A Zinc-Finger-Family Transcription Factor, *AbVf19*, Is Required for the Induction of a Gene Subset Important for Virulence in *Alternaria brassicicola*

Akhil Srivastava,¹ Robin A. Ohm,² Lindsay Oxiles,¹ Fred Brooks,¹ Christopher B. Lawrence,³ Igor V. Grigoriev,² and Yangrae Cho¹

¹Plant and Environmental Protection Sciences, University of Hawaii at Manoa, 3190 Maile Way, St. John 317, Honolulu 96822, U.S.A.; ²United States Department of Energy Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek, CA 94598, U.S.A.; and ³Virginia Bioinformatics Institute and Department of Biological Sciences, Virginia Tech, Blacksburg 24061, U.S.A.

Submitted 26 October 2011. Accepted 2 December 2011.

Alternaria brassicicola is a successful saprophyte and necrotrophic plant pathogen with a broad host range within the family Brassicaceae. It produces secondary metabolites that marginally affect virulence. Cell wall-degrading enzymes (CWDE) have been considered important for pathogenesis but none of them individually have been identified as significant virulence factors in *A. brassicicola*. In this study, knockout mutants of a gene, *AbVf19*, were created and produced considerably smaller lesions than the wild type on inoculated host plants. The presence of tandem zinc-finger domains in the predicted amino acid sequence and nuclear localization of *AbVf19*-reporter protein suggested that it was a transcription factor. Gene expression comparisons using RNA-seq identified 74 genes being downregulated in the mutant during a late stage of infection. Among the 74 downregulated genes, 28 were putative CWDE genes. These were hydrolytic enzyme genes that composed a small fraction of genes within each family of cellulases, pectinases, cutinases, and proteinases. The mutants grew slower than the wild type on an axenic medium with pectin as a major carbon source. This study demonstrated the existence and the importance of a transcription factor that regulates a suite of genes that are important for decomposing and utilizing plant material during the late stage of plant infection.

Alternaria brassicicola is the causal agent of black spot disease of cultivated brassicas, such as cabbage, canola, and mustard. It has also been used in studies of host–pathogen interactions on *Arabidopsis* (Oh et al. 2005; Thomma et al. 1999). Pathogenesis in necrotrophic fungi is generally described as a two-step process of initially killing host cells directly (necrosis) or inducing programmed cell death with toxins, followed by or in conjunction with decomposing tissues with cell wall-degrading enzymes (CWDE).

Nucleotide sequence data for *AbVf19* (*Alternaria brassicicola* virulence factor 19) is available in the GenBank database under accession number JN835469 and RNA-seq data GEO Series accession number GSE33148.

Corresponding author: Y. Cho; E-mail: yangrae@hawaii.edu; Telephone: +1.808.956.5305;

*The e-Xtra logo stands for “electronic extra” and indicates three supplementary figures and three supplementary tables are published online and Figure 6 appears in color online.

The importance of toxins in disease development has been clearly demonstrated in other plant–pathogen systems with necrotrophic fungi (Churchill et al. 1995; Ciuffetti et al. 1997; Del Sorbo et al. 2000; Yun et al. 1998). Several *A. alternata* pathotypes produce toxic, host-specific secondary metabolites synthesized by polyketide synthases or nonribosomal peptide synthases that are pathogenicity factors (Ito et al. 2004; Johnson et al. 2000; Spassieva et al. 2002; Tanaka et al. 1999). Several secondary metabolites and proteins in *A. brassicicola* have been identified as toxins, such as the polyketide depudecin (Wight et al. 2009), the diterpenoid brassicenones (Hashimoto et al. 2009), Brassicicolin A (Pedras et al. 2009a), and two proteins (Oka et al. 2005; Otani et al. 1998). All toxins produced by *A. brassicicola*, however, are modest virulence factors that only marginally affect pathogenesis.

The importance of CWDE genes in virulence has been supported by the identification of mutants with impaired derepression mechanisms of carbon catabolite repression in *Cochliobolus carbonum* and *Fusarium oxysporum* (Ospina-Giraldo et al. 2003; Tonukari et al. 2000). Mutation of the sucrose nonfermenting factor 1 (SNF1) homolog showed severely reduced virulence in these two fungi. It has been speculated that coordinated expression of many CWDE genes is needed for successful pathogenesis. In contrast, mutants of the homologous gene *AbSnf1* and its downstream gene, *AbCreA*, were as virulent as the wild type (Cho et al. 2009). These findings suggested that *A. brassicicola* uses a different regulatory mechanism for expression of CWDE genes during pathogenesis.

No single CWDE has been identified as important for pathogenesis in *A. brassicicola* despite knockout of over a dozen individual genes predicted to encode lipases, pectate lyases, cutinases, xylanases, and proteases (Y. Cho and C. B. Lawrence, unpublished results). Furthermore, inefficient utilization of xylan due to loss of the transcription factor *AbXlnR* that regulates the multiple xylanase genes did not affect virulence (Cho et al. 2009). Slow growth of a deletion mutant of the pathogenicity factor gene *AbSte12*, with pectin as a major carbon source, suggested that this gene was necessary for the induction of pectin digestion enzymes. Its upstream mitogen-activated protein kinase gene, *Amk1*, was necessary for the induction of several hydrolytic enzyme-coding genes (Cho et al. 2007).

In addition to the loss of pathogenicity, mutation of *Ste12* homologs caused developmental defects such as impaired conidia maturation in most pathogenic fungi, including *A. brassicicola* (Cho et al. 2009; Wong Sak Hoi and Dumas 2010). Mutants of the *Amk1* gene (*amk1*) showed more severe phenotypes

than those of *Δabste12*. The highly pleiotropic nature of *Δabste12* and *amk1* mutants made it difficult to interpret the importance of CWDE genes in pathogenesis. To date, no transcription factors have been identified that regulate CWDE genes during pathogenesis without affecting fungal development. In this study, we identified mutants that exhibited severely reduced virulence but did not show developmental defects. Our study provides an example of a zinc-finger-family

transcription factor gene that plays a critical role in regulating virulence by coordinated expression of a suite of genes, including putative CWDE genes, in the necrotrophic plant pathogen *A. brassicicola*.

RESULTS

Disruption mutants of the *AbVf19* gene.

We systematically created mutants for over 200 genes by a single homologous recombination-based gene disruption method described previously (Cho et al. 2006). During our pathogenicity assays of the knockout mutants, we discovered one gene whose mutants showed a 90% decrease in virulence. The gene of interest was predicted to encode 564 amino acids with nuclear localization signals (probability 95%) at two locations predicted by PSORT (Horton and Nakai 1997) and two C₂H₂-zinc-finger domains (Pfam ID: PF00096) (Bateman et al. 2004). Only one copy of the gene was predicted to be present in the draft genome sequence of *A. brassicicola*. The tandem putative DNA-binding domains are highly conserved among fungi in the phylum Ascomycota, including *Saccharomyces cerevisiae* (Supplementary Fig. S1). All ascomycete fungi in the Pezizomycotina with genome sequences publically available had a homolog in their genomes (Supplementary Fig. S2). Its homolog was involved in but not essential for the osmotic stress response in *Trichoderma atroviride* (Seidl et al. 2004). The functions of its homolog were annotated as a hypothetical protein in most fungal genomes and as a cutinase G-box binding protein in four genomes, but without experimental evidence. We named this gene *AbVf19* (*A. brassicicola* virulence factor 19).

Replacement of the *AbVf19* gene with a *HygB* cassette.

We created additional null mutants of *AbVf19* by replacing the coding region with a hygromycin B transferase (*HygB*) gene cassette. Southern hybridization with three probes against the genomic DNA extracted from 11 transformants and purified by single-spore isolation confirmed that the *AbVf19* gene was absent in 4 of 11 transformants (Fig. 1). The *AbVf19* coding region was replaced by a single copy of the *HygB* resistance cassette in one mutant (*Δabvf19-1*) and by multiple copies in three gene-deletion mutants (*Δabvf19-8*, *Δabvf19-9*, and *Δabvf19-10*).

Decreased virulence of the *Δabvf19* mutants on green cabbage.

We performed pathogenicity assays to further characterize virulence attributes associated with *AbVf19*. All four deletion mutants produced significantly smaller lesions ($P < 0.001$) than the wild type (77 to 92%) in each assay using various concentrations of inoculum on detached leaves (Table 1). The mutants consistently produced smaller lesions at concentrations of 1,000 and 2,000 conidia compared with the wild type (Fig. 2A). The relative reduction in virulence was slightly less with 2,000 conidia on detached leaves. To conservatively estimate mutation effects on virulence, we used 2,000 conidia for the pathogenicity assays on whole plants to compare the lesions produced by the wild type and *Δabvf19*. The lesions produced by *Δabvf19-1* were similarly reduced compared with the wild type on whole plants (Fig. 2B); therefore, we used detached leaves in the rest of our assays for speed and convenience. We also compared lesions produced by the wild type, *Δabvf19* mutant, and a mutant complemented with a wild-type allele (*Δabvf19:AbVf19*). Lesions produced by the complemented mutant were similar to those produced by the wild type, while the *Δabvf19* mutant showed a severe reduction in virulence as evidenced by the smaller lesions (Fig. 2C).

Fig. 1. Deletion of the *AbVf19* gene. **A**, Schematic diagram of the wild-type (wt) locus, replacement construct, and mutant locus are shown in order. The mutant locus represents a scenario of replacement of the coding region of the *AbVf19* gene by a single copy of a selectable marker hygromycin B transferase (*HygB*) cassette. **B**, Lane 1 shows replacement of the gene by a single copy. Lanes 8, 9, and 10 show replacement of the gene by multiple copies of the replacement constructs. Dots (•) indicate DNA lanes for pathogenicity assay results presented in this study. Probe regions are marked by P-*AbVf19*, P-3', and P-*HygB*. B = *Bam*HI enzyme digestion site.

No difference in germination and appressorium-like structure formation.

We compared spore germination between the *Abvf19-1* and the wild-type strains on minimal medium agar (MMA) and potato dextrose agar (PDA). Germination for both the mutant and wild type 3 h after inoculation on the two types of media was approximately 100%. The length of germ tubes was highly variable within each group (wild type and mutant). There was no statistical difference in average length between the mutant and wild-type germ tubes on MMA or PDA (Fig. 3A).

Germination was slower on *Brassica oleracea* (green cabbage) than on synthetic media for the wild type and the *Abvf19* mutant. Germination rates for both were approximately 50% at 12 h postinoculation (hpi) and 100% at 24 hpi (Fig. 4). Germ tube length on host plant leaves varied within each group but average length was similar between the two groups at 12 hpi. The mutant and wild type formed small, swollen structures (appressorium-like structures hereafter) at the tips of germ tubes at a similar frequency by 24 hpi (Fig. 5).

Expression and localization of AbVf19 protein.

To survey the expression and localization of the AbVf19 protein, we tagged its gene at the C-terminus, right before the stop codon, with a green fluorescent protein (GFP) coding sequence (Lorang et al. 2001). The tagging construct was designed for the GFP protein to be expressed as a fusion protein with an *AbVf19* gene that was regulated by its native promoter elements. As a negative control, we also made mutants that expressed GFP regulated by an *Amr1* promoter (Y. Cho, unpublished data). All GFP signals in the negative control mutant remained in the cytoplasm (Fig. 5A). In contrast, the GFP signals were located in the nuclei of the mutant expressing AbVf19-GFP fusion proteins (Fig. 5B and C). The AbVf19-GFP protein was expressed and located in the nuclei of all fungal tissues during all stages of host infection from 24 to 96 hpi. The AbVf19-GFP protein was also expressed in hyphae during growth in axenic media, although it was expressed at a lower level than during plant infection.

Genes regulated by AbVf19.

To study the regulatory roles of *AbVf19* in *A. brassicicola*, we compared gene expression profiles between the wild type and the *Abvf19* mutant at 96 hpi, a late stage of infection, using an RNA-seq approach. In total, 27 and 74 genes among *A. brassicicola*'s 10,688 predicted genes were significantly up- or downregulated, respectively, more than twofold ($P < 0.05$) in the *Abvf19* mutant compared with the wild type (Supplementary Table S1). This represents 0.25 and 0.69% of the predicted genes, respectively, in the current *A. brassicicola* genome. Functional categories that were overrepresented in the downregulated genes included 15 glycoside hydrolases, 6 pectate lyases, and 6 peptidases (Supplementary Table S2). In addition, one of nine cutinases was also downregulated 73 times. In total, 27 genes were upregulated in the mutant. These genes included one pectate lyase and three glucoside hydrolase genes in contrast to the six other pectate lyase and 14 glucoside

hydrolase genes that were downregulated. We examined the reliability of the gene expression data produced by RNA-seq with quantitative real-time polymerase chain reaction (qRT-PCR), using three differentially expressed genes and two non-differentially expressed genes. The qRT-PCR results were very similar to the RNA-seq data.

Conserved sequence motifs shared among downstream genes.

To discover common motifs at the putative promoter region among downregulated genes in the mutant, we surveyed the putative promoter regions of genes with expression values over 300 mapped fragments per kilobase of exon model per million (FPKM) in wild-type *A. brassicicola* and below 100 FPKM in *Abvf19*. Nine genes matched these criteria. Comparison of the promoters of these genes revealed a conserved motif shared by all nine genes (Fig. 6). Next, occurrences of the same motif were counted within the promoter regions of 56 other downregulated genes in the mutant. Of the 56 genes, 17 had at least one conserved motif in their promoter region. The occurrence of

Fig. 2. Decreased virulence of *Abvf19* mutants on host plants (*Brassica oleracea*). **A**, Lesions on *B. oleracea* 5 days after inoculation with 1,000 or 2,000 conidia of the wild type and *Abvf19-1* mutant on a detached leaf. **B**, One leaf showing lesions produced by 2,000 conidia of the wild type and *Abvf19-1* mutant on a whole plant. The leaf was detached and photographed at the end of the assay. **C**, Similar lesion size produced by a complemented mutant and the wild type compared with the small lesions produced by the *Abvf19* mutant. **D**, Lesions produced by 2,000 conidia of the wild type (top left) and 2,000, 5,000, and 10,000 conidia of a *Abvf19* mutant. wt = wild-type *Alternaria brassicicola*; *Abvf19* = *AbVf19* deletion mutant; compl = mutant complemented with a native allele of the *AbVf19* gene (*Abvf19:AbVf19*).

Table 1. Decreased virulence of four strains of *Abvf19* deletion mutants compared with wild-type *Alternaria brassicicola*^a

Mutants	Number of spores (wt/mut)	df	Lesion size (mm)		Virulence decrease (%)	P
			Wild type	Mutant		
<i>Abvf19-1</i>	1,800/1,900	8	19.6 ± 2.6	4.3 ± 3.2	78	4.0E-09
<i>Abvf19-1</i> *	2,000/2,000	8	10.4 ± 5.3	0.9 ± 0.9	92	7.0E-05
<i>Abvf19-8</i>	2,000/2,025	8	9.2 ± 4.6	2.1 ± 3.0	77	1.0E-04
<i>Abvf19-10</i>	2,000/2,025	8	9.4 ± 4.5	1.8 ± 1.9	81	8.0E-04

^a Abbreviations: wt = wild type, mut = mutant, df = degrees of freedom, and P = probability. Lesion size indicates the average lesion diameter. Results marked with an asterisk (*) were from pathogenicity assays on whole plants, others were from assays with detached leaves.

this motif among the 56 promoters was significantly greater than can be randomly selected from a pool of 1,071 promoters with this motif among a total of 8,274 promoters ($P = 5 \times 10^{-4}$). The motif showed similarity to the previously described binding site of a zinc-finger transcription factor in the JASPAR CORE database (MA0036.1, $P = 4 \times 10^{-3}$). The cutinase-encoding gene *CutAb1* that expressed 73 times more transcripts in the wild type than in the mutant had two copies of the putative binding motif whereas the other eight cutinase genes did not have this motif.

Plant responses.

The sequence tags used to identify differentially expressed genes in the $\Delta abvf19$ mutant and wild-type *A. brassicicola*

were also aligned to 14,000 full-length cDNA sequences of *B. oleracea*. Several putative defense-related genes were highly expressed in plants that were inoculated with either the wild type or the mutant. They consisted of genes annotated as lectin domain-containing protein, pathogenesis-related protein 1, reticulon oxidase-like protein, psi-producing oxygenase, cytochrome P450, and D-glucan cellobiohydrolase. In our analysis, however, no plant genes in the 14,000-gene set were identified as being differentially expressed when challenged by the wild type or the $\Delta abvf19$ mutant.

Reduced virulence in *Arabidopsis* PAD3 mutants.

Gene expression profiles at the late stage of infection suggested that the reason for the decrease in virulence of $\Delta abvf19$

Fig. 3. Comparisons of vegetative growth between the $\Delta abvf19$ mutant and wild-type *Alternaria brassicicola*. **A**, Length of germ tubes on two different media measured at 3 and 5 h postinoculation. **B**, Effect of pectin on vegetative growth of the $\Delta abvf19$ mutant and wild type. Bars represent dry weight of mycelia in milligrams. Error bars represent standard deviation in both charts. wt = wild type, MMA = minimal media agar, PDA = potato dextrose agar, ** = $P < 0.01$.

Fig. 4. Germination and formation of appressorium-like structures. Images represent infection courts at 24 h postinoculation by the wild type (left) and $\Delta abvf19$ mutant (right). Fungal tissues were stained with Trypan blue. Appressorium-like structures are marked with arrows.

was the reduced expression of several enzymes putatively important for depolymerization of plant material. We compared the virulence of the $\Delta abvf19$ mutants on the *pad3* mutant and the wild-type *Arabidopsis thaliana* to test the effects of a lack

Fig. 5. Confocal microscopic images showing AbVf19 protein expression and localization during pathogenesis. **A**, Negative control of green fluorescent protein (GFP) localization. Composite image of green fluorescence representing GFP and pink autofluorescence representing chloroplasts in plant tissues. **B**, Green fluorescence represents AbVf19-GFP fusion protein 24 h postinoculation. The green fluorescence signal is variable because fungal hyphae are imbedded in plant tissues. Deeply imbedded signals appear weaker than those on the plant surface. X- and Y-axis rotation views provide a sense of the depth of fungal penetration. **C**, Infected plant tissue was squashed to get a better view of penetrating fungal hyphae at 96 h postinoculation. The inset picture shows nuclear localization of green fluorescence.

of phytoalexin and the delayed response of plant resistance genes on the severe reduction of the mutant's virulence. We expected that *pad3* plants were very susceptible to $\Delta abvf19$ if *AbVf19* was associated with detoxification of the camalexin (the Discussion section discusses reasons). The $\Delta abvf19$ mutant, however, failed to cause lesions or was only weakly virulent on *pad3* plants, such as on wild-type *A. thaliana*. Lesion size caused by the wild-type *Alternaria brassicicola* was a little smaller on the wild type than on the *pad3* mutant *Arabidopsis thaliana* (Fig. 7).

Inefficient use of pectin by the mutant.

To determine the reason for reduced virulence in the mutant, fungal growth rates were compared in axenic broth media containing different carbon sources. We grew the mutants and wild type in a minimal medium broth supplemented with glucose as a major carbon source. Biomass, measured as dry mycelial weight, was similar for the mutant and the wild type (Fig. 3B), suggesting that glucose did not affect vegetative growth of the mutant. Both $\Delta abvf19$ and wild-type *Alternaria brassicicola* grew very slowly in the presence of α -cellulose or xylan as a major carbon source; thus, we did not investigate their effect on mutant growth. Both the wild type and the mutant produced almost 300% more mycelial biomass in glucose-yeast extract broth (GYEB) than in a minimal medium supplemented with glucose. The difference between the two in GYEB was not statistically significant. Notably, there was a significant difference when a pectin supplement was added to the minimal medium. The biomass of the wild type increased by 58% while the mutant biomass decreased by 11%. The mutant grew significantly less than the wild type ($P = 0.0012$, $df = 3$) with pectin as the major carbon source.

DISCUSSION

Multiple strains of disruption and deletion mutants of the *AbVf19* gene consistently displayed an approximately 90% reduction in virulence compared with wild-type *A. brassicicola*. The reduced virulence was restored by complementing a $\Delta abvf19$ mutant with the wild-type allele of its *AbVf19* gene. These pathogenicity assay results showed that the severe reduction of virulence in the mutants was due to the loss of the *AbVf19* functions.

The predicted amino acid sequence of the AbVf19 protein contains nuclear localization signals and tandem C_2H_2 -zinc-finger domains (Pfam ID: PF00096) (Bateman et al. 2004).

Fig. 6. Putative transcription factor binding site. This motif is overrepresented in promoters among the downregulated genes in $\Delta abvf19$ mutants.

The nuclear localization of AbVf19 protein was confirmed by accumulated green fluorescence signals from AbVf19-GFP fusion proteins. The C₂H₂-zinc finger is best known for its binding in the major groove of DNA and is typically spaced at 3-bp intervals. The α -helix expanding two conserved histidines in each domain can make sequence-specific contacts to DNA bases (Iuchi 2001). The accumulation of AbVf19 proteins in nuclei and the presence of DNA-binding motifs in the predicted amino acid sequence indicated that AbVf19 is a transcription factor.

Mechanisms of reduced virulence in the $\Delta abvf19$ mutant.

The $\Delta abvf19$ mutant expressed fewer transcripts for 74 genes than wild-type *A. brassicicola* during the late stage of infection, as was made evident initially by RNA-seq experiments. Among these 74 downregulated genes, 26 hydrolytic enzyme genes were overrepresented. Their predicted functions suggested depolymerization of cell wall components or extracellular proteins. Although we cannot rule out the possible importance of other genes, the 74 genes included neither peroxisomal enzymes such as catalases or dismutases, nor detoxification enzymes such as glucosyltransferase genes (Sexton et al. 2009) and dithiocarbamate hydrolase (Pedras et al. 2009b). The gene expression comparisons between the mutant and wild-type *A.*

brassicicola suggested that inefficient detoxification of reactive oxygen species or phytoalexins in the host plant was not the main cause for the decrease in $\Delta abvf19$ mutant virulence.

The *pad3* mutant does not produce the phytoalexin camalexin and is more susceptible to wild-type *A. brassicicola* (Narusaka et al. 2003; Zhou et al. 1999). The $\Delta abvf19$ mutant was expected to be more virulent on the *pad3* mutant than on the Col-0 ecotype if the $\Delta abvf19$ mutant could not detoxify camalexin. The $\Delta abvf19$ mutant, however, was equally less virulent on the phytoalexin-deficient *pad3* and wild-type *Arabidopsis thaliana*. Likewise, there was no difference in the gene expression profile of *B. oleracea* in response to the $\Delta abvf19$ mutant or wild-type *Alternaria brassicicola*. These two independent data consistently suggested that the reduced expression of 26 hydrolytic enzymes (putative CWDE and proteases) were the major reason for the approximately 90% reduction in mutant virulence.

Plant infection by fungi is characterized by the catabolism of internal lipid stores in the early stage and by the use of readily available external sugars and amino acids during the middle stage of infection (Solomon et al. 2003). Relatively little is known about the major nutrient sources of fungal metabolism during the late stage of infection. The regulation of CWDE expression is orchestrated by SNF1 homologs in two distantly related fungi (Ospina-Giraldo et al. 2003; Tonukari et al. 2000). A kinase *ccSNF1* in *C. carbonum* regulates a suite of CWDE genes during pathogenesis. Its mutants were less able to digest the main components of the host plant's cell walls and had a severe reduction in virulence. However, the primary role of genes regulated by *ccSNF1* was in the penetration of host leaves, not in utilization of plant materials as nutrients. Unlike $\Delta ccSNF1$ mutants, the $\Delta abvf19$ mutant was similar to wild-type *A. brassicicola* in its appressorium-like structure formation and leaf penetration. Although we did observe somewhat shorter germination tubes in the $\Delta abvf19$ mutants, the main difference in our study was in the much smaller lesions caused by mutants compared with the wild type.

Specialized hydrolytic enzyme genes for pathogenesis.

Hydrolytic enzyme-coding genes with reduced expression in the mutant during late infection represented only a few genes within each gene family. For example, only 1 of 9 cutinase genes, 15 of 171 glycoside hydrolase genes, 6 of 235 peptidases, and 5 of 18 pectate lyase genes were downregulated in the mutant during late-stage infection (Table 2). The vegetative growth rate of the mutant was similar to the wild type in an axenic broth medium containing either glucose as a major carbon source or yeast extract as a major nitrogen source (Fig. 3).

Fig. 7. Decreased virulence of $\Delta abvf19$ mutants on *Arabidopsis thaliana*. **A**, Lesions caused by approximately 2,000 conidia of the $\Delta abvf19-1$ mutant and the wild-type *Alternaria brassicicola* on whole plants of wild-type *Arabidopsis thaliana* (Col-0). **B**, Lesions caused by the wild type and $\Delta abvf19-1$ mutant on whole plants of the *pad3* mutant of *A. thaliana* (Col-0 background). The leaf was detached and photographed at the end of the assay. The wild type and $\Delta abvf19-1$ mutant were inoculated on the left and right side of the leaf midvein, respectively.

Table 2. Downregulated genes in the $\Delta abvf19$ mutant of *Alternaria brassicicola*

Gene function	Downregulated in mutant	Total numbers in the genome
Glycoside hydrolase	14	171
Pectate lyase	5	18
Pectinesterase	1	4
Cutinases	1	9
Peptidases	6	235
P450	4	79
Ribonucleases	2	19
Unknown ^a	26	6,008
Miscellaneous ^b	14	4317
Total	74	10,680

^a Genes in this category were annotated with hypothetical protein-coding genes that do not have similar sequences with known functions.

^b Genes in this category were annotated with functions that we did not see meaningful connections among their functions.

This result suggested that *AbVf19* and its downstream hydrolytic enzyme genes were not associated with utilization of these nutrients derived from non-plants. The slower growth of the mutant than the wild type in the presence of pectin as a major carbon source suggested that these genes encode proteins important in depolymerizing plant substrates for use as nutrients. Similarly, some hydrolytic enzymes, such as extracellular proteases, cellulases, and xylanases, are dominantly expressed during late-stage infection in other phytopathogenic fungi (Dean et al. 2005; Hane et al. 2007).

Importance of a coordinated expression of individual genes.

Downregulated genes in the $\Delta abvf19$ mutant included *Cbh7* and a chymotrypsin-coding gene that showed 21- and 2.6 times fewer transcripts, respectively, than wild-type *A. brassicicola*. These genes were previously known to be induced during pathogenesis (Cho et al. 2007; Cramer et al. 2006; Lev and Horwitz 2003). They were considered important for pathogenesis based on their highly elevated expression level. However, mutation of the chymotrypsin-coding gene (*Cbh7*) did not lead to a significant reduction in virulence (Y. Cho, unpublished data). Similarly, the cutinase gene (*CutAb1*) had 73 times fewer transcript in the $\Delta abvf19$ mutant in this study but *cutab1* mutants in a previous study did not affect virulence (Yao and Köller 1995). These mutation studies suggested that each enzyme-coding gene either contributed little (<5%) to virulence or their functions were redundant, as proposed previously based on *ccSNF1* and *foSNF1* studies (Ospina-Giraldo et al. 2003; Tonukari et al. 2000). Regardless of the functional relationship among the products of each gene, the current $\Delta abvf19$ mutant study supports the importance of coordinated regulation of those genes during pathogenesis.

Mechanistic roles of AbVf19.

We expected AbVf19-GFP fusion proteins to be localized only during the late stage of infection or in the presence of pectin in an axenic medium. However, the proteins were consistently localized in wild-type *A. brassicicola* during all stages of plant infection and during saprophytic growth in axenic media containing either glucose or pectin. Deletion of this gene affected only pathogenicity, not saprophytic growth in axenic media containing nutrients derived from non-host plants. These two contrasting results suggested that the regulatory functions of AbVf19 depended on either its modification or the presence of cofactors, and that localization of the protein in the nuclei was insufficient for the regulation of virulence-associated genes.

The transcription factor binding motif (Fig. 6) was compatible with cutinase expression data. Only one of nine genes in the family had a putative *cis*-element shared among downregulated genes and its expression level was 73 times lower in the mutant than in the wild type. The other eight cutinase genes did not have the binding motif and were not differentially expressed. However, the same binding site was not compatible with pectate lyase expression. We have not found a correlation between the presence or absence of this motif and the differential expression of pectate lyase. The AbVf19 proteins might have required additional transcription factors (cofactors) and each cofactor acted as a determinant for the regulation of downstream genes. Many hydrolytic enzymes are sequentially induced in *A. brassicicola* during pathogenesis (Fan and Köller 1998; Thomma 2003). The AbVf19 proteins may be involved in sequential regulation of a range of genes with the aid of various cofactors. Regardless of the regulation mechanisms of AbVf19, its amino acid sequence is highly conserved among *Alternaria* spp. but highly divergent from animal transcription

factors. It is a potential target for the control of plant-pathogenic species of *Alternaria*, with little or no impact on animals.

MATERIALS AND METHODS

Transformation, maintenance of fungal strains, and primers.

Growth and maintenance of the fungus and its transformation, nucleic acid isolation, mutant purification, and mutant verification by Southern hybridization were performed as described previously (Cho et al. 2009). Three probes for Southern hybridization were produced. Wild-type *A. brassicicola* and each of the mutant strains created during this study were purified by two rounds of single-spore isolation to obtain a more uniform genetic background. The cultures were maintained as glycerol stock in separate tubes, with one tube used for each assay. All primers used in this study are listed in Supplementary Table 3.

Determination of the full-length sequence.

Using the partial sequence of the *AbVf19* gene in the draft annotated genome sequence, we designed one primer, AbVf19Fseq, in the coding region of the gene at the end of the contig. Another primer, AbVf19Rseq, was designed at the adjacent contig. These two primers were used to amplify the gap between the two contigs. The PCR products were used as template DNA and the sequence was determined with the two PCR primers. The sequence data have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database (JN835469).

Generation of deletion mutants for AbVf19.

All transformation constructs described in this work were produced by a double-joint PCR method (Yu et al. 2004), with modifications described previously (Cho et al. 2009). We made *AbVf19* deletion mutants by replacing the gene with a HygB cassette. The replacement construct was produced with the following three sets of primers. The primer set P1 and P2 and the primer set P5 and P6 were used to amplify the 5' and 3' flanking regions, respectively, of the targeted locus. Another set of primers, P3 and P4, was used to amplify the HygB-selectable marker gene cassette (1,436 bp) from pCB1636. The final transformation constructs were produced by PCR amplification using the mix of products from three reactions as template DNA and two primers, P1 and P6 (Supplementary Fig. S3).

Generation of mutants expressing AbVf19-GFP fusion proteins.

In order to create mutants expressing GFP fused to the C terminal of AbVf19, the *AbVf19* coding region (1,209 bp) and 3' flanking region (192 bp) were amplified with P1' and P2', and P5 and P6', respectively. Another set of primers, P3' and P4, was used to amplify the 2,384 bp that covered the coding regions of the GFP and the HygB cassette. The final transformation constructs were produced by PCR amplification from the mixture of the PCR products using P1' and P6'.

Complementation of the $\Delta abvf19$ mutants.

The $\Delta abvf19$ mutant was complemented with the wild-type *AbVf19* allele with its native promoter. We used two primers to reintroduce wild-type *AbVf19* into the $\Delta abvf19$ -8 mutant, 1zAbVf19-5F and 6z9219-3R. These primers amplified the 4,200-bp wild-type allele of the *AbVf19* gene using *A. brassicicola* genomic DNA as a template. The PCR product included a 1,223-bp 5' flanking region, 1,747-bp complete coding region, and a 1,230-bp 3' flanking region. Separately, pNR-20F and pNR-775R were used to amplify a 2,226-bp-long nourseo-

thricin-resistant cassette as a selectable marker gene, using a pNR vector as the template (Malonek et al. 2004). These two products (7 µg of *AbVf19* and 3 µg of *NTC* cassette) were mixed to transform the *Δabvf19* mutant. Complemented mutant clones were purified by two rounds of single-spore isolation.

Pathogenicity assays.

Pathogenicity assays were performed on either whole plants or detached leaves harvested from 5- to 8-week-old *B. oleracea* (green cabbage) or *Arabidopsis thaliana*. *A. thaliana* plants included the Columbia ecotype and its *pad3* mutant (Zhou et al. 1999). For most detached-leaf assays, leaves were removed, placed in mini-moist chambers, and randomly located on a laboratory bench. For the pathogenicity assays on whole plants, potted plants were placed in a semitransparent plastic trough with adequate water. The troughs with plants inside were sealed with saran wrap after inoculation to keep the relative humidity close to 100%. The decreased virulence of each mutant was calculated using the formula $[\Sigma(Dwi - Dmi)/\Sigma(Dwi)] \times 100$, where *Dwi* was the lesion diameter created by the wild-type for the *i*th sample and *Dmi* was the lesion diameter produced by the mutant for the *i*th sample.

Confocal microscopy.

Infected plant tissues were trimmed with a razor blade, placed on microscope slides, and covered with Gold Seal cover glasses. Confocal images were acquired using a 633 C-Apochromat (numerical aperture 1.2) water-immersion objective lens and an Olympus Fluoview 1000 Laser Scanning Confocal System on an IX-81 inverted microscope. Spectra for fungal tissues expressing standard green fluorescence and for plant cells emitting autofluorescence were collected by simultaneous 488- and 543-nm excitation using 30-mW argon and 1-mW helium/neon lasers, respectively. The standard GFP spectrum was collected through 488-nm excitation using a 20-nm window from 505 to 525 nm. Plant tissue, including chloroplasts, was visualized using a 543-nm excitation with a 560-nm-long pass filter. Images of fungal tissue grown in nutrient media were captured with 488-nm excitation and differential interference contrast (DIC)-transmitted light. All fluorescent images were composed of multiple layers acquired with the Confocal System.

Synthetic media and vegetative growth assays.

We made a minimal medium broth containing 0.5% (NH₄)₂SO₄, 0.05% yeast extract, salts (0.15% KH₂PO₄, 0.06% MgSO₄, and 0.06% CaCl₂), and trace amounts of metals (0.0005% FeSO₄ 7H₂O, 0.00016% MnSO₄ H₂O, 0.00014% ZnSO₄ 7H₂O, and 0.00037% CoCl₂). Agar (2% wt/vol) was added to the broth to make MMA. Flasks (250 ml) containing 50 ml of minimal medium broth were supplemented with either 1% glucose or 1% of another complex carbon source, such as α-cellulose, xylan, or citrus pectin (Sigma-Aldrich, St. Louis). Each flask was inoculated with 5 × 10⁵ conidia of either the *Δabvf19* mutant or the wild type. The flasks were incubated in the dark at 25°C with continuous agitation at 100 rpm. Mycelia were harvested at 4 days postinoculation, washed with distilled water, and dried at 105°C overnight. The decrease in biomass of each mutant compared with the wild type was calculated using the formula $[\Sigma(Wwi - Wmi)/\Sigma(Wwi)] \times 100$, where *Wwi* was the dry weight of the wild type for the *i*th sample and *Wmi* was the weight of the mutant for the *i*th sample.

RNA-seq data generation and gene expression analysis.

Nine detached leaves harvested from three plants were inoculated with 10 µl of water containing either 1,500 conidia

from the *Δabvf19* mutant or 1,500 conidia from the wild type. Tissue samples containing both host plant tissue and fungal hyphae were harvested and immediately frozen in liquid nitrogen to fix gene expression profiles. The frozen tissues were ground and total RNA was extracted using an RNeasy kit. Residual DNA was digested in columns with RNase-free DNase following the manufacturer's protocol (Qiagen, Palo Alto, CA, U.S.A.). Three biological replicates were prepared for the mutant and three for the wild type and their gene expression profiles compared.

We constructed strand-specific sequencing libraries using the TruSeq small RNA Sample Prep Kit (Illumina, San Diego, CA, U.S.A.) from total RNA following the manufacturer's protocol and size selecting libraries in 4% agarose gels to obtain fragment sizes between 100 and 150 bases in length. Each sample RNA was used to construct a library with a unique index primer. In total, six index primers were used to construct six libraries. All six libraries were mixed and determined 40 nucleotide-long sequence tags using Illumina HiSeq2000 (Illumina) according to the manufacturer's protocols. Image analysis, base-calling, and quality checks were performed with the Illumina data analysis pipeline CASAVA v1.8.0. The sequence data have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar et al. 2002) and are accessible through GEO Series accession number GSE33148.

The sequenced reads were mapped to the genome sequence of *A. brassicicola* and the UniGene sequences of *B. oleracea* using the programs Tophat 1.3.1 (Trapnell et al. 2009) and Bowtie 0.12.7 (Langmead et al. 2009). Default settings were used, with these exceptions: the segment length was set at 20 nucleotides and the number of allowed segment mismatches was set at 1 nucleotide. Additionally, intron length was designated as a minimum of 10 nucleotides and a maximum of 400 nucleotides. The program Cuffdiff (version 1.0.3, part of Cufflinks) (Trapnell et al. 2010) was used to identify reads overlapping with previously predicted genes. The expression levels of each predicted gene were determined and normalized by the mapped FPKM. Differentially expressed genes between the wild type and the mutant were determined by comparing FPKM from three biological replicates for both the wild type and the mutant. We also applied a cutoff of at least a twofold change in expression value for differential expression. The bias correction method was used while running Cuffdiff (Roberts et al. 2011). Custom scripts were written in Python to analyze the data.

Representation analysis.

Custom scripts were developed in Python and R to analyze over- and underrepresentation of functional annotation terms in sets of differentially regulated genes using the Fisher Exact test. The Benjamini-Hochberg correction was used to correct for multiple testing using a *P* value of 0.05.

Identification of transcription factor binding sites.

MEME Suite 4.5.0 (Bailey and Elkan 1994) was used to identify conserved motifs in the promoters of genes. Promoters are defined here as the nucleotide sequence 750 bp upstream of the translation start site of predicted genes. Incomplete promoters (with less than 750 bp of available sequence or containing gaps) were excluded from the analysis. Parameters were chosen to identify conserved motifs of up to 10 nucleotides, with zero or one expected occurrence in each promoter on both strands. The nucleotide frequencies of all promoters were used as background frequencies. The identified motifs were manually inspected. Next, occurrences of the relevant motifs in the full promoter set were counted using FIMO, which is part of the MEME Suite. Significance of overrepresentation of the

identified motifs in the promoter subset was determined with the Fisher Exact test. To identify the similarity of the identified motifs with previously published motifs, TOMTOM (part of the MEME Suite) (Gupta et al. 2007) was used to search the JASPAR CORE database of transcription factor-binding profiles (Bryne et al. 2008).

qRT-PCR.

The *Δabvf19* mutant and wild type were used for qRT-PCR. Total RNA (2 μg) was transcribed to cDNA in a final volume of 20 μl using 50 ng of random pentamers and 200 ng of poly(T)₂₀N with Superscript III (Invitrogen, Carlsbad, CA, U.S.A.). Each cDNA was diluted 1:20. Subsequent qRT-PCR reactions were performed in a 20-μl volume containing 120 nM each primer, 1 μl of diluted cDNA, and 10 μl of FastStart SYBRGreen Master (Roche, Mannheim, Germany). Each reaction was run in triplicate in a Bio-Rad i-cycler (Bio-Rad, Hercules, CA, U.S.A.) as described previously (Cho et al. 2006). Relative amounts of the transcript of each gene were calculated as $2^{-\Delta C_t}$ using a threshold cycle (C_t), where $\Delta C_t = (C_{t_{\text{genei}}} - C_{t_{\text{actin}}})$. Fold changes of each gene between the wild type and *Δamr1* strain were calculated as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{\text{genei}}} - C_{t_{\text{actin}}})_{\Delta abvf19} - (C_{t_{\text{genei}}} - C_{t_{\text{actin}}})_{\text{wild type}}$.

ACKNOWLEDGMENTS

We thank T. M. Carvalho, W. Borth, A. Alvarez, and J. Hu for generously sharing their research equipment. This research was supported by United States Department of Agriculture TSTAR 2009-34135-20197 and HATCH funds to Y. Cho, administered by the College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu. Analysis of RNA-seq data and downstream analyses were done by the U.S. Department of Energy Joint Genome Institute that is supported by the Office of Science of the U.S. Department of Energy under contract number DE-AC02-05CH11231.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Department of Energy *Alternaria brassicicola* genome portal page:
jgi.doe.gov/Abbrassicicola
 NCBI GEO database: www.ncbi.nlm.nih.gov/geo/info/linking.html
 UniGene sequence repository for *Brassica oleracea*:
ftp.ncbi.nih.gov/repository/UniGene/Brassica_oleracea