.13 Mb that appeared to carry the genes. Additionally multiple "sets' of homologs were also identified on the 1.05 Mb chromosome of strain NAF-8 and appeared to be organized into clusters with a different open reading frame order.

Characterization of several *A. alternata* pathotypes found that the fungi carrying the additional chromosomes could be cured of them or lose them through repeated subculture, suggesting that they are not required for normal saprophytic growth, and were thus coined as "conditionally dispensable" to imply that genes located on these elements may confer selective advantages in certain situations or ecological niches. For example, it has been shown that an apple pathotype that loses its conditionally dispensable chromosome (CDC) becomes indistinguishable to saprophyte strains, implying that this plant pathogen is basically is a saprophyte that has acquired the CDC carrying apple pathogenicity factors (Johnson et al., 2000). Subsequent studies on these CD chromosomes show that the DNA found here does not hybridize to any other host genomic DNA sequence, indicating that these sequences are unique in the genome and suggesting their acquisition through horizontal gene transfer (HGT). Hatta et al. (2002) showed that the repeated patterns of DNA sequences on CDCs have a different evolutionary history from those of essential chromosomes in the same genome, and that they may have been acquired by HGT. Natural evidence for horizontal transfer of CDCs was best illustrated by Masunaka et al. (2005). This group examined citrus-infecting isolates of *Alternaria*, namely pathogens of tangerine (producers of ACT-toxin) and rough lemon (producers of ACR-toxin). Both can be recovered from healthy rough lemon tissue in Florida, but most isolates are pathogenic to only one or the other host. The proximity of both the hosts and the *Alternaria* isolates provides the opportunity for interaction on a common host (rough lemon) because citrus production often involves cultivation of multiple species (e.g. lemon and tangerine) in the same geographical region. They iso-

lated a strain (BC3-5-1-OS2A) from a lesion on rough lemon capable of producing both ACT- and ACR-toxins, and this strain was able to infect tissues of both rough lemon and tangelos. They found 9 chromosomes in the tangerine pathotype (estimated genome size=30 Mb), and 12 in BC3-5-1-OS2A (also approximately 30 Mb). A probe specific to a 1.5 Mb chromosome of rough lemon pathotype hybridized to two chromosomes in the hybrid of sizes 1.05 and 2.0 Mb. Probes designed from the ACT-toxin biosynthetic region of the 1.9 Mb chromosome of the tangerine pathotype hybridized to a 1.05 Mb chromosome in the hybrid strain. This work clearly documented the natural occurrence of a genetic hybrid.

The *Alternaria brassicicola*-Brassicaceae Pathosystem

Brassicaceae, the crucifer plant family, consists of approximately 3,500 species in 350 distinct genera. However, the most important crop species from an economic perspective are found within the single genus, *Brassica*. These crop species include *B. oleracea* (vegetables), *B. rapa* (vegetables, oilseeds, and forages), *B. juncea* (vegetables and seed mustard), and *B. napus* (oilseeds) (Westman et al., 1999). *A. brassicicola* causes black spot disease (also called dark leaf spot) on virtually every important *Brassica* spp. (Sigareva and Earle, 1999a; Westman et al., 1999). Black spot disease is of worldwide economic importance (Humpherson-Jones, 1983, 1985, 1989; Humpherson-Jones and Maude, 1982a, 1982b; Humpherson-Jones and Phelps, 1989; Rotem, 1994; Sigareva and Earle, 1999a). For example, black spot can be a devastating disease resulting in 20-50% yield reductions in crops such as canola or rape (Rotem, 1994). Like other diseases caused by *Alternaria* species, black spot appears on the leaves as necrotic lesions, which are often described as black and sooty with chlorotic yellow halos surrounding the lesion sites (Fig. 1) (Rotem, 1994). *A. brassicicola*, however, is not limited to infection of leaves, and can infect all parts of the plant including pods, seeds, and stems, and is

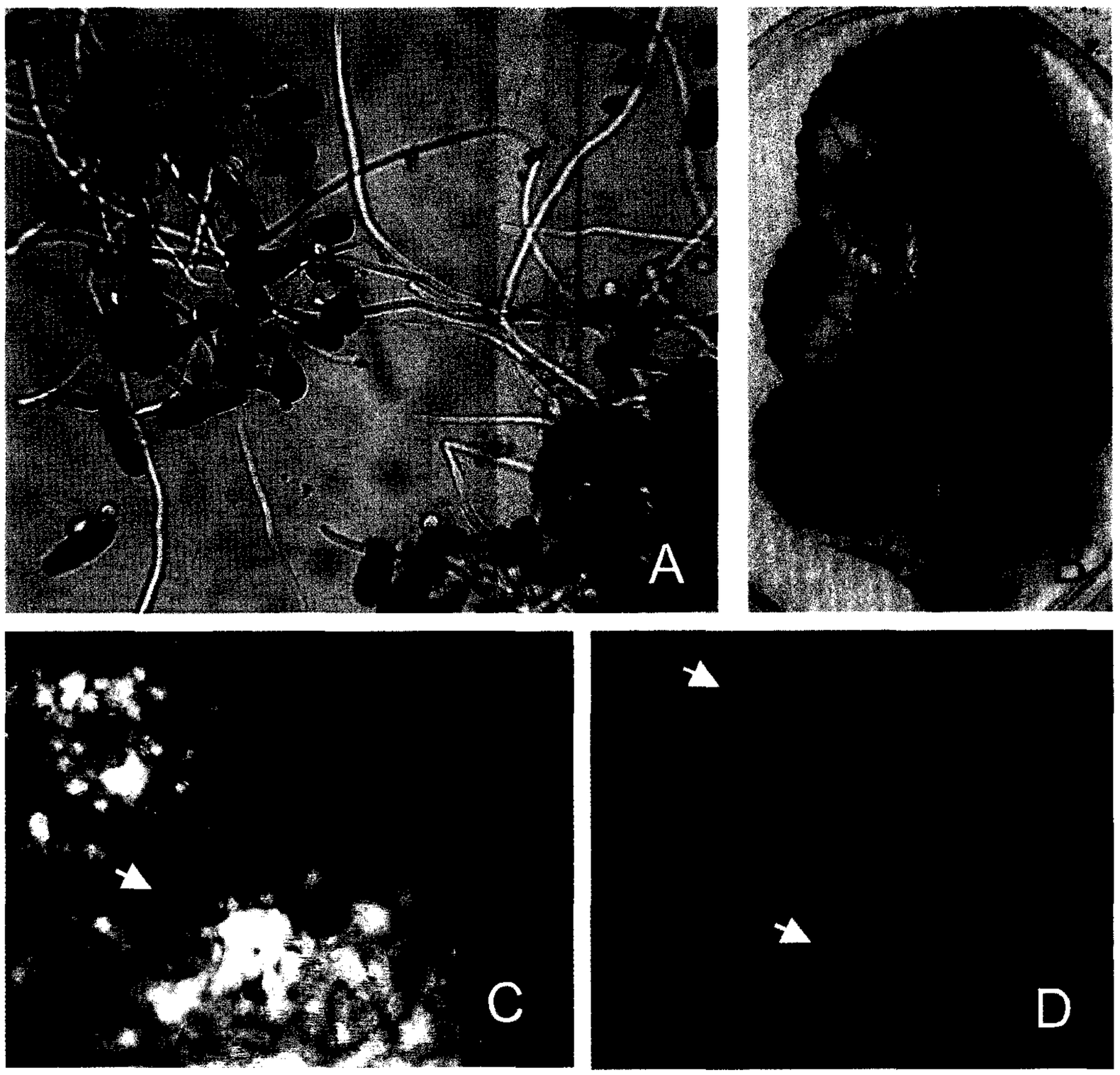

Fig. 1. *A. brassicicola* infection of Brassicas. A. Germinating spores of *A. brassiciola in vitro*, B. Black spot symptoms on cultivated mustard, C. Trypan blue staining of infected cabbage tissue. Arrow points to an invasive hypha. Note dark blue stain in cells surrounding hypha suggesting damaged walls and membranes of host cells due to the action of toxic secreted proteins and metabolites, D. GFP tagged *A. brassicicola* germinating on a cabbage leaf. Arrows depict entry through stomata and appresorium like structures.

of particular importance as a post-harvest disease (Rimmer, 1995). The necrotrophic nature of *A. brassicicola* typically leads to extensive damage of the plant and harvest product, with seedlings seldom surviving an attack (Rimmer, 1995, Humpherson-Jones, 1985). *A. brassicicola* is typically seed transmitted, though transmission by insects has also been reported (Dillard et al., 1998). Between 1976 and 1978 in the United Kingdon, 86% of the commercial *Brassica* seed produced was contaminated with *A. brassicicola* (Maude and Humpherson-Jones, 1980). The primary disease consistently affecting the cabbage seed industry in the U.S. is also *Alternaria* leaf spot, caused by *A. brassicicola* and the related species *A. brassicae*. Infection decreases seed yield, quality, and germination. Seed lots may be rejected if germination drops below 85 to 90% and infected seed lots must be treated accordingly, increasing costs associated with seed production. Spread of the disease during the growing season is typically by rain and wind dislodged spores. Optimal conditions for sporulation and infection include a minimum wet period of 13 hours and ambient temperatures of 20-30°C (Humpherson-Jones and Phelps, 1989; Rotem, 1994). Consequently, black spot disease has been of particular importance in regions of the world with cool, wet weather during the growing season, such as in the United Kingdom, Thailand, and the northeastern United States (Pattanamahakul and Strange, 1999). High-levels of resistance/immunity to this fungus have been reported in weedy cruciferous plants such as *A. thaliana*, *C. sativa* and *C.bursa-pastoris*, but no satisfactory source of resistance has been identified among cultivated *Brassica* species (Conn et al., 1988; Sigareva and Earle, 1999a, 1999b; Westman et al., 1999). Of the very few *Brassica* species or breeding lines that have been reported to possess some level of resistance, the genetic basis appears to involve additive and dominant gene action (King, 1994). Importantly, necrotrophs are traditionally hard to control with host resistance, which is usually quantitative in nature.

Alternaria brassicicola is an important pathogen for studies with Arabidopsis

Despite our limited understanding of *A. brassicicola* pathogenesis mechanisms, a substantial amount of work has been done to characterize resistance mechanisms to *A. brassicicola* using the model plant *Arabidopsis thaliana*. Natural variation in resistance to *A. brassicicola* exists in *Arabidopsis* ecotypes and several mutants have been identified that confer increased susceptibility to this fungus (Kagan and Hammerschmidt, 2002; King, 1994; Mengiste et al., 2003; Oh et al., 2005; Thomma, 2003; Thomma et al., 1997, 1998, 1999a, 1999b, 2000; Tierens et al., 2002; Zheng et al., 2006; Zhou et al., 1999). Furthermore, the enormous genomic resources that are available for *Arabidopsis* make it an ideal host candidate to identify important signaling pathways for resistance to necrotrophic fungal pathogens and determine what pathogen-derived components activate them. *A. brassicicola* is incompatible on the *A. thaliana* ecotype Columbia (Col-0) and rapidly induces jasmonate-inducible defense genes such as the defensin pdf1.2, PR-3, and PR-4 (Thomma et al., 1998). In this study the jasmonic acid (JA)-insensitive mutant (*coi1-1*) in the Col-0 background is more susceptible to *A. brassicicola* and failed to express these defense-associated genes during infection with *A. brassicicola*. This indicates that JA-mediated defenses are important for resistance as has been found for other necrotrophic fungi such as *Pythium* species and *Botrytis cinerea* (Staswick et al., 1998; Vijayan et al., 1998). It has been demonstrated that the phytoalexin camalexin deficient mutant, *pad-3* is more susceptible to *A. brassicicola* than wild-type plants (Zhou et al., 1999; Thomma et al., 1999b). It has also been found that *NahG* plants (markedly reduced salicylic acid (SA) levels) remain resistant to *A. brassicicola* indicating that SA is not required for resistance (Thomma et al., 1998). Additional evidence that camalexin plays a major role in resistance came from the observation that different Arabidopsis ecotypes with varying levels of camalexin show correlative differential resistance (Kagan and Hammerschmidt, 2002). Finally, the esa1 mutation affects resistance against *A. brassicicola* through a severe reduction in both camalexin-production as well jasmonate-dependent gene induction although the *esa1* gene has yet to be cloned (Tierens et al., 2002). Other important Arabidopsis genes important for resistance to *A. brassicicola* include a lipase identified using a proteomics approach (Oh et al., 2005), BOS1 (*Botrytis* Susceptible 1) found to encode a R2R3MYB transcription factor protein (Mengiste et al., 2003), MAP kinases (Brader et al., 2007; Brodersen et al., 2006), and specific WRKY transcription factors (Li et al., 2006; Zheng et al., 2006).

Several large-scale gene expression studies have been undertaken to dissect *Arabidopsis* resistance to *A. brassicicola*. In an initial microarray analysis study, Schenk et al. (2000) identified 168 genes up-regulated during an interaction between the Arabidopsis ecotype Col-0 and *A. brassicicola*, but the role of these genes in resistance has yet to be determined. This same group has since examined gene expression in distal uninoculated tissues during *A. brassicicola* infection and found 35 genes with altered gene expression (Schenk et al., 2003). Most recently, Van Wees et al. (2003) have identified 645 genes induced by *A. brassicicola* infection in wild-type Columbia (Col-0) and *pad3* plants indicating that *pad3* does not have a major effect on early stages of defense signaling. Interestingly, 265 of the 645 *A. brassicicola* induced genes identified in

this study required *COI1-1* for full expression, perhaps supporting the role of JA signaling in resistance to *A. brassicicola*. Cramer et al. (2006) performed a study to characterize expressed sequence tags (ESTs) derived from a compatible interaction between *A. brassicicola* and green cabbage. In this study, a suppression subtractive hybridization (SSH) cDNA library enriched for *A. brassicicola* and *Brassica oleracea* genes expressed in infected leaf tissue was created, along with a fungal cDNA library representing genes expressed during nitrogen starvation. Interestingly, many host resistance gene homologs appeared to be up-regulated during the compatible interaction suggesting that SA-mediated defenses were activated. Thus, it seems clear that jasmonic acid and camalexin biosynthetic pathways both play critical roles in resistance to *A. brassicicola,* and novel genes (e.g. *BOS1*) are being identified that play important roles in basal resistance. These results collectively indicate that the *A. brassicicola – Arabidopsis* interaction is a valuable system to study necrotrophic fungal pathogenesis and defense signaling pathways. The *A. brassicicola* genome sequence will allow the identification of putative secreted pathogen effector proteins *in silico* that could be used in a variety of functional experimental analyses.

The *Alternaria brassicicola* genome sequencing project

In 2004 the USDA Microbial genome sequencing program funded random shotgun sequencing of the *A. brassicicola* genome (isolate ATCC 96836) at 6x coverage, generation of a physical map (fingerprinting of BACs), sequencing of BAC and fosmid ends to help facilitate assembly (0.4x coverage), and an MPSS experiment (Massively Parallel Signature Sequencing) as a novel means for genome annotation (PI - C. Lawrence). The 6.4X shotgun sequencing, BAC and fosmid end sequencing, physical map construction, and MPSS experiments were completed at Washington University Genome Center (St. Louis, USA) and Solexa Inc., Hayward, USA. A genome assembly (V1.0) was generated and is publicly available (http://www.genome.wustl.edu/genome.cgi?GENOME=Alternaria%20brassicicola) The assembly is composed of 838 supercontigs averaging 36,147 bp in length and having an N50 value of 2,400,717 bp (the length such that 50% of all nucleotides contained in supercontigs are of at least this size). The supercontigs are composed of 4,039 contigs with 84% of the contigs longer than 1000 bp and 98.8% of the bases having a phred quality above 20. The total length of the sequenced portion is ~31 Mb, consistent with the previous estimate of 29.6 Mb for genome size using pulse-field gel electrophoresis. Approximately 80% of the assembled genome (25 Mb) is distributed on 12 supercontigs suggesting a relatively robust assembly for only 6x coverage. Bioinformatic analyses of the genome were carried out at the Virginia Bioinformatics Institute (http://www.vbi.vt.edu). For the initial prediction of genes in the genome assembly, version 2.4 of the FGENESH software (http://www.softberry.com) was used with an *Alternaria* trained parameter matrix. A total of 10,688 genes was predicted when using the contig sequences as input, or 9,814 using the supercontigs. Similar values were obtained when using other gene prediction programs such as SNAP. MegaBLAST was used to map a set of 6,430 ESTs (from fungus grown *in vitro* and plant infection libraries) to support the *ab-initio* gene prediction and included BLASTX results from similarity searches against the Uniprot protein database and against other related gene models from taxonomically-related fungi. Results of MPSS of an mRNA library derived from late stage *Alternaria*-infected cabbage leaves have provided additional experimental evidence of transcription for a subset of the gene models (~4,500). HMMER analysis of predicted proteins based on gene models has been performed utilizing protein functional domain databases individually (Pfam, Tigrfam, Superfam) and part of the Interpro suite of tools (http://www.ebi.ac.uk/interpro/). Collectively, all annotation data are currently deposited into a local SQL-queryable database and are being transferred to the web-accessible, community annotation database system in final stages of development. Public release and access to the annotated genome is expected to occur in late 2008.

Alternaria brassicicola functional genomics: production and optimization of linear disruption and replacement knockout (KO) mutants

A transformation method has been established for *A. brassicicola* in order to generate targeted gene disruption mutants at 80-100% efficiency for diverse genes using linear minimal element (LME) constructs (Cho et al., 2006). This was the first study in which PEG-mediated protoplast transformation of this fungus was reported to be successful. Targeted gene disruption was accomplished using a single homologous recombination event. The targeting efficiency was consistently high for the constructs harboring 250 bp homologous sequences corresponding to target genes. Shorter sequences have not been evaluated. In a recent regulatory gene study described in more detail below, the transformation method was used not for disruption but for deletion of target genes by replacing them with the Hygromycin B phosphotransferase (*HygB*) cassette (selectable marker) using linear replacement constructs containing ~1 kb genomic sequences that flank the target gene. The efficiency of targeted gene KO (gene disruption or gene replacement) in *A. brassicicola* is quite high when compared to

that observed in other fungi with sequenced genomes, and this high efficiency makes targeted functional genomics experiments in *A. brassicicola* physically possible and economically feasible. The targeted gene KO via disruption and/or replacement by homologous recombination is high enough for a high throughput approach; however, as expected most of the targeted gene mutants produced so far have shown little or no reduction of virulence (Lawrence et al., unpublished data). These genes were selected based on in plant expression levels or based upon machine annotated features of interest (e.g. histidine kinases, secondary metabolite biosynthesis, transcription factors, cell wall degrading enzymes, etc.). Interestingly, there were some genes identified in current studies that can be considered exciting exceptions as described hereafter.

Identification of *A. brassicicola* pathogenicity factors

Previously an examination of the role of cutinase genes in *A. brassicicola* pathogenesis was conducted (Yao and Koller, 1994, 1995). In these studies, biolistic transformation was used to disrupt the *CUTAB1* gene. Disruption of *CUTAB1* affected saprophytic growth since cutin was no longer able to be utilized as a sole carbon source, but this disruption had no significant effect on *A. brassicicola* pathogenicity. An extracellular lipase was found to be produced by *A. brassicicola in vitro* (Berto et al., 1999). In this study anti-lipase antibodies were found to significantly decrease *A. brassicicola's* ability to cause disease on cauliflower leaves. However, disruption of four predicted *A. brassicicola* lipase genes expressed during plant infection did not result in reduced virulence on cabbage (Cho et al., 2006; Lawrence et al., unpublished data). These results suggest functional redundancy of lipases in regards to pathogenicity especially since there are 12 predicted lipases in the *A. brassicicola* genome (Lawrence et al., unpublished data). It is also possible the polyclonal anti-lipase antibodies (Berto et al., 1999) recognized and blocked the action of multiple lipases simultaneously or blocked the action of a yet to be analyzed gene product. One area of interest regarding *A. brassicicola* pathogenicity lies in the area of secondary metabolite biosynthesis. Recently a non-ribosomal peptide synthase gene (*NPS6*) in *Cochliobolus heterostrophus* and *A. brassicicola* was found to direct the biosynthesis of a siderophore metabolite important for oxidative stress tolerance and pathogenicity (Oide et al., 2006). In another study, a nonribosomal peptide synthase gene (*AbNPS2*) was found to be important for cell wall integrity, conidial viability, and virulence of aged spores of *A. brassicicola* (Kim et al., 2007). The secondary metabolite corresponding to or synthesized via *AbNPS2* has yet to be characterized. Clearly more research is needed to further characterize secondary metabolite biosynthetic genes and their role in pathogenicity and fungal development.

Another area ripe for exploration in the *A. brassicicola*-Brassicaceae pathosystem is fungal signal transduction mechanisms. Disruption of the Fus3/Kss1 MAP kinase homolog (*Amk1*) in *A. brassicicola* resulted in a complete loss of pathogenicity as observed in other fungi (Cho et al., 2006, 2007). Interestingly, in the latter study it was shown that addition of long polypeptide nutrients partially restored pathogenicity to the mutants. In contrast to the null mutants of other phytopathogenic fungi, *A. brassicicola amk1* mutants were capable of partially infecting wounded tissues. In another recent study, targeted gene deletion mutants for 21 putative regulatory genes were produced in *A. brassicicola* (Cho et al., 2008). In this study the SNF1 (sucrose non-fermenting 1) kinase, several histidine kinases, and transcription factors such as Ste12 and Pro1 were subjectively selected from the machine annotated *A. brassicicola* genome. The SNF1 kinase plays a central role in carbon catabolite repression in *Saccharomyces cerevisiae* (Palecek et al., 2002). Deletion of homologous genes in *Cochliobolus carbonum* and *Fusarium oxysporum* resulted in reduced virulence due to decreased expression of cell wall degrading enzyme (CWDE) genes under derepressive conditions including growth in media utilizing complex carbohydrates and not simple sugar as carbon source. (Ospina-Giraldo et al., 2003; Tonukari et al., 2000). Deletion of the *A. brassicicola SNF1* homolog did not significantly affect virulence in contrast to the observations made in several other pathogenic fungi (Ospina-Giraldo et al., 2003; Tonukari et al., 2000). Deletion of the STE12 homolog, a transcription factor downstream of Amk1, resulted in loss of pathogenicity as was described in other pathogenic fungi (for review see Xu, 2000). However, Δ*abste12* mutants were capable of infecting wounded plants. In addition, two novel virulence factors were discovered as part of the study by Cho et al. (2008) predicted to encode a transcription factor (*AbPro1*) and a two-component histidine kinase gene (*AbNIK1*). Deletion of *AbPro1* resulted in a 70% reduction in virulence and also exhibited a 25% reduction in vegetative growth rates *in vitro*. Deletion of *AbNIK1* resulted in a near complete loss of virulence without changes in vegetative growth rates *in vitro*. Importantly, the addition of long polypeptides to spores of both Δ*abste12* and Δ*abnik1* during plant inoculations resulted in a complete restoration of pathogenicity. These results strongly suggest once again the presence of a previously undescribed nutrient- or polypeptide-sensing pathway downstream of Amk1/AbSte12 signaling pathways and a putative AbNIK1 osmoregulation pathway. In addition to *AbNIK1* and *Amk1*, the *A. brassicicola* Slt2 MAP kinase and HOG MAP kinase homologs have been knocked out and characterized (Law-

rence, unpublished). Both of these kinases are pathogenicity factors in phytopathogenic fungi (for review see Xu, 2000). Slt2 has been shown to be associated with cell wall integrity and HOG with oxidative stress tolerance (for review see Xu, 2000). The Slt2 homolog is a major virulence determinant in *A. brassicicola* (Cho, Scott, and Lawrence, unpublished). However, knock out of the HOG kinase homolog in *A. brassicicola* did not result in reduced virulence suggesting that this fungus has evolved or developed alternative mechanisms for oxidative stress tolerance (Lawrence, unpublished). In a recent study disruption of *Aso-1*, a gene required for hyphal fusion (anastomosis) is also required for pathogenicity in *A. brassicicola* (Craven et al., 2008). Interestingly, it was also show in this study that the *amk1* mutants also failed at hyphal fusion suggesting a link between MAP kinase signaling and anastomosis. In contrast to these studies, the Δ*abste12* mutant exhibited a hyper anastomosis phenotype but was non-pathogenic on plants (Lawrence and Mitchell, unpublished). In summary, considerable progress has been made over the last several years regarding identification of *A. brassicicola* virulence genes. Well over a hundred genes have been functionally analyzed through gene knockout and overexpression experiments making *A. brassicicola* the species of choice for functional genomics research to define conserved virulence mechanisms for this important genus of fungi (Cho, 2008; Cho et al., 2007; Cho et al., 2006; Kim et al., 2007; Oide et al., 2006).

Alternaria pathogenomics and human airway disorders

In medical mycology, *Alternaria* species are gaining importance as emerging human invasive pathogens, particularly in immuno-compromised patients (Morrison and Weisdorf, 1993; Vartivarian et al., 1993). Several *Alternaria* species and numerous uncharacterized *Alternaria* taxa have been found associated with infections of the cornea, oral and sinus cavities, respiratory tract, skin, and nails (Arrese et al., 1996; Barbasso et al., 2005; Barnes et al., 2007; Romano et al., 2001; Mirkin, 1994; Machet et al., 1994; Hazouard et al., 1999). In a number of cases, these infections have been fatal or associated with certain cancers (Brugger et al., 2006; Hazouard et al., 1999; Liu et al., 1992; Neumeister et al., 1994; Yekeler et al., 2001). Perhaps more importantly from an economic and chronic health perspective, *A. alternate* spores are one of the most common and potent airborne sources of allergens yet very little is known regarding the immunological properties of and human host response to *Alternaria* proteins (Black et al., 2000; Bush and Prochnau, 2004). In the US alone over 3 billion dollars are spent annually for the relief of allergic rhinitis, much of which is due to sensitization to *Alternaria* spores (Bush and Prochnau, 2004). Additionally, *A. alternate* sensitization has been determined to be one of the most important factors in the onset of childhood and fatal asthma (Black et al., 2000; Bush and Prochnau, 2004; Ohollaren et al., 1991; Plaza et al., 2003). Currently eight major and minor proteinaceous allergens have been identified in *A. alternate* including the major allergen Alta1 (Thaker et al., 1995; Yunginger and Jones, 1978). In a recent study it was demonstrated that *A. brassicicola* expresses *Altb1*, a highly conserved homolog of *Alta1 in vitro* and it was found to be highly up-regulated during pathogenesis of Arabidopsis (Cramer and Lawrence, 2003, 2004). This study was the first to show that this major allergen gene was present in another species of *Alternaria*. Since this study it has now been shown that over 52 species of *Alternaria* and very closely related fungi possess highly conserved Alta1 homologs suggesting that virtually every species within the genus is potentially allergenic (Hong et al., 2005). Alta1 has been recently shown to possess phosphatase activity (Saenz-de-Santamaria et al., 2006). Preliminary data also suggests that this protein has phytotoxic properties and is a putative virulence factor (Lawrence, unpublished data). Finally, in several recent studies related to chronic rhinosinusitis (CRS), antigen extracts derived from *A. alternate* appear to have more potent immunostimulatory activity towards CRS patient lymphocytes, eosinophilis, and in a nasal epithelium explant model than other fungi examined including *Aspergillus*, *Cladosporium*, and *Penicillium* (Shin et al., 2004, 2006). Research is currently underway using a combination of fungal functional genomics and immunology to identify new *A. alternate* proteins and enzymes that contribute to allergic airway inflammation with several candidates already identified (Kita and Lawrence, unpublished results).

Conclusion

The genus *Alternaria* contains many economically important species from plant and human health perspectives. Substantial progress has been made regarding the evolution and molecular basis for the biosynthesis of phytotoxic secondary metabolites and their role in plant disease development. Molecular manipulation of *Alternaria* spp. has proven to be very efficient from a targeted gene disruption or replacement standpoint and will allow for identification of additional virulence mechanisms as well as investigate fundamental questions in fungal biology. The *A. brassicicola* genome sequence will undoubtedly serve as the reference genome for this important genus of fungi and will aid researchers in both the plant pathology and human allergic diseases areas. This genome sequence has already proven valuable for the identification of putative plant

virulence factors and proteins causing inflammatory responses in humans and has paved the way for sequencing the *A. alternate* genome. Comparative studies between the genomes of *A. alternate*, the species clinically associated with human airway disorders, and the plant pathogen *A. brassicicola*, may soon prove very valuable for identifying candidate human antigenic proteins and additional plant virulence factors, respectively.

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