

# **Fungal-Specific Transcription Factor AbPf2 Activates Pathogenicity in *Alternaria brassicola***

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August 2013

The work conducted by the U.S. Department of Energy Joint Genome Institute is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231

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Fungal-Specific Transcription Factor *AbPf2* Activates Pathogenicity in *Alternaria brassicicola*

Running head: Rapid conidial response to a host

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*AbPf2* GenBank accession number: JQ899199

RNA-seq data: GEO Series Accession No. GSE38984

## Abstract

*Alternaria brassicicola* is a successful saprophyte and necrotrophic plant pathogen. Molecular determinants of its life style shift between saprophyte and pathogen, however, are unknown. To identify these determinants we studied nonpathogenic mutants of a transcription factor-coding gene, *AbPf2*. Frequency and timing of germination and appressorium formation on host plants were similar between the nonpathogenic  $\Delta abpf2$  mutants and wild-type *A. brassicicola*. The mutants were also similar *in vitro* to wild-type *A. brassicicola* in vegetative growth, conidium production, and responses to chemical stressors, such as a phytoalexin, reactive oxygen species, and osmolites. The mutants, however, did not penetrate host plant tissues, though their hyphae continued to grow on the plant surface. Transcripts of the *AbPf2* gene increased exponentially soon after wild-type conidia encountered their host plants. A small amount of AbPf2 protein, monitored by fused green fluorescent protein, was located in both the cytoplasm and nuclei of young, mature conidia. The protein level decreased during saprophytic growth but increased several-fold during pathogenesis. Levels of both the proteins and transcripts sharply declined following colonization of host tissues beyond the initial infection site. When the transcription factor was expressed at an induced level in the wild type during early pathogenesis, the expression of 106 fungal genes was down-regulated in the  $\Delta abpf2$  mutants. Notably, 33 of the 106 genes encoded secreted proteins, including eight putative effector proteins. Plants inoculated with  $\Delta abpf2$  mutants expressed higher levels of genes associated with photosynthesis, the pentose phosphate pathway, and primary metabolism, but lower levels of defense-related genes. Our results suggest that conidia of *A. brassicicola* are programmed as saprophytes, but become parasites upon contact with their hosts. AbPf2 coordinates this transformation by expressing pathogenesis-associated genes, including those coding for effectors.

## Author summary

There are more than a thousand secretion proteins and hundreds of putative effector proteins in the genomes of most plant-pathogenic fungi and oomycetes. Several effector proteins, previously known as *Avr* genes, are putative pathogenesis factors that modulate the gene expression profiles of susceptible host plants. Some effector proteins are recognized by resistant host plants and trigger strong immune responses. Many effector proteins are secreted during pathogenesis, suggesting that their expression and secretion are coordinated. In this study we

identified a transcription factor that orchestrates the expression of 8 putative effectors and 25 other secretion proteins. These proteins are likely important during the early-stages of pathogenesis. It is of note that only 8 of 137 putative effector genes appeared to be regulated by the transcription factor that is essential for pathogenicity. Gene expression profiles suggested that wild-type *A. brassicicola*, but not mutants of the transcription factor, modulates the expression of plant genes associated with photosynthesis and primary metabolisms. We speculate and have been testing a hypothesis that the putative effector proteins are responsible for changes in plant gene expression profiles.

## Introduction

Some biotrophic plant-pathogenic fungi are obligate parasites of living plant tissue. They overcome host plant immunity by suppressing plant defenses (Buttner and Bonas, 2003; Glazebrook, 2005; Sexton and Howlett, 2006). In contrast, necrotrophic pathogens use nutrients derived from dead host plant tissue, or kill host tissue before colonizing it. They are facultative parasites, able to complete their life cycles either as saprophytes on dead organic matter, or as pathogens in killed host plant tissue. It is unclear which gene or genes determine whether a facultative fungus will be saprophytic or parasitic.

Both biotrophic and necrotrophic fungi respond to environmental conditions, such as nutrient deprivation, osmotic stress, defense metabolites and reactive oxygen species (ROS) in host plant tissue. The reactions of pathogenic fungi on host plants have been of special interest to plant pathologists as the reactions are directly associated with the mechanisms of pathogenesis (Shlezinger et al., 2011; Williams et al., 2011). Several molecular sensing mechanisms in fungi lead to complex changes in their cell physiology and in infection structures that promote fungal pathogenesis (Hoch et al., 1987; Kulkarni et al., 2005; Kloda et al., 2008). The speed of the fungal response to its host plants at the gene level and the regulation of this response, are two fascinating but challenging areas of study.

*Alternaria brassicicola* is a necrotrophic fungus that causes black spot disease of cultivated brassicas, such as cabbage, canola, and mustard. It is also pathogenic on *Arabidopsis thaliana* and the *A. brassicicola* – *Arabidopsis thaliana* system is occasionally used to study host-

1 pathogen interactions (Thomma et al., 1999; Oh et al., 2005). A conserved mitogen-activated  
2 protein (MAP) kinase, *Amk1*, and its downstream transcription factor, *AbSte12*, are essential for  
3 pathogenesis (Cho et al., 2007; Cho et al., 2009). Mutation of either *Amk1*, *AbSte12*, or their  
4 homologs in other fungi, however, caused defects in other cellular processes in addition to the  
5 loss of pathogenicity (Caracuel et al., 2003; Cho et al., 2007; You and Chung, 2007; Zhao et al.,  
6 2007; Cho et al., 2009; Wong Sak Hoi and Dumas, 2010). Most transcription factors associated  
7 with pathogenesis in other fungi are also linked to additional cellular processes (Kim et al., 2009;  
8 Guo et al., 2011; Son et al., 2011; Wang et al., 2011). Because of the multiple functions of  
9 transcription factors, it is difficult to identify the pathogenicity genes regulated by them.  
10 Recently, we discovered that *A. brassicicola* induces a group of hydrolytic enzymes during the  
11 late stage of colonization that are important for the digestion of plant tissue (Srivastava et al.,  
12 2012). Aside from this limited information, the regulation mechanism of pathogenesis in *A.*  
13 *brassicicola* is elusive, especially during the early stages.

14  
15 In this study, we identified a transcription factor gene that was essential for initiating  
16 pathogenesis in *A. brassicicola*. Targeted mutants of the gene were nonpathogenic and grew on  
17 the surface of host tissue without causing disease symptoms. We tested three hypotheses:  
18 whether the transcription factor was important for fungal development, for defense from the  
19 harsh environment of the infection court, or for aggressiveness towards the host plant. This  
20 study revealed that conidia of the necrotrophic fungus *A. brassicicola* were programmed to  
21 germinate as saprophytes, but with a potential to rapidly switch to parasitism on contact with  
22 their host plants.

## 23 24 **Results**

### 25 **Initial screening of mutants for the *AbPf2* gene**

26 We systematically created and screened gene knockout mutants of fungal-specific transcription  
27 factors, using gene disruption or gene deletion methods previously described (Cho et al., 2006;  
28 Cho et al., 2009). During pathogenicity assays, we found a gene whose mutants had a phenotype  
29 comparable to wild-type *A. brassicicola*. These mutants, however, were nonpathogenic. The  
30 mutated gene (AB06533.1) was predicted to encode 630 amino acids. It included a GAL4  
31 (Zn<sub>2</sub>Cys<sub>6</sub>) fungal-specific DNA binding domain (IPR001138, PF00172) predicted by Interpro

(Bateman et al., 2004). Homologs of the gene were annotated as either a hypothetical protein or a C6 zinc finger domain protein in other fungal genomes. We named this gene, *AbPf2* (*Alternaria brassicicola* pathogenicity factor 2). We also found a paralogous gene with 320 amino acids (960 nucleotides). The nucleotide sequences were very similar, with 81% identity, between the two homologous genes in *A. brassicicola* (Table S1).

### **Replacement of the *AbPf2* gene with a *HygB* resistance cassette**

To confirm the involvement of *AbPf2* in pathogenesis, we created additional mutants of the gene. We replaced the gene with a Hygromycin B (*HygB*) resistance cassette. Southern hybridization verified that the *AbPf2* coding region was replaced by two copies of the *HygB* resistance cassette in one mutant ( $\Delta abpf2$ -5), and by one copy in six of the seven gene-deletion mutants (Figure S1, left blots). We also produced a second set of mutants whose *AbPf2* gene was replaced by a *GFP-HygB* resistance cassette. Southern hybridization verified three replacement mutants and three ectopic insertion mutants (Figure S1, right blots).

### **Loss of pathogenicity in the $\Delta abpf2$ mutants**

Pathogenicity assays were initially performed using five mutants ( $\Delta abpf2$ -1, -2, -4, -5, and -s5) on green cabbage (*Brassica oleracea*). All five mutants were nonpathogenic on the leaves of 6-8-week-old plants. We performed further experiments with  $\Delta abpf2$ -2 and  $\Delta abpf2$ -s5, representing two groups of mutants. We also tested the mutants' pathogenicity on wild-type *Arabidopsis thaliana*, ecotype Col-0, and the *Arabidopsis thaliana* mutant, *pad3*. The *pad3* mutant lacks the phytoalexin, camalexin (Zhou et al., 1999). Inoculations with  $1-2 \times 10^3$  conidia of wild-type *A. brassicicola* produced large lesions on the leaves of green cabbage (Figure 1A) and leaves of the *pad3* mutants were extensively colonized by 5 days postinoculation (dpi) (Figure 1B). The wild type killed whole plants of the Col-0 ecotype when they were drenched with a conidial suspension of  $5 \times 10^5$ /ml (Figure 1C). In contrast, none of the  $\Delta abpf2$  mutants caused disease symptoms on healthy young leaves of green cabbage, the *pad3* mutant, or wild-type *Arabidopsis* (Figure 1). The  $\Delta abpf2$  mutants occasionally caused mild disease symptoms on senescing leaves. In these rare cases, the symptoms were not apparent until 4-6 dpi, compared to 24 hours postinoculation (hpi) with wild-type inoculum. In addition, the small spots caused by the mutants did not expand beyond the initial infection site (Figure 1B). The mutants

1 did colonize host leaves wounded before inoculation, but lesion expansion was so slow that they  
2 rarely grew beyond the wounds during the 6-day assay period (Figure 1A, second and third  
3 images).

#### 4 5 **Restoration of pathogenicity by complementation**

6 Two strains of the nonpathogenic mutants,  $\Delta abpf2-2$  and  $\Delta abpf2-s5$ , were independently  
7 complemented with the wild-type allele of the *AbPf2*-coding gene. The  $\Delta abpf2-s5$  mutant was  
8 complemented with either single or multiple copies of the wild-type allele (Figure S1).

9 Pathogenicity of the complemented mutants,  $\Delta abpf2-s5:AbPf2$ , was restored on green cabbage  
10 and *Arabidopsis thaliana* (Figure 1). The  $\Delta abpf2-2$  mutant was complemented with multiple  
11 copies of the wild-type allele (data not shown). A complemented mutant,  $\Delta abpf2-2:AbPf2$  also  
12 restored pathogenicity on green cabbage and *Arabidopsis thaliana* (Figure 1). We quantified the  
13 virulence of the complemented mutants by comparing the diameters of lesions caused by the  
14 complemented mutants and wild-type *A. brassicicola* on green cabbage leaves. The lesion size  
15 was comparable (Figure 1D, Table 1). These results indicated that *AbPf2* was essential for  
16 pathogenicity and full virulence.

#### 17 18 **Germination and appressorium formation**

19 We tested whether the loss of pathogenicity in  $\Delta abpf2$  was associated with developmental  
20 defects. Spore germination by the mutants and wild type was ~100% at 3 hpi. There were no  
21 differences in either germination, vegetative growth (Figure 2, PDA), or spore production on  
22 PDA (Table 2). Germination rates for both the wild type and the  $\Delta abpf2$  mutants were also  
23 similar on host plant leaves. Germination was ~100% by 8 hpi on 6-8-week-old green cabbage  
24 (data not shown), and 12 hpi on and a 5-6-week-old *Arabidopsis* (Figure 3 A, E). The lengths of  
25 their germ tubes and hyphae were so variable that the differences in average lengths were  
26 statistically insignificant at 12, 18, and 20 hpi (Table 3). A small, swollen structure  
27 (appressorium hereafter) was occasionally formed at the tips of germ tubes at 8-12 hpi on green  
28 cabbage and 12-24 hpi on *Arabidopsis*. By 20 hpi, appressoria were formed on about 45% of the  
29 germ tubes produced by the mutants and the wild-type fungus on the two plants (Figure S2,  
30 Table 4). In summary, the mutants germinated and formed appressoria similar to the wild type  
31 on the surface of both host plants.

## **Mutant growth on plant surfaces**

We compared the growth habits of the  $\Delta abpf2$  mutants and wild-type *A. brassicicola* on green cabbage and *Arabidopsis*. Wild-type *A. brassicicola* created sunken infection sites on *Arabidopsis* leaves by ~48 hpi. At this time, plant tissue and fungal hyphae growing within the tissue were stained with trypan blue (Figure 3 D). In contrast, the  $\Delta abpf2$  mutants grew only on the surface of *Arabidopsis thaliana* and plant tissue was not stained by the trypan blue (Figure 3 H). It is of note that the mutants did not penetrate, but continued to grow on the surface of *Arabidopsis* (Figure 3 E-H) and green cabbage (Figure S3).

## **No changes in colony or conidial response to stressors**

Plants produce ROS and phytoalexins in response to pathogen infection (VanEtten et al., 1995; Thomma et al., 1999; Torres et al., 2006; Ahuja et al., 2012). In addition, the infection court creates various osmolites. To indirectly evaluate the importance of the *AbPf2* gene in managing stressors, we evaluated the effects of the oxygen stressors  $H_2O_2$  and  $KO_2$ ; the phytoalexin brassinin, produced by green cabbage; and the osmolites; NaCl and sorbitol, on the vegetative growth of  $\Delta abpf2$  mutants. Colony size and characteristics of the  $\Delta abpf2-2$  and  $\Delta abpf2-s5$  mutants were comparable to the wild type and their complemented mutants on PDA containing any of the tested chemicals (Figure 2).

## ***AbPf2* expression during plant infection**

The mutants' loss of pathogenicity was not accompanied by developmental defects or an inability to detoxify plant chemicals. Therefore, we investigated the expression pattern of *AbPf2* transcripts in wild-type *A. brassicicola* during pathogenesis. The level of *AbPf2* transcripts was lowest during saprophytic growth in necrotic tissue (late-stage colonization) and slightly higher in conidia harvested from PDA (Figure 4). Compared to conidia before inoculation, transcription levels increased over 30-fold ( $p < 0.001$ ) in pre-germination conidia at 4 hpi on both green cabbage and *Arabidopsis thaliana*. These increased levels were maintained only until penetration was completed and the initial infection site was colonized. Gene expression levels fell sharply by the time lesions had expanded beyond the initial infection site. This occurred at 24 hpi on green cabbage and 48 hpi on *Arabidopsis*. Gene expression remained low throughout

1 lesion expansion and saprophytic growth in necrotic tissue. Expression of the gene increased  
2 again during conidiation at about 9 dpi on green cabbage leaves. The increase in gene  
3 expression was not obvious on *Arabidopsis*, where fewer conidia were produced. In general, the  
4 expression pattern of *AbPf2* was similar during infection on both green cabbage and *Arabidopsis*  
5 when it was normalized with the two housekeeping genes, *GAPDH* and *Ef1- $\alpha$*  (Figure 4).

### 7 **Localization of AbPf2 protein during saprophytic growth**

8 We monitored the expression and localization of AbPf2 protein using an AbPf2-GFP fusion  
9 protein. The mutant expressing the fusion protein was similar to wild-type *A. brassicicola* in  
10 germination and vegetative growth on PDA. To reveal the nuclei in fungal cells, we used a  
11 fusion protein of mCherry and a nuclear localization signal (mCherry-NLS) (Khang et al., 2010).  
12 In hyphal tips growing on PDA, mCherry-NLS was moderately expressed, but the AbPf2-GFP  
13 protein was hard to detect (Figure 5A, Table 5). Unlike the growing hyphal tips, little AbPf2  
14 protein accumulated in young mature conidia still attached to conidiophores above the medium  
15 on 3-day old plates. The fusion protein concentration was ~2-fold higher in nuclei than in the  
16 cytoplasm of conidia at this stage (Table 5). When the conidia were inoculated on PDA, they  
17 germinated and began to grow in about three hours. During this time, the GFP signal moved into  
18 the cytoplasm of the germ tube and became weaker as the germ tube grew (Figure 5 C D), until  
19 the signal was barely detectable (Table 5).

### 21 **Expression and localization of AbPf2 protein during pathogenesis**

22 We also monitored AbPf2 protein expression on *Arabidopsis thaliana*. On both *Arabidopsis* and  
23 aged green cabbage plants, germination of the mutant expressing AbPf2-GFP fusion protein was  
24 delayed as much as 9 hours compared to the wild type. The mutant expressing AbPf2-GFP was  
25 still pathogenic, but was about 50% less virulent than the wild type (Table 6). We investigated  
26 the protein accumulation pattern based on the fungal development stages and disease progress.  
27 When conidia of the AbPf2-GFP-expressing mutant were inoculated on *Arabidopsis thaliana*,  
28 the AbPf2 protein increased ~2-fold ( $p < 2.3E-04$ ) compared to the level in the inoculum before  
29 germination at similar stage to 4hpi in Figure 4B (Figure 6 A, B, Table 7). The AbPf2-GFP  
30 protein increased both in the cytoplasm and nuclei. After germination at similar stage to 8-12  
31 hpi in Figure 4B, the protein increased even more in the germ tubes and in the appressoria

(Figure 6 C, D). The protein concentration was ~3-fold higher in the nuclei than in the cytoplasm at the appressorium-forming stage (Table 7). When the initial infection site was sunken but fungal hyphae did not grow beyond the inoculation site at similar stage to 24 hpi in Figure 4B, the AbPf2-GFP protein was reduced over 2-fold and stayed mainly in nuclei of the hyphae (Figure 6E, Table 7). By the time hyphae colonized dead plant tissue beyond the initial infection site at similar stage to 48 hpi in Figure 4B, the GFP signal had decreased further and was undetectable in the nuclei of the hyphae (Figure 6F and Table 7). The expression pattern of AbPf2-GFP on green cabbage was similar to that on *Arabidopsis thaliana* (Figure 7). As a negative control in this experiment, we monitored GFP alone, which was constitutively expressed under the control of the *ToxA* promoter (Ciuffetti et al., 1997; Lorang et al., 2001). The GFP was evenly distributed in the cytoplasm (Figure 7, top panel) and did not co-localize with DAPI in the nuclei (Figure 7I).

#### **Gene expression changes in $\Delta abpf2$ mutants**

To identify pathogenicity-associated genes regulated by the transcription factor, we compared gene expression profiles between the  $\Delta abpf2$  mutant and wild-type *A. brassicicola* at two time points, 12 hpi and 48 hpi. From tissue samples representing 12 hpi, when *AbPf2* transcripts reached their highest level (Figure 4), a total of 154.5 and 177.8 million reads were produced for the wild type and  $\Delta abpf2$  mutant, respectively. Of these,  $8.5 \times 10^5$  (0.55%) and  $9.3 \times 10^5$  (0.53%) were mapped to the genome of *A. brassicicola* and  $158.4 \times 10^6$  (82.5%) and  $189.8 \times 10^6$  (86.0%) were mapped to the genome of *Arabidopsis thaliana*, respectively. We identified differentially expressed genes using fungal tags. Among 10,688 predicted genes in the *A. brassicicola* genome, 106 genes were significantly down-regulated and 62 genes up-regulated more than two-fold ( $p < 0.05$ ) in the  $\Delta abpf2$  mutant compared to the wild type (Figure 8, Table S2). These 106 and 62 genes respectively represented 0.99% and 0.58% of the predicted genes in the current *A. brassicicola* genome. Although sequence tags of the *AbPf2* gene were in the  $\Delta abpf2$  mutants, all of them were mapped at the 3' side of the coding region that was part of the deletion construct (Figure S1). There were no full-length transcripts. Among the 106 down-regulated genes, 33 genes encoded proteins with secretion signal peptides (Figure 8 marked as S). They included genes encoding two pectate lyases, a necrosis-inducing factor, and eight small proteins with 88 to 147 amino acids.

1  
2 The pectate lyase genes in the wild type were highly expressed (0.67% of total expression)  
3 among the differentially expressed genes. Five of the eight small proteins contained over six  
4 cysteine residues, which are often needed for the formation of disulfide bonds in effector  
5 proteins (Luderer et al., 2002; Doehlemann et al., 2009). These proteins were also expressed at  
6 high levels, representing up to 0.5% of the sequence tags in the wild type but very few in the  
7 mutant (Table S2). Their small size, the presence of secretion signal peptides, and frequent  
8 cysteine residues, met the conditions for fungal effector proteins. The 62 up-regulated genes in  
9 the mutant included seven transporters and several putative detoxifying enzymes, such as  
10 cyanide hydrolase, indolamine dioxygenase, glutamine aldoltransferease, and lactamase. They  
11 also included four glycoside hydrolases, three bacterial rhodopsins, and a short protein with eight  
12 cysteine residues and secretion signal peptides. The expression level of the short protein gene  
13 was 0.5% in the wild type and 2.7% in the  $\Delta abpf2$  mutants. Of note, was that genes encoding  
14 enzymes, such as superoxide dismutase, peroxidases, catalase, laccases, and polyphenol oxidases  
15 were not differentially expressed at 12 hpi.

16  
17 At 48 hpi, the level of *AbPf2* transcripts had returned to almost the same level as before the  
18 induction. At this time, a total of  $119.6 \times 10^6$  and  $100.2 \times 10^6$  reads were produced for the wild  
19 type and the  $\Delta abpf2$  mutant, respectively. Of these,  $4.9 \times 10^6$  (4.1%) and  $1.1 \times 10^6$  (1.1%) were  
20 mapped to the genome of *A. brassicicola* and  $98.3 \times 10^6$  (82.5%) and  $85.9 \times 10^6$  (85.7%) were  
21 mapped to the genome of *Arabidopsis thaliana*. Among 10,688 fungal genes, a total of 252  
22 genes were significantly down-regulated over 2-times ( $p < 0.05$ ) in the mutant compared to the  
23 wild type. Of the 252 genes, 40 were also found among the 106 genes down-regulated at 12 hpi  
24 (Figure 8D). Noticeably, the expression level of these 40 genes decreased sharply in wild-type  
25 *A. brassicicola* at 48 hpi. Furthermore, 525 genes were up-regulated in the mutant compared to  
26 wild-type *A. brassicicola* (Table S3). Of these up-regulated genes, only 41 were also up-  
27 regulated in the mutant at 12 hpi (Figure 8E, Table 8). In addition, a total 232 genes were  
28 expressed more at 48 hpi than at 12 hpi in the mutant during plant infection. The differentially  
29 expressed genes were unique with little overlap at 12 hpi and 48 hpi (Figure S4 compare the  
30 color codes).

## Expression pattern of selected genes during pathogenesis

We next wanted to understand the functional importance of the genes at each stage of infection and to evaluate the reliability of the gene expression profiles. For this, we surveyed the relative amount of transcripts and the expression patterns of five genes in the wild type and  $\Delta abpf2$  mutant during the first week of the disease cycle. The selected genes encoded two pectate lyases (AB01332.1, AB04813.1), two effector proteins (AB04512.1, AB09024.1), and an amidohydrolase (AB09632.1), which were down regulated in the  $\Delta abpf2$  mutant at 12 hpi. In conidia, the expression levels of all five genes were low before inoculation, and then increased exponentially in the wild type at 4 and 8 hpi. During this time, the expression of *AbPf2* had increased, but the wild-type conidia had not yet germinated. Expression of the five genes in the wild-type conidia was over 1,000-times higher in the germinated conidia at 12 hpi than in the conidia before inoculation (Figure 9 A-E). This expression level was maintained in the invading hyphae at 24 hpi, and then fell rapidly in the colonizing hyphae at 48 hpi (Figure 3). At 48 hpi, the expression of *AbPf2* was at the same level as before induction (Figure 4). The *AbPf2* gene was induced slightly before the other five genes. Each of the five genes was also induced in the  $\Delta abpf2$  mutants during early infection, although to a lesser extent. In summary, the expression of all five genes was from 16- to >1,000-times higher in the wild type than in the mutant at 12 hpi (Figure 9F).

We also examined three previously studied genes as controls for the qRT-PCR survey (Cho et al., 2012). The expression patterns of these three genes were very different from the five genes and the *AbPf2* gene (Figure 9 G-I). The expression of one of these genes, a pectate lyase (AB05514.1), was ~2-fold higher in the wild type than in the mutant during the infection process, including the time when *AbPf2* was not induced (Figure 9G). Another of the genes, *Cbh7* (AB06252.1), was induced at a low level until 24 hpi in both the wild type and mutants, then increased dramatically during the late stage of infection (Figure 9H). Expression of the chymotrypsin gene (AB01734.1) was higher in the wild type during infection and expressed in the greatest amounts during the late stage of infection, as described previously (Cho et al., 2012). Its expression, however, fluctuated greatly in the mutants without regard to the *AbPf2* expression pattern (Figure 9I). The qRT-PCR results were similar to the RNA-seq data at 12 hpi and 48 hpi for all 8 genes although RNA-seq was slightly less sensitive than qRT-PCR in some cases.

## Conserved sequence motifs shared among promoters of down-regulated genes

We surveyed the putative promoter regions of genes that were significantly down regulated in the *Δabpf2* mutant compared to the wild type for common motifs, and for which a full-length promoter was available. Fifty genes matched these criteria. The comparison revealed a conserved motif shared by the promoters of 25 of these genes (Fig. 10). The occurrence of this motif was significantly greater than can be randomly selected from the total pool of 1,044 promoters with this motif among a total of 8,237 promoters ( $p < 1 \times 10^{-4}$ ). The motif contained a CGG subsequence, which is characteristic of a binding site for a fungal-specific transcription factor. The motif showed similarity to the previously described binding sites of several fungal-specific transcription factors in the JASPAR CORE Fungi database (e.g. MA0429.1,  $p = 6 \times 10^{-3}$ ).

## Plant responses

Differentially expressed plant genes were also identified by aligning the sequence tags to the *Arabidopsis* genome. A total of 1,277 of *Arabidopsis thaliana*'s 34,134 predicted genes were significantly ( $p < 0.05$ ) up-regulated and 2,117 genes were down-regulated more than two-fold in the plants inoculated with the *Δabpf2* mutant compared to the wild type at 12 hpi (Table S4). They represent 3.4% of the up- and 6.2% of the down-regulated genes predicted in the current *Arabidopsis thaliana* genome. The functional categories over-represented among the up-regulated plant genes encoded were chloroplast proteins (Table S5). They included genes associated with light harvesting and electron transport in photosystems I and II and with CO<sub>2</sub> fixation. Many other up-regulated genes with unknown functions also encoded proteins located in chloroplasts. The over-represented groups among up-regulated genes also included genes associated with the pentose-phosphate pathway, transcription, translation, cell differentiation, cell morphogenesis, meristem growth, cell tip growth, and a response to auxin. Down-regulated plant genes that were over-represented included many genes associated with defense against fungi. Their functions were related to a response to chitin, ethylene stimulus, wounding, jasmonic acid, hydrogen peroxide, and oxidative stress, among others (Table S5). Interestingly, 21 genes encoding leucine-rich receptor (LRR) proteins were also included in this group (Table S6).

## Discussion

### Recent gene duplication and functional independence of *AbPf2*

The *AbPf2* gene was duplicated in *A. brassicicola*. The ortholog (*AbPf2*) and paralog (*AbEf1*) shared a 57% similarity in their promoter sequences within 300 nucleotides to the start codon and were over 80% identical in their coding region (Table S1). The *AbPf2* gene was moderately expressed, but the *AbEf1* was not expressed in either the wild type or the  $\Delta abpf2$  mutants. All verified mutants of the *AbPf2* gene, such as  $\Delta abpf2$ -1, -2, -4, -5, and -s5 were nonpathogenic. These consistent results indicated that the *AbPf2* gene was essential for pathogenicity. The paralogous gene remained unchanged in the  $\Delta abpf2$  mutants (Figure S1), but the mutants were still nonpathogenic. This result indicated that *AbEf1* did not compensate for the loss of pathogenicity in the  $\Delta abpf2$  mutants. It suggested that their functions were independent of each other regardless of the history of the gene duplication and its subsequent evolution.

### Transcription factor AbPf2

Bioinformatics analysis by Interpro (Bateman et al., 2004) suggested that the AbPf2 protein was a transcription factor with a GAL4 fungal-specific DNA binding domain (IPR001138, PF00172). There was no obvious nuclear localization signal predicted by PSORT (Horton et al., 2007). AbPf2 protein, monitored by AbPf2-GFP fusion proteins, however, accumulated in nuclei of the conidia and invading hyphae during early pathogenesis (Figure 6 and 7). The data indicated that AbPf2 had entered the nuclei. A predicted DNA binding domain and the nuclear localization during early pathogenesis suggest that AbPf2 is a transcription factor.

### *AbPf2* mutations affect only pathogenesis

Several transcription factor genes are associated with pathogenesis in other fungi. Mutation of these genes, or their downstream genes, displays multiple problems in addition to defects in pathogenesis (Kim et al., 2009; Guo et al., 2011; Son et al., 2011; Wang et al., 2011). These additional problems affect vegetative growth, conidium production, or colony morphology. Other pathogenesis-associated genes in conserved signal pathways (Xu and Hamer, 1996; Lev et al., 1999; Xue et al., 2002) or primary metabolism (Seong et al., 2005; Oide et al., 2006; Lee et al., 2009) also affect multiple traits. In addition, an appressorium is required for early penetration and full virulence in several plant pathogenic fungi (Lev et al., 1999; Thines et al.,

2000). Thus, we carefully examined the mutant phenotypes to determine the possible role of *AbPf2* gene in traits other than the pathogenesis. All  $\Delta abpf2$  mutants were similar to the wild type in conidia production (Table 2), vegetative growth, and colony morphology *in vitro* (Figure 2). Germination rates and the frequency of appressorium formation on host plant tissue were also similar (Figure S2 and Table 3). These results suggest that *AbPf2* is dispensable for fungal development and primary metabolism in *A. brassicicola*, and is associated exclusively with pathogenesis.

### **Mutants' ability to overcome stressors**

Pathogenic fungi must overcome diverse plant defense mechanisms and toxic metabolites to successfully infect their host plants. Two of the most prominent plant defenses are phytoalexins (VanEtten et al., 1995; Thomma et al., 1999; Ahuja et al., 2012) and reactive oxygen species (ROS) (Cessna et al., 2000; Mayer et al., 2001; Molina and Kahmann, 2007; Lin et al., 2009; Guo et al., 2011). Most brassicaceous plants produce phytoalexins, such as camalexin in *Arabidopsis thaliana*, and brassinin in cultivated brassicas (Pedras et al., 2004; Pedras et al., 2011). The *Arabidopsis thaliana* mutant, *pad3*, does not produce camalexin and is very susceptible to *A. brassicicola* (Zhou et al., 1999). All  $\Delta abpf2$  mutants, however, failed to infect the *pad3* mutant (Figure 1). In addition, the phytoalexin brassinin, produced by *B. oleracea*, had similar effect on the vegetative growth of the  $\Delta abpf2$  mutants and wild-type *A. brassicicola*. Neither was there difference in an observed effect on the mutants or wild type when exposed to  $KO_2$ ,  $H_2O_2$ , sorbitol, NaCl, or KCl (Figure 4). Notably,  $\Delta abpf2$  mutants expressed similar amounts of transcripts of putative ROS scavenging enzymes, such as superoxide dismutase, peroxidases, catalase, laccases, and polyphenol oxidases as the wild type during pathogenesis. Furthermore, putative detoxification enzymes, such as cyanide hydrolase, indolamine dioxygenase, glutamine aldoltransferease, and lactamase were up-regulated in the  $\Delta abpf2$  mutant. These results suggest that *AbPf2* is also dispensable for the detoxification of phytoalexins, unknown phytotoxins, or reactive oxygen species, or for osmoregulation. In other words, the loss of pathogenicity was not due to the mutants' inability to cope with phytoalexins, phytotoxins, and ROS produced by these host plants.

### **Functional importance of *AbPf2* in pathogenesis**

Changes in gene expression profiles of the mutant when in contact with its host plants provided a clue to its loss of pathogenicity. Of 10,688 genes, 106 were down-regulated in the  $\Delta abpf2$  mutant during the early penetration stage (12 hpi). They included 13 genes encoding hydrolytic enzymes and 8 genes encoding putative effector proteins. The hydrolytic enzyme-coding genes included 2 of 18 pectate lyase genes in the genome, AB04813.1 and AB01332.1. These two pectate lyase genes were exponentially induced in wild-type *A. brassicicola* during early infection (Figure 9). They were different from six pectate lyase genes (AB05514.1, AB00904.1, AB10322, AB06838.1, AB03608, AB10575.1) that were highly induced during the late stage of plant infection (Srivastava et al., 2012). Reduced expression in the mutants of these two pectate lyases and other cellulases might have been detrimental to the initial penetration of host tissue due to an inefficient digestion of plant cell walls. This reduction of cell wall-degrading enzymes by the mutant might have slowed down the colonization process once it was initiated, but not stopped it. Thus, the reduced expression of hydrolytic enzyme genes was insufficient to explain both the loss of pathogenicity and the failure of lesion expansion at wound sites or on senescent host tissue (Figure 1).

The eight putative effector proteins might have had crucial roles in pathogenesis. Notably, five of the eight genes were highly expressed in the wild type but only occurred at low levels in the  $\Delta abpf2$  mutant at 12 hpi (Table S2). The importance of effectors in the interactions between various host plants and their fungi and fungus-like oomycete pathogens has been established (Kale et al., 2010; Kale and Tyler, 2011). In compatible interactions many effector proteins reengineer host gene expression, causing a suppression of the plant's defenses (Doehlemann et al., 2009; Djamei et al., 2011). In the incompatible interactions between biotrophic pathogens and their host plants, effector proteins are usually recognized by host receptor proteins. These receptor proteins have nucleotide-binding and leucine-rich repeat (NB-LRR) domains and are known as resistance (R) genes (Dangl and Jones, 2001). Most effector-related research has been done using biotrophic pathogens. However, a few NB-LRR proteins are known to interact with secondary metabolites or small proteins secreted by necrotrophic fungi. For example, the host-specific toxin victorin is recognized by a NB-LRR protein, which makes oats and *Arabidopsis thaliana* susceptible to *Cochliobolus victoriae* (Lorang et al., 2007). The small ToxA protein is also recognized by a receptor with a NB-LRR that makes wheat susceptible to *Stagonospora*

*nodorum* (Faris et al., 2010). In our study, 21 NB-NRR genes were down regulated in the host plants inoculated with  $\Delta abpf2$  mutants (Table S6). The interaction between NB-LRR proteins and effector proteins might have made the host plants susceptible to *A. brassicicola*. These effector proteins may be toxins, like ToxA in *S. nodorum*. Putative toxins also include a necrosis-inducing-factor (AB09384.1), two pectate lyases, and other cellulases, like a xylanase (*Xyn11A*) in *Botrytis cinerea* (Noda et al., 2010). A hypersensitive reaction in plants causes local cell death, and is an efficient defense mechanism against biotrophic pathogens (Gilchrist, 1998). However, necrotrophic pathogens may take advantage of hypersensitive reactions, absorbing nutrients from the dead plant tissue (Govrin and Levine, 2000). We suspect that a low-level expression of the eight putative effectors, the necrosis inducing factor, and hydrolytic enzymes was insufficient to make the host cells susceptible to wild-type *A. brassicicola*.

### **Other plant genes affected by *AbPf2***

Some plant genes were expressed at lower levels following a challenge by the  $\Delta abpf2$  mutants than when challenged by wild-type *A. brassicicola*. These included genes associated with host responses to chitin, jasmonic acids, ethylene, wounding, oxidative stress, and fungi. These genes are important in the defense against necrotrophic fungi (McDowell and Dangl, 2000). They also included genes associated with lignin synthesis and transporter proteins. Reduced expression of these genes suggested that the  $\Delta abpf2$  mutant did not activate, or only marginally activated, the host plant defense mechanisms against the pathogenic fungus. Plants activate an initial defense after recognition of microbe-associated molecular patterns and a full defense response after triggered by fungal effectors. It is possible that the plants in our study could not sense, or neglected the presence of, the  $\Delta abpf2$  mutants growing on their surface (Figure 3).

Alternatively, defense reactions were activated after microbe associated pattern recognition, but very weak compared to the effector triggered immune responses because the  $\Delta abpf2$  mutant did not secrete effectors.

Up-regulated genes in plants inoculated with  $\Delta abpf2$  mutants were associated with primary metabolism. They included transcription, translation, cell division, cell differentiation, and the biosynthesis of membranes and cell walls. Differential expression of these genes suggested that wild-type *A. brassicicola* damaged the primary metabolism and tissue growth of the host plant,

but the  $\Delta abpf2$  mutants did not. We excluded the possibility that plant gene expression data were the result of a complete arrest in growth and metabolism of the  $\Delta abpf2$  mutant for two reasons. First, mutant conidia germinated and grew on the host plant surface (Figure 3 and S3), and second, many genes were highly expressed at 48 hpi during growth in the host tissue (Table 8). Interestingly, plants infected by the mutant expressed a higher level of genes associated with chloroplasts and photosynthesis compared to the wild type. Plants infected by the wild type might have caused an increased expression of host defense genes at the expense of photosynthesis. We prefer an alternative possibility that wild-type *A. brassicicola* manipulated its host plants to slow photosynthesis, as implied in other systems. Photosynthesis was reduced when conidia of certain pathogens or fungal elicitors were applied to intact leaves (Govrin et al., 2006; Bolton, 2009). Also, the ToxA protein secreted by *S. nodorum* and *Pyrenophora tritici-repentis* interacted directly with a chloroplast protein and indirectly with another protein, Tsn1, which is involved in circadian rhythms and photosynthesis (Manning et al., 2007; Faris et al., 2010). The gene expression data suggested that wild-type *A. brassicicola* suppressed primary metabolism and photosynthesis during early infection, but  $\Delta abpf2$  mutant did not.

### **Infectivity of *Alternaria brassicicola* conidia**

A small amount of AbPf2 protein was present in the young conidia of *A. brassicicola* (Figure 5). The amount of this protein decreased and eventually disappeared when the conidia germinated on a nutrient-rich synthetic medium. In contrast, the amount of AbPf2 protein increased both in the cytoplasm and in the nuclei of conidia upon contact with their host plants. This high concentration was maintained in the nuclei of conidia, germ tubes, appressoria, and invading hyphae during early pathogenesis (Figures 6 and 7). The AbPf2 protein disappeared from the nuclei, however, during the early colonization stage when host tissue developed obvious necrotic spots (Figure 6F and Figure 7J). Furthermore, the gene deletion mutants did not penetrate host tissue, but grew on the surface of the plant. These data and our observations suggested that *AbPf2* was essential for plant penetration and the subsequent establishment of infection by *A. brassicicola*.

### **Implications of this study**

We have screened targeted gene mutants of over 200 genes that encode transcription factors in *A. brassicicola* (Cho et al., 2009; Cho et al., 2012). *AbPf2* is the only transcription factor gene with null mutants that were nonpathogenic but did not show other changes in phenotype. *Fusarium graminearum* ([http://www.broadinstitute.org/annotation/genome/fusarium\\_graminearum](http://www.broadinstitute.org/annotation/genome/fusarium_graminearum)) has nine transcription factors with a high sequence similarity to *AbPf2* (Son et al., 2011) (Table S7). However, mutants of the nine individual genes in *F. graminearum* remained pathogenic. Both *A. brassicicola* and *F. graminearum* are necrotrophic plant pathogens, able to kill host plant tissue to obtain nutrients. If the ability to cause infection and use host nutrients is important for adaptation to their host plants, or to niche expansion, it might suggest that regulators of this function were conserved. Homologous transcription factors, however, evolved with unique functions in each of these two fungi. Similarly, the transcription factor *MoAp1* is important in coping with oxidative stress and is important for pathogenesis in *Ustilago maydis*, *Alternaria alternata*, and *Magnaporthe oryzae* (Molina and Kahmann, 2007; Lin et al., 2009; Guo et al., 2011), but dispensable in *Cochliobolus heterostrophus* (Lev et al., 2005). Most pathogenesis-associated genes are unique to their distantly-related taxa. The extent of functional conservation of essential pathogenesis regulators, including *AbPf2*, is an important area to investigate for logical targets to manage fungal diseases.

## Materials and Methods

### Transformation, maintenance of fungal strains, and confocal microscopy

Growth and maintenance of *A. brassicicola* Schweinitz & Wiltshire (ATCC96836) 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 were produced for Southern hybridization and their primers listed in Table S8. 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 uniform genetic background. Cultures were maintained as glycerol stock in separate tubes with one tube used for each assay. Our method of capturing images of fungal tissue by confocal microscopy was described previously (Srivastava et al., 2012).

### Determination of the full-length sequence of *AbPf2*

Using the partial sequence of the *AbPf2* gene in the draft annotated genome sequence (<http://jgi.doe.gov/Abrassicicola>), we designed one primer, P1, in the coding region of the gene at the end of the contig. Another primer, P2, was designed in the adjacent contig. These two primers were used to amplify the ~5-kilobase-pair gap between the two contigs. The PCR products were used as template DNA and the sequence was determined with the 2 PCR primers and 14 additional primers (P3-P16) that were subsequently designed during sequencing (Table S8). The completed sequence was used to identify a predicted stop codon. The sequence data were deposited in the NCBI GenBank (JQ899199).

### **Generation of deletion mutants for *AbPf2***

All transformation constructs used in this work were produced as described previously (Cho et al., 2009). We made *AbPf2* deletion mutants by replacing the 891 nucleotides (nt) spanning the partial promoter (233 nt) and partial protein-coding region (658 nt) with a HygB resistance cassette (Figure S1). The replacement construct was produced with the three sets of primers (Figure S5A), P17 plus P18, P21 plus P22, and P19 plus P20 to amplify 1,056, 951, and 1,436 base pairs (bp) as described previously (Cho et al., 2009). All primer information is provided in Table S8. We used two primers, P23 and P24, to make 476-bp-long Southern hybridization probes.

### **Complementation of the $\Delta abpf2$ mutants**

The  $\Delta abpf2$ -2 and  $\Delta abpf2$ -s5 mutants were complemented with the wild-type *AbPf2* allele and its native promoter. We used two primers to reintroduce wild-type *AbPf2* into the two mutants, P17 and one of the sequencing primers, P15 (Figure S5B). These primers amplified the 4,496-bp-wild-type allele of the *AbPf2* gene using *A. brassicicola* genomic DNA as a template. The PCR product included a 1,291 bp 5' flanking region, 2,308 bp complete coding region, and an 897 bp 3' flanking region. Separately, pNR-20F and pNR-775R were used to amplify a 2,226-bp-long nourseothricin-resistant (NTC) cassette as a selectable marker gene, using a pNR vector as the template (Malonek et al., 2004). These two products (7  $\mu$ g of *AbPf2* and 3  $\mu$ g of NTC cassette) were mixed to transform the  $\Delta abpf2$ -2 and  $\Delta abpf2$ -s5 mutants. Two rounds of single-spore isolation purified the clones of the complemented mutants.

## **Generation of mutants expressing AbPf2-GFP fusion proteins**

To investigate the expression pattern of AbPf2 proteins and their localization, we tagged the gene at the C-terminus, right before the stop codon, with a 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 *AbPf2* gene that was regulated by its native promoter elements. To make a transformation construct, the *AbPf2* coding region (1,054 bp) and 3' flanking region (448 bp) were amplified with primers P25 and P26, and P27 and P28, respectively (Figure S5C). Another set of primers, P29 and P30, was used to amplify the 2,384 bp that covered the coding regions of the GFP and the resistance cassette. The final transformation constructs were produced by PCR amplification from a mixture of the three PCR products using primers P25 and P28.

## **Generation of mutants expressing mCherry-NLS fusion proteins**

To investigate the localization of AbPf2 proteins in nuclei, we made a construct with a ToxA promoter and mCherry-NLS fusion protein. The tagging construct was designed for the mCherry-NLS protein to be constantly expressed under the control of the ToxA promoter. To make a transformation construct, the mCherry-NLS was amplified with primers P35 and P36 from pBV579 (Khang et al., 2010). Another set of primers, P33 and P34, was used to amplify the ToxA promoter. The final transformation constructs were produced by PCR amplification from a mixture of the three PCR products and using primers P33 and P36 (Figure S5D).

## **Pathogenicity assays**

Either whole plants or detached leaves harvested from 5-to-8-week-old *Brassica oleracea* (green cabbage) were inoculated with  $1-2 \times 10^3$  conidia in 10  $\mu$ l of water. To minimize plant effects on disease severity, we inoculated the mutants and the wild-type *A. brassicicola* onto opposite sides of the upper surface of the same leaf. *Arabidopsis thaliana*, ecotype Col-0, and the *pad3* mutant of the ecotype (Zhou et al., 1999) were also used for the pathogenicity assays. The *Arabidopsis* plants were either spot-inoculated or spray-inoculated. Spot inoculation was performed with 2,000 conidia in 10  $\mu$ l of water. Spray inoculations were performed by spraying to runoff with a concentration of  $5 \times 10^5$  conidia/ml. For most detached-leaf assays, the leaves were removed, inoculated, placed in mini-moist chambers, and randomly located on a laboratory bench. For pathogenicity assays on whole plants, potted plants were placed in a semi-transparent plastic

trough with adequate water. The troughs and plants were sealed with plastic wrap after inoculation to keep the relative humidity close to 100%. We measured pathogenicity by the presence or absence of visible lesions at the inoculation sites.

#### **Examination of germ tubes and appressoria**

To examine germ tubes and the formation of appressoria at the tips of germ tubes, trypan blue staining was performed as described previously (Srivastava et al., 2012). Infected plant tissue was trimmed with a razor blade and mounted on microscope slides. Images were recorded with an Infinity 2 camera mounted on a compound microscope (BX41 TF) and analyzed with Infinity Analyze (Olympus, Japan).

#### **Quantitative real-time PCR**

Expression of the *AbPf2* gene in wild-type *A. brassicicola* was measured with qRT-PCR. We collected mature conidia before inoculation (0 hpi), conidia attached to the host plant (4 hpi), and conidia at ~100% germination (8 hpi on cabbage and 12 hpi on *Arabidopsis*). Other fungal tissues were collected that represented host-plant penetration (12 hpi on cabbage and 24 hpi on *Arabidopsis*), early hyphal colonization (24 hpi on cabbage and 24 hpi on *Arabidopsis*), saprophytic growth on necrotic host tissues (48-72 hpi on cabbage and 48-114 hpi on *Arabidopsis*), and the conidiation stage (216 hpi on cabbage). All tissues collected from inoculation courts were a mixture of fungal and host plant tissues. The tissues were immediately frozen in liquid nitrogen and total RNA from each tissue extracted with an RNeasy kit (Qiagen, Palo Alto, CA). One microgram of total RNA was transcribed to cDNA in a final volume of 20  $\mu$ l using 50 ng of random pentamers and 200 ng of poly(T)<sub>20</sub>N with Superscript III (Invitrogen, Carlsbad, CA). Each cDNA was diluted 1:10. Subsequent qRT-PCR reactions were performed in a 20- $\mu$ l volume containing 120 nM of each primer, 1.0  $\mu$ l of dilute cDNA, and 10  $\mu$ l of FastStart SYBRGreen Master (Roche, Mannheim, Germany). Each reaction was run in triplicate in a Biorad I-cycler (Bio-Rad, Hercules, CA, USA) as described previously (Srivastava et al., 2012). Relative amounts of the transcripts of *AbPf2* were calculated as  $2^{-\Delta Ct}$  using a threshold cycle (Ct), where  $\Delta Ct = (Ct_{AbPf2} - Ct_{\text{average of two genes}})$ . Two genes were regarded as housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and elongation factor 1- $\alpha$  (*Ef1- $\alpha$* ). Using the same set of cDNA, we investigated the expression patterns of eight genes. Primer

1 sequences for each gene are listed in Table S8. Relative amounts of the transcript of each gene  
2 were presented as difference of Ct values [ $\Delta Ct = (Ct_{\text{genei}} - Ct_{\text{average of } Ef1-\alpha \text{ and } GAPDH})$ ], which are  
3 equivalent to the relative amounts of transcripts on a log 2 scale. Relative transcripts between  
4 the wild type and  $\Delta abpf2$  strain were presented as difference of  $\Delta Ct$  ( $\Delta \Delta Ct$ ) between the mutant  
5 and the wild type, where  $\Delta \Delta Ct = [(Ct_{\text{genei}} - Ct_{\text{average of } Ef1-\alpha \text{ and } GAPDH})_{\Delta abpf2} - (Ct_{\text{genei}} - Ct_{\text{average of } Ef1-\alpha$   
6  $\text{and } GAPDH})_{\text{wild type}}]$ . Primers for each gene are listed in Table S8.

## 8 **RNAseq data generation and gene expression analysis**

9 To identify pathogenicity-associated genes regulated by the transcription factor, we compared  
10 gene expression profiles between the  $\Delta abpf2$  mutant and wild-type *Al. brassicicola* at two time  
11 points, 12 hpi and 48 hpi. For this experiment, we used *Arabidopsis thaliana*. This plant was  
12 better choice than cabbage to investigate plant responses to the pathogen because the genome is  
13 well annotated. Twenty-seven plants of *Arabidopsis thaliana* ecotype Col-0 were spray-  
14 inoculated until run-off with  $5 \times 10^5$  conidia per milliliter of  $\Delta abpf2-2$ ,  $\Delta abpf2-s5$ , or the wild  
15 type. Tissues containing both host plant leaves and fungal hyphae were harvested from three  
16 plants for each sample and immediately frozen in liquid nitrogen to fix gene expression profiles.  
17 The frozen tissues were ground and total RNA extracted using an RNeasy kit. Residual DNA  
18 was digested in columns with RNase-free DNase following the manufacturer's protocol (Qiagen,  
19 Palo Alto, CA). Three biological replicates were prepared for the mutant and three for the wild  
20 type and their gene expression profiles compared.

21  
22 We constructed strand-specific sequencing libraries from total RNA using the TruSeq™ RNA  
23 Sample Prep Kit (Illumina, San Diego, CA) following the manufacturer's protocol. Each RNA  
24 sample was used to construct a library with a unique index primer. A total of six index primers  
25 were used to construct six libraries. All six libraries were mixed and 100 nucleotide-long  
26 sequence tags were determined using Illumina Hiseq2000 (Illumina, San Diego, CA, USA)  
27 according to the manufacturer's protocol. Image analysis, base-calling, and quality checks were  
28 performed with the Illumina data analysis pipeline CASAVA v1.8.0. The data have been  
29 deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through  
30 GEO Series Accession No. GSE38984 (<http://www.ncbi.nlm.nih.gov/geo/info/linking.html>).

The sequenced reads were mapped to the genome sequence of *A. brassicicola*, which can be accessed through the interactive JGI fungal portal MycoCosm (Grigoriev et al., 2012) at <http://jgi.doe.gov/Abrassicicola> (<http://jgi.doe.gov/Abrassicicola>) and the latest genome release of *Arabidopsis thaliana* (TAIR10 ) (<ftp://ftp.arabidopsis.org/home/tair/Genes/>) (Lamesch et al., 2012) using the programs Tophat 2.0.0 (Trapnell et al., 2009) and Bowtie 2.0.0 (Langmead et al., 2009). Default settings were used, except in the case of *A. brassicicola* the intron length was designated as a minimum of 10 nucleotides and a maximum of 500 nucleotides. The program Cuffdiff version 1.3.0 (which is part of Cufflinks, (Trapnell et al., 2010)) was used to identify reads overlapping with previously predicted genes. The mapping bias correction method was used while running Cuffdiff (Roberts et al., 2011). The expression levels of each predicted gene were determined and normalized to mapped Fragments Per Kilobase of exon model per Million (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 using the default-allowed false discovery rate (FDR) of 0.05. In addition to this we also applied a cutoff of at least a two-fold change in expression value for differential expression. Custom scripts were written in Python for data analysis.

### **Representation analysis of functional annotation terms**

Custom scripts were developed in Python and R to analyze over- and under-representation 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 over-representation 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 ((Gupta et al., 2007), part of the MEME Suite) was used to search the JASPAR CORE Fungi database of transcription factor-binding profiles (Bryne et al., 2008).

## Supplemental materials

Figure S1. Deletion of the AbPf2 gene

Figure S2. Germination and germ tube growth of wild-type *Alternaria brassicicola* and mutant conidia on green cabbage leaves

Figure S3. Hyphal growth of the  $\Delta abpf2$  mutant and wild-type *Alternaria brassicicola* on the leaves of green cabbage.

Figure S4. Hierarchical clustering of fungal RNA-seq data

Figure S5. Schematic diagram of the PCR strategy used to make all constructs

Table S1. Table S1. Sequence similarity between orthologous and paralogous genes in *Alternaria brassicicola* and *Pyrenophora tritici-repentis*

Table S2. List of fungal genes differentially expressed in the  $\Delta abpf2$  mutants compared to the wild type at 12 hours postinoculation.

Table S3. List of fungal genes differentially expressed in the  $\Delta abpf2$  mutants compared to the wild type at 48 hours postinoculation

Table S4. List of plant genes differentially expressed in plant inoculated with the  $\Delta abpf2$  mutants compared to the plant inoculated wild-type *Alternaria brassicicola* at 12 hours postinoculation

Table S5. Statistically over-represented functional annotation terms among plant genes differentially expressed at 12 hours postinoculation

Table S6. List of genes with leucine-rich repeat domains

Table S7. Mutant phenotypes of nine AbPf2 homologs in *Fusarium graminearum* (Excerpted from (Son et al., 2011))

Table S8. List of primers used for quantitative real-time polymerase chain reaction, sequencing, or transformation constructs

#### Acknowledgements

We thank Wayne Borth, Anne Alvarez, and John Hu for generously sharing their research equipment; Tina M. Carvalho for assisting with the confocal microscopy, Johnson Siu and Hui Trung for plant growth; and Fred Brooks for a critical reading of the manuscript.

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## Figure legends

**Figure 1. Loss of pathogenicity in  $\Delta abpf2$  mutants.** A. Lesions on *Brassica oleracea* leaves at 5 days postinoculation (dpi) with 1,000 conidia in 10  $\mu$ l of water. B. Lesions on the *Arabidopsis thaliana pad3* mutant (Col-0 background) caused by 2,000 spores in 10  $\mu$ l of water. The leaves were detached from four plants for each assay and photographed at 5 dpi. C. Leaves of *Arabidopsis thaliana*, ecotype Col-0, were sprayed to runoff with a concentration of  $5 \times 10^5$  spores/ml. D. Charts showing complete restoration of virulence in two mutants by complementing them with the wild-type allele of the *AbPf2* gene. Abbreviations: wt = wild-type *Alternaria brassicicola*;  $\Delta abpf2$  = *AbPf2* deletion mutant; c-2 and c-s5 = complemented mutants  $\Delta abpf2$ -2:*AbPf2* and  $\Delta abpf2$ -s5:*AbPf2*, respectively; injured = injured by scratching with a pipette tip; not injured = not injured; Col-0 = *Arabidopsis thaliana* ecotype Columbia-0.

**Figure 2. Pharmacological tests.** Each fungal strain from glycerol stocks was grown on PDA containing an appropriate selectable agent and cultured in the dark for 5 days at 25°C. In order to test the fungal cultures for sensitivity to osmotic stress and oxygen radicals, wild-type and mutant conidia were pipetted onto PDA containing 0.1 mM brassinin, 2 M sorbitol, 15 mM  $H_2O_2$ , 10 mM  $KO_2$ , 0.7M KCl, or 0.5 M NaCl. The experiment was conducted three times.

**Figure 3. Hyphal growth of the  $\Delta abpf2$  mutant and wild-type *Alternaria brassicicola* on the leaves of *Arabidopsis thaliana*.** Fungal tissues were stained with trypan blue. Scale bar = 100  $\mu$ m.

**Figure 4. Expression of the *AbPf2* gene during pathogenesis.** A. host plant green cabbage. B. *Arabidopsis thaliana* (Col-0). The Y-axes illustrate the relative quantity of the transcripts compared to the average of two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase and elongation factor 1- $\alpha$ . We arbitrarily set the lowest expression level as zero to estimate the extent of induction of the transcripts during pathogenesis. The X-axes show the number of hours postinoculation at which biological samples were collected. Three biological replicates (N=3) were used for each sample. Error bars indicate standard deviation. Stages of pathogenesis marked at the bottom of the charts are based on morphological changes in the fungus and status of the plant tissue during sample collection. Numbers below the charts: 1 = before germination,

2 = germination, 3 = appressorium formation, 4 = lesion expansion; early stage of saprophytic growth, 5 = saprophytic growth, 6 = extensive conidium formation.

**Figure 5. AbPf2 protein expression and localization during germination on potato dextrose agar (PDA).** A. Hyphal tip growing in PDA. B. Conidia before inoculation. C. Germinating conidium at 4 hours after transfer. D. Hyphal growth at 24 hours after transfer. All images were acquired under the same scanning conditions. Green color represents AbPf2-GFP fusion protein and pink color represents nuclei marked by mCherry with nuclear localization signal (mCherry-NLS). Composites represent overlays of green, pink, and light microscope images after sequential scanning.

**Figure 6. Confocal microscope images showing AbPf2-GFP fusion protein expression and localization on *Arabidopsis thaliana*.** A. Conidia before inoculation. B. Conidia near germination time. C. Conidia immediately after germination. D. Conidium with a germ tube and an appressorium. Arrow marks an appressorium at the tip of a germ tube. Arrowheads mark autofluorescence of plant tissue. E. Invading hyphae in the plant tissue before disease spots expanded beyond the initial infection site. F. Conidia and colonizing hyphae in necrotic plant tissues. Pink color indicates mCherry-NLS in nuclei. Abbreviations: GFP = green fluorescent protein; AbPf2-GFP = AbPf2 and GFP fusion protein; mCherry-NLS = mCherry and simian nuclear localization signal fusion protein. Composites represent overlays of green, pink, and light microscope images after sequential scanning.

**Figure 7. Confocal microscope images showing GFP and AbPf2-GFP fusion protein expression and localization.** All images were acquired during host plant infection on 4-week-old green cabbage. A-B. Conidia before inoculation. C-D. Conidia near germination time. E-F. Conidium with a germ tube and an appressorium. Arrowheads mark appressoria at the tip of each germ tube. G-H. Hyphae invading the plant tissue. I-J. Conidia and colonizing hyphae. Arrows in image I mark nuclei stained with DAPI that are not overlapped with GFP. K. Co-localization of AbPf2-GFP and DAPI at 24 hpi. Abbreviations: DAPI = 4',6-diamidino-2-phenylindole; GFP = green fluorescent protein; ToxA-GFP = GFP expressed under regulation of a ToxA promoter; AbPf2-GFP = AbPf2 and GFP fusion protein.

**Figure 8. Hierarchical clustering of fungal RNA-seq data from plants 24 and 48 hours**

**postinoculation (hpi).** A. Set of 168 genes that show differential expression patterns between the  $\Delta abpf2$  mutant and wild-type *A. brassicicola* at 12 hpi. The color key represents the log<sub>2</sub> ratio of Fragments Per Kilobase of exon model per Million (FPKM). Red indicates higher and green indicates lower expression levels in the  $\Delta abpf2$  mutant than in wild-type *A. brassicicola*. B. Expanded view of up-regulated genes in the mutants at 12 hpi. C. Expanded view of down-regulated genes at 12 hpi. “S” indicates putative secretion proteins predicted by HMM and signal P. D and E. Comparisons of down- and up-regulated genes at 12 and 48 hpi.

**Figure 9. Expression of eight genes during pathogenesis in *Arabidopsis thaliana* (Col-0).** A-

E. Comparison of transcripts ( $\Delta Ct$ ) of each gene in the  $\Delta abpf2$  mutant and wild-type *A. brassicicola*. They were normalized by the average of two house-keeping genes, glyceraldehyde 3-phosphate dehydrogenase and elongation factor 1- $\alpha$ . We added 10 to  $\Delta Ct$  to make the expression values positive at most time points. F. Relative transcripts ( $\Delta \Delta Ct$ ) of five genes that are differentially expressed at 12 hours postinoculation (hpi) in the  $\Delta abpf2$  mutant compared to wild-type *Alternaria brassicicola*. G-I. Y-axes indicate the relative quantity of the transcripts on the Log<sub>2</sub> scale. The X-axes show the number of hours postinoculation at which biological samples were collected. J. Relative transcripts ( $\Delta \Delta Ct$ ) of three genes during pathogenesis that are not differentially expressed at 12 hpi. Three biological replicates (N=3) were used for each sample. The grey line in each chart represents the expression pattern of the *AbPf2* gene. Error bars indicate standard deviation. The grey curve indicates the *AbPf2* expression pattern. *Amhy* = Amidohydrolase AB09633.1, *EF4512* = Effector AB04512.1, *PL4813* = pectate lyase AB04813.1, *EF9024* = Effector Ab09024.1, *PL1332* = Pectate lyase AB01332.1, *PL5514* = Pectate lyase AB05514.1, *AbCbh7* = cellobiohydrolase AB06252.1, *Chymo* = Chymotrypsin AB01734.1.

**Figure 10. Putative transcription factor binding sites.** Over-represented sequence motif in promoters among the down-regulated genes in  $\Delta abpf2$  mutants.

**Figure S1. Deletion of the *AbPf2* gene.** Top panel shows a schematic diagram of the wild-type locus, replacement construct, and mutant locus, in order. The mutant locus represents replacement of the coding region of the *AbPf2* gene with a single copy of a selectable marker, Hygromycin B (*HygB*) resistance cassette. Bottom panel shows Southern blots. Lanes 1-7 (except lane 5) show replacement of the targeted gene by a single copy of the *HygB* resistance cassette. Lanes s1-s3 show ectopic insertion mutants and lanes s4-s6 show replacement of the gene. Asterisks (\*) indicate DNA lanes for mutants complemented with a wild-type allele in this study. P5', Pg, and Ph indicate locations of Southern probes. Abbreviations: X = *Xba*I enzyme digestion site.

**Figure S2. Germination and germ tube growth of wild-type *Alternaria brassicicola* and mutant conidia on green cabbage leaves.** Fungal tissues were stained with trypan blue. Appressorium-like structures are marked with arrows.

**Figure S3. Hyphal growth of the  $\Delta abpf2$  mutant and wild-type *Alternaria brassicicola* on the leaves of green cabbage.** Fungal tissues were stained with trypan blue. Scale bar = 100  $\mu$ m.

**Figure S4. Hierarchical clustering of fungal RNA-seq data.** A. Set of 1,288 genes that show differential expression patterns in at least one of the four comparisons indicated at the top of the columns. The color key represents a log2 ratio of Fragments Per Kilobase of exon model per Million (FPKM). Numbers on the internal node indicate the number of proteins with a secretion signal among all proteins included in each node. B. Expression pattern of 777 genes that are differentially expressed in the  $\Delta abpf2$  mutant and wild-type *Alternaria brassicicola* at 48 hpi. Red indicates higher and green indicates lower expression levels in the tissues indicated above the slash than the tissues indicated below the slash.

**Figure S5. Schematic diagram of the PCR strategy used to make all constructs.** A. Construct for replacement of the *AbPf2* gene with a Hygromycin B resistance cassette. B. Amplification of the wild-type allele of the *AbPf2* gene. C. Construct for the *AbPf2*-GFP fusion protein. D. Construct for the mCherry-NLS fusion protein with a *ToxA* promoter. nos = nos terminator.

1 Table 1 Restoration of pathogenicity and full virulence by complemented mutants

|             | Wild type | $\Delta abpf2-s5$ | $\Delta abpf2-s5:AbPf2-c10$ |
|-------------|-----------|-------------------|-----------------------------|
| Lesion size | 17.3±4.9  | 0.0±0.0           | 18.5±3.6                    |
|             | Wild type | $\Delta abpf2-2$  | $\Delta abpf2-s5:AbPf2-c2$  |
| Lesion size | 19.8±3.7  | 0.0±0.0           | 16.9±3.9                    |

2 Numbers indicate mean diameters of lesions (mm) and their standard deviations on 6-week-old  
3 green cabbage plants.

4

5

6

7 Table 2. Number of conidia produced by  $\Delta abpf2$  and wild-type *Alternaria brassicicola* during  
8 saprophytic growth on potato dextrose agar.

|      | Wild type             | $\Delta abpf2-s5$     | $\Delta abpf2-2$      | F-statistic | p-value | F-critical |
|------|-----------------------|-----------------------|-----------------------|-------------|---------|------------|
| Mean | 1.60 x10 <sup>6</sup> | 1.60 x10 <sup>6</sup> | 1.66 x10 <sup>6</sup> | 0.0174      | 0.983   | 19         |
| s.d. | 5,5346                | 5,9856                | 4,6231                |             |         |            |

9 Conidia in 78.5 square millimeters of colony on potato dextrose agar were released in water and  
10 the total number counted using a hemacytometer. s.d. represents standard deviation. F-statistic  
11 was calculated by ANOVA implemented in Excel.

12

1 Table 3. Germ tube growth (micrometers) over time for the  $\Delta abpf2$  mutants and wild-type *Alternaria brassicicola* on the host plant,  
2 *Brassica oleracea*.

|      | 12 hpi           |      | 18 hpi           |                  |      | 20 hpi           |                  |                  |       |
|------|------------------|------|------------------|------------------|------|------------------|------------------|------------------|-------|
|      | $\Delta abpf2-5$ | wt   | $\Delta abpf2-2$ | $\Delta abpf2-5$ | wt   | $\Delta abpf2-1$ | $\Delta abpf2-2$ | $\Delta abpf2-5$ | wt    |
| N    | 35               | 37   | 13               | 35               | 43   | 22               | 28               | 26               | 37    |
| Mean | 75.5             | 89.4 | 91.7             | 91.9             | 99.7 | 118.5            | 138              | 136.1            | 172.4 |
| s.d. | 52.1             | 56.9 | 28.0             | 49.4             | 57.6 | 67.4             | 62.0             | 84.2             | 98.8  |

3 The data were combined from three separate experiments. N = total number of samples, s.d. = standard deviation, hpi = hours  
4 postinoculation, wt = wild-type *Alternaria brassicicola*

5  
6  
7  
8 Table 4. Frequency of appressorium formation during early pathogenesis.

|                   | Total number of<br>germ tubes | Number of<br>appressoria | Percent of germ tubes<br>forming an appressorium |
|-------------------|-------------------------------|--------------------------|--------------------------------------------------|
| $\Delta abpf2-2$  | 36.0±3.5                      | 16.0±1.0                 | 44.9±7.2                                         |
| $\Delta abpf2-s5$ | 33.5±0.7                      | 16.5±0.7                 | 49.3±3.1                                         |
| Wild type         | 55.7±11.6                     | 25.1±4.6                 | 45.2±3.7                                         |

9 Numbers indicate mean and standard deviations. Numbers of germ tubes and appressoria were counted from 3 separate experiments.

Table 5. AbPf2-GFP accumulation in the nuclei and cytoplasm of conidia, germ tubes, and hyphae during growth on potato dextrose agar

|                              | Hyphal tips in PDA |               | Conidia (0 hpi) |               | Germ tubes (4 hpi) |               | Hyphae (24 hpi) |               |
|------------------------------|--------------------|---------------|-----------------|---------------|--------------------|---------------|-----------------|---------------|
| Nuclei                       | AbPf2-GFP          | NLS - mCherry | AbPf2-GFP       | NLS - mCherry | AbPf2-GFP          | NLS - mCherry | AbPf2-GFP       | NLS - mCherry |
| Average <sup>1</sup>         | 97.4               | 745.8         | 552.1           | 889.0         | 397.1              | 268.5         | 384.3           | 466.1         |
| sd                           | 58.4               | 302.5         | 236.3           | 700.0         | 77.5               | 88.6          | 171.6           | 349.7         |
| <i>p</i> -value <sup>2</sup> | -                  | -             | 0.0             | 0.5           | 0.0                | 0.0           | 0.8             | 0.0           |
| % change <sup>3</sup>        | -                  | -             | 466.9           | 19.2          | -28.1              | -69.8         | -3.2            | 73.6          |

| Cytoplasm                    | AbPf2-GFP | NLS - mCherry | AbPf2-GFP | NLS - mCherry | AbPf2-GFP | NLS - mCherry | AbPf2-GFP | NLS - mCherry |
|------------------------------|-----------|---------------|-----------|---------------|-----------|---------------|-----------|---------------|
| average                      | 0.0       | 18.5          | 254.3     | 18.7          | 227.4     | 1.4           | 100.1     | 67.5          |
| sd                           | 0.0       | 9.5           | 62.5      | 17.2          | 59.5      | 2.3           | 33.8      | 80.4          |
| <i>p</i> -value <sup>1</sup> | -         | -             | 0.0       | 0.3           | 0.5       | 0.0           | 0.0       | 0.0           |

- Numbers indicate fluorescence signal strength measured by ROI pixels using the FV10-ASW 2.0 viewer software.
- p*-value: Probability of statistical significance of the difference in average signal strength between the indicated stage and its preceding stage.
- % Change: Relative signal strength in pixel at the indicated stage compared to the preceding stage.

Table 6. Decreased virulence of the AbPf2-GFP-expressing mutant compared to wild-type *Alternaria brassicicola* on 8-week-old *Brassica oleracea*.

| d.f. | Lesion diameter (mm) |                         | Decrease in lesion diameter | p-value |
|------|----------------------|-------------------------|-----------------------------|---------|
|      | Wild type            | <i>AbPf2-GFP</i> mutant |                             |         |
| 14   | 16.2±2.3             | 8.1±4.2                 | 50%                         | 7.4E-08 |

Lesion diameter = mean ± standard deviation

1 Table 7. AbPf2-GFP accumulation in the nuclei and cytoplasm of conidia and hyphae during growth on *Arabidopsis thaliana*

|                              | Conidia (0 hpi) |             | Before germination |             | germ tube + appressorium |             | Infection at initial site |             | colonization of large area |             |
|------------------------------|-----------------|-------------|--------------------|-------------|--------------------------|-------------|---------------------------|-------------|----------------------------|-------------|
| Nuclei                       | AbPf2-GFP       | NLS-mCherry | AbPf2-GFP          | NLS-mCherry | AbPf2-GFP                | NLS-mCherry | AbPf2-GFP                 | NLS-mCherry | AbPf2-GFP                  | NLS-mCherry |
| Mean <sup>1</sup>            | 552.1           | 889.0       | 1168.3             | 834.6       | 2299.0                   | 1005.4      | 943.6                     | 721.5       | 117.7                      | 421.8       |
| s.d.                         | 236.3           | 700.0       | 596.4              | 420.3       | 499.7                    | 557.4       | 500.5                     | 425.9       | 62.4                       | 211.9       |
| <i>p</i> -value <sup>2</sup> |                 |             | 0.0                | 0.8         | 0.0                      | 0.2         | 0.0                       | 0.0         | 0.0                        | 0.0         |
| % change <sup>3</sup>        |                 |             | 111.6              | -6.1        | 96.8                     | 20.5        | -59.0                     | -28.2       | -87.5                      | -41.5       |

2

| Cytoplasm                    | AbPf2-GFP | NLS-mCherry | AbPf2-GFP | NLS-mCherry | AbPf2-GFP | AbPf2-GFP | NLS-mCherry | AbPf2-GFP | AbPf2-GFP | NLS-mCherry |
|------------------------------|-----------|-------------|-----------|-------------|-----------|-----------|-------------|-----------|-----------|-------------|
| mean                         | 250.3     | 28.5        | 570.6     | 59.8        | 777.6     | 4.1       | 271.7       | 9.9       | 12.6      | 92.1        |
| s.d.                         | 60.8      | 24.2        | 450.5     | 70.8        | 285.1     | 6.9       | 231.7       | 17.9      | 9.4       | 58.1        |
| <i>p</i> -value <sup>1</sup> |           |             | 0.1       | 0.6         | 0.5       | 0.5       | 0.0         | 0.9       | 0.0       | 0.8         |
| % change <sup>2</sup>        |           |             | 128.0     | 109.4       | 36.3      | -93.2     | -65.1       | 141.9     | -95.4     | 832.9       |

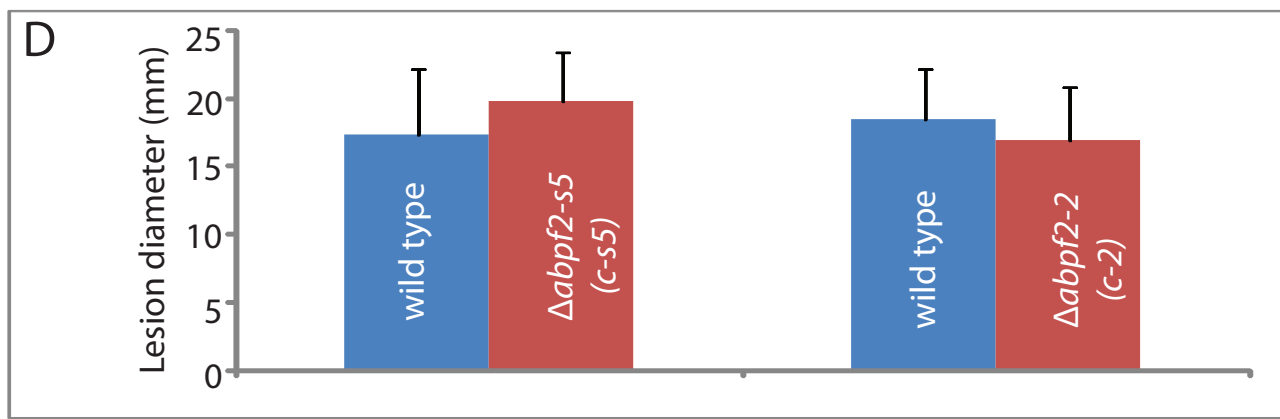
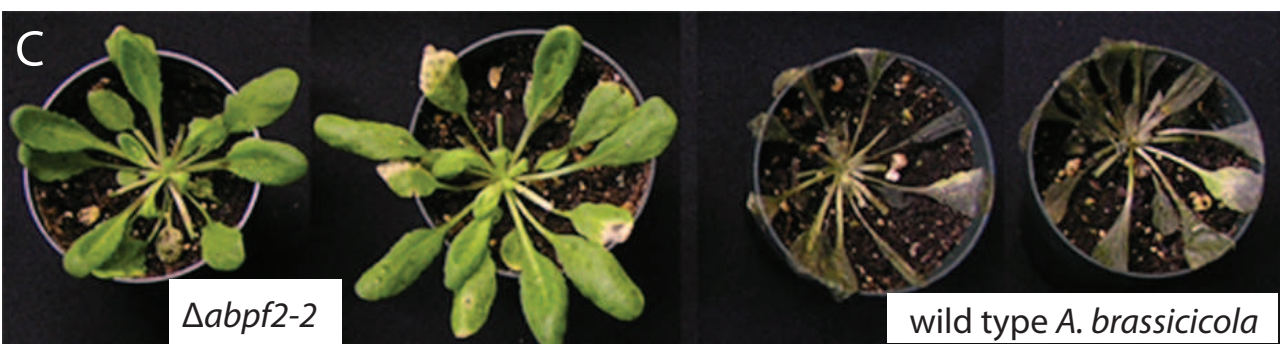
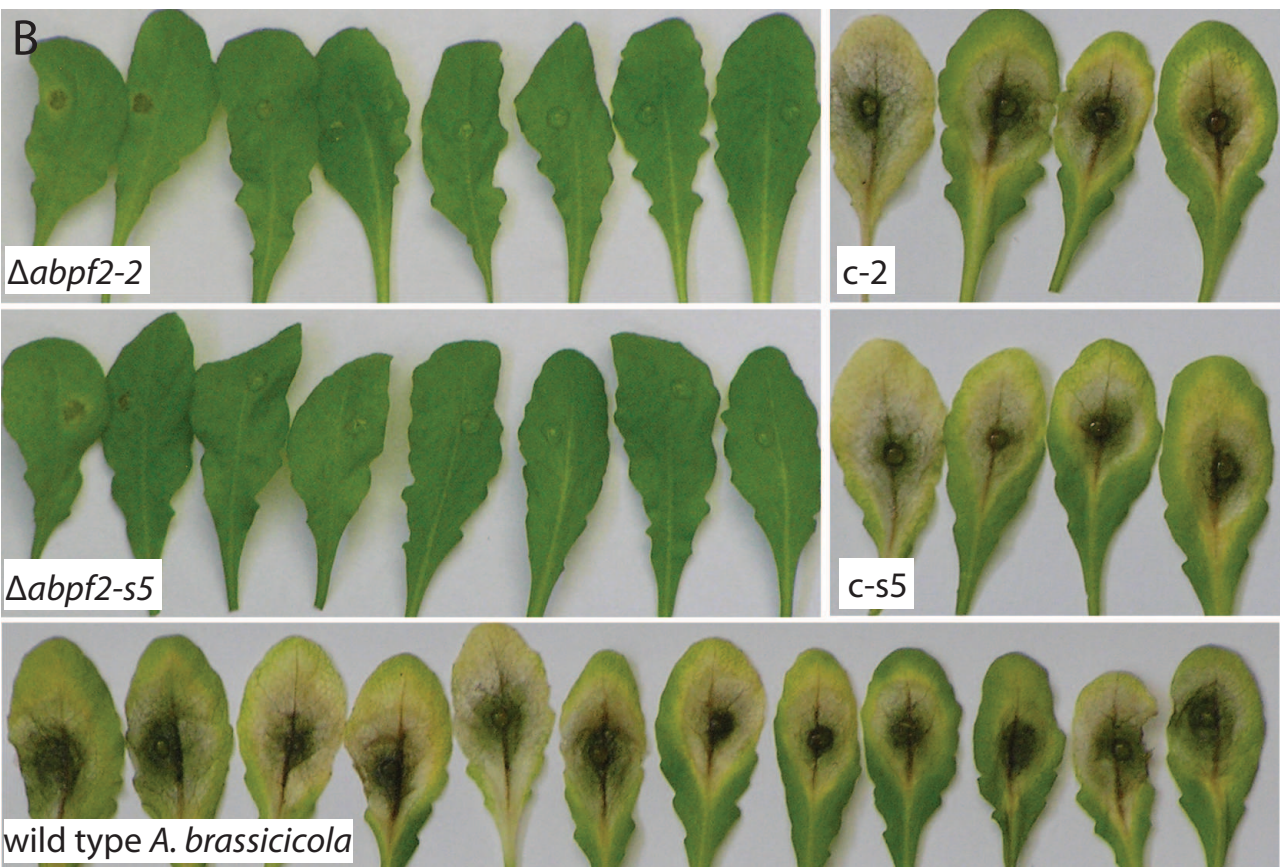
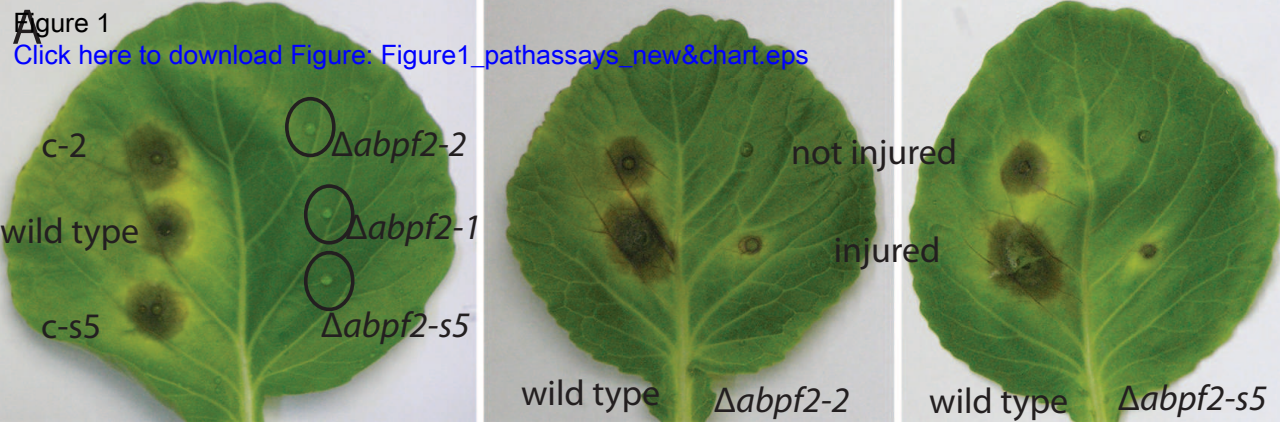
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- 4 1. Numbers indicate fluorescence signal strength measured by ROI pixels using the FV10-ASW 2.0 viewer software.
- 5 2. *p*-value: Probability of statistical significance of the difference in average signal strength between the indicated stage and its
- 6 preceding stage.
- 7 3. % Change: Relative signal strength in pixel at the indicated stage compared to the preceding stage.

1    Table 8. Number of differentially expressed fungal genes during plant infection

|                                                           | Up-regulated<br>genes | Down-<br>regulated<br>genes | Total |
|-----------------------------------------------------------|-----------------------|-----------------------------|-------|
| $\Delta ftf2$ mutant 12 hpi / wild type 12 hpi            | 62                    | 106                         | 168   |
| $\Delta ftf2$ mutant 48 hpi / wild type 48 hpi            | 525                   | 252                         | 777   |
| wild type 48 hpi / wild type 12 hpi                       | 243                   | 267                         | 510   |
| $\Delta ftf2$ mutant 48 hpi / $\Delta ftf2$ mutant 12 hpi | 232                   | 107                         | 339   |

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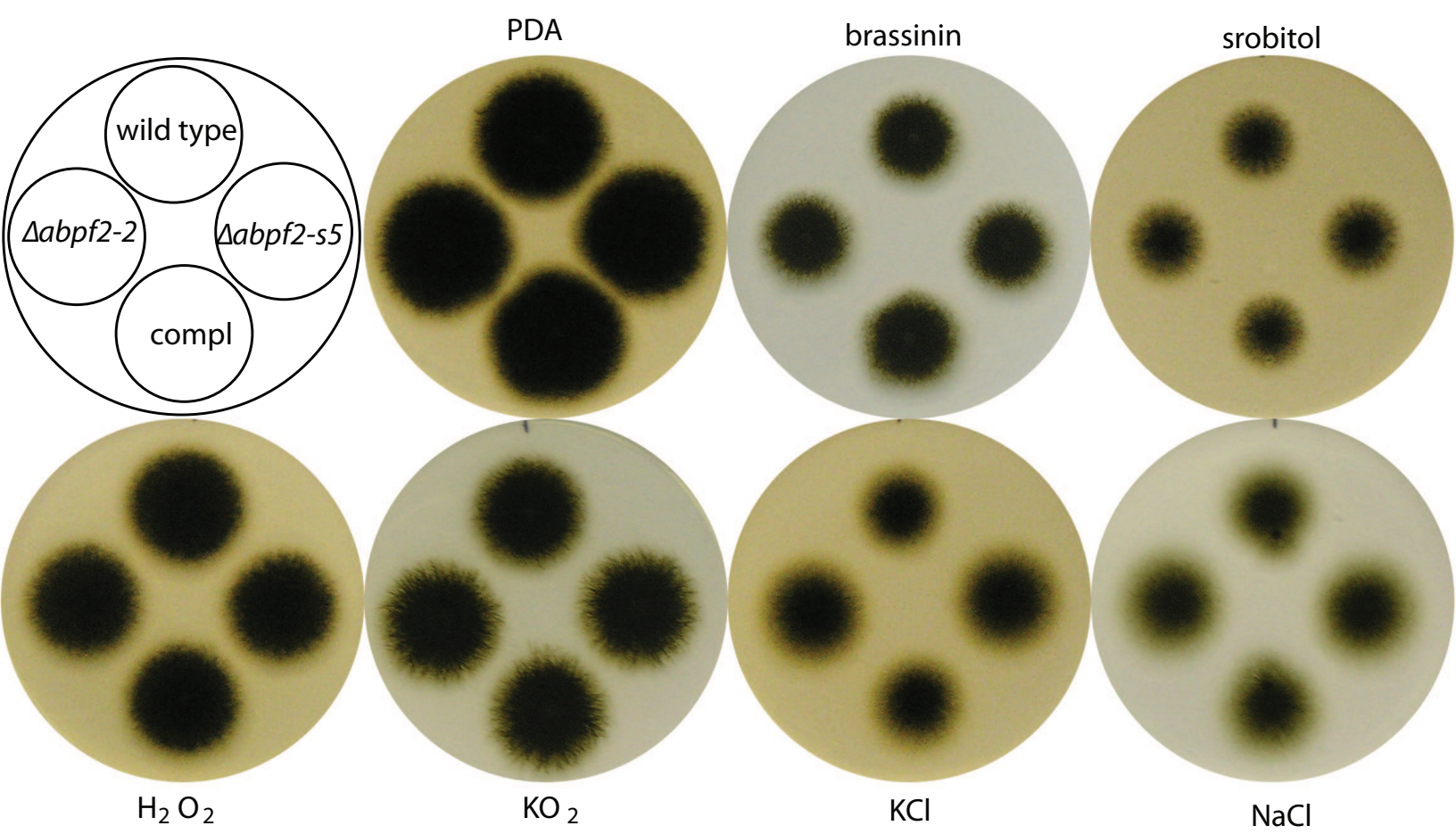


Figure 3

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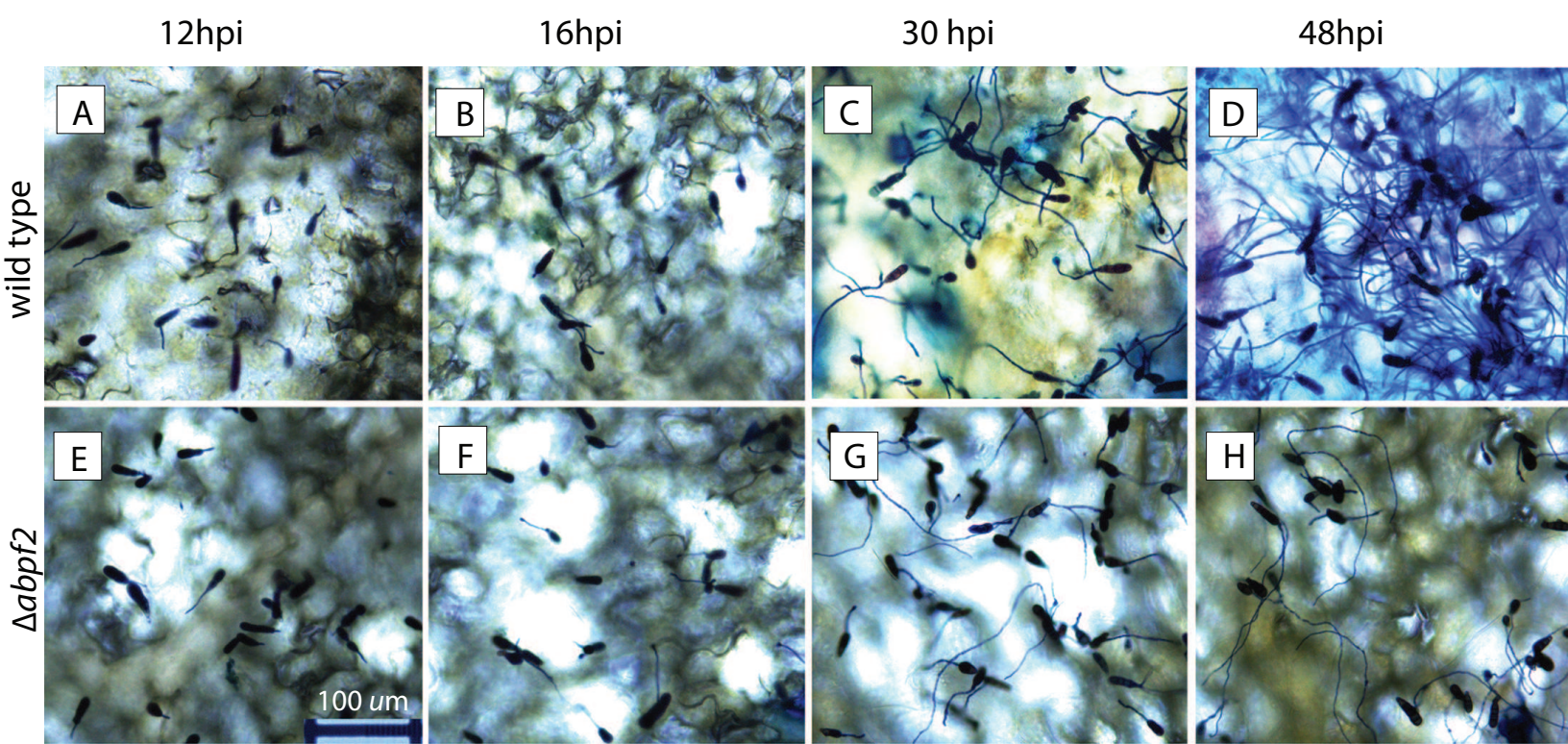


Figure 4  
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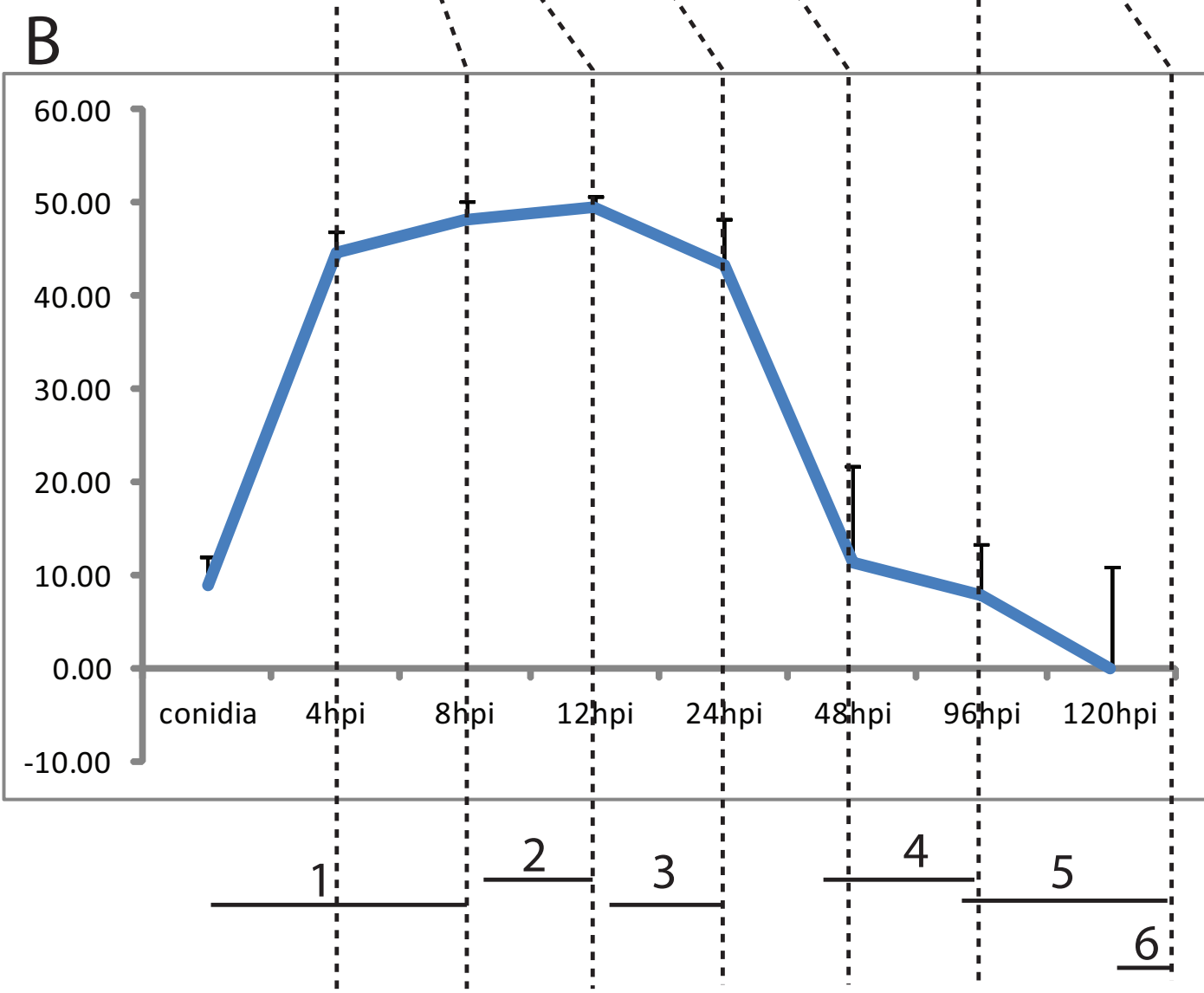
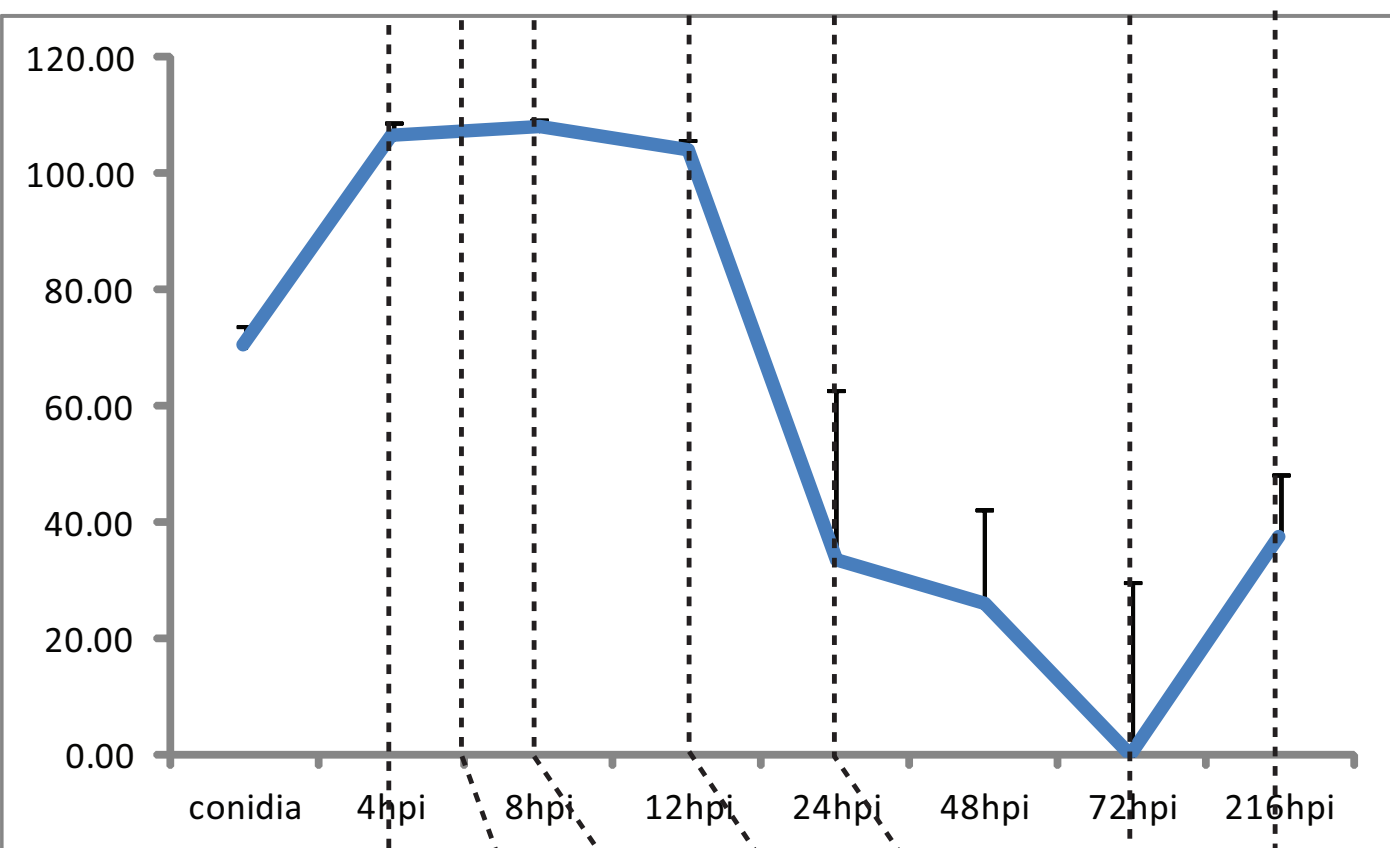


Figure 5  
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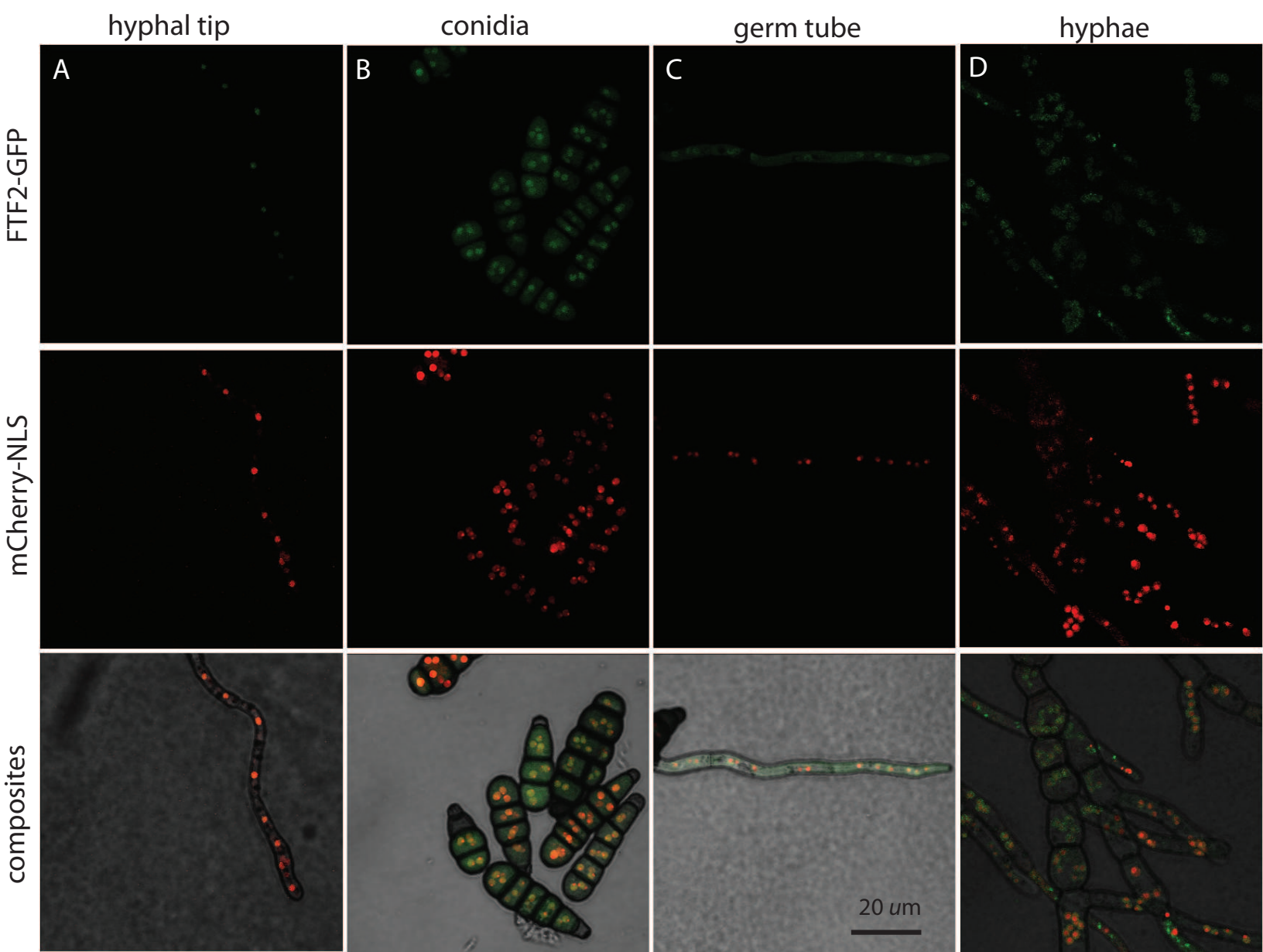


Figure 6 Abpf2-GFP mCherry-NLS composites

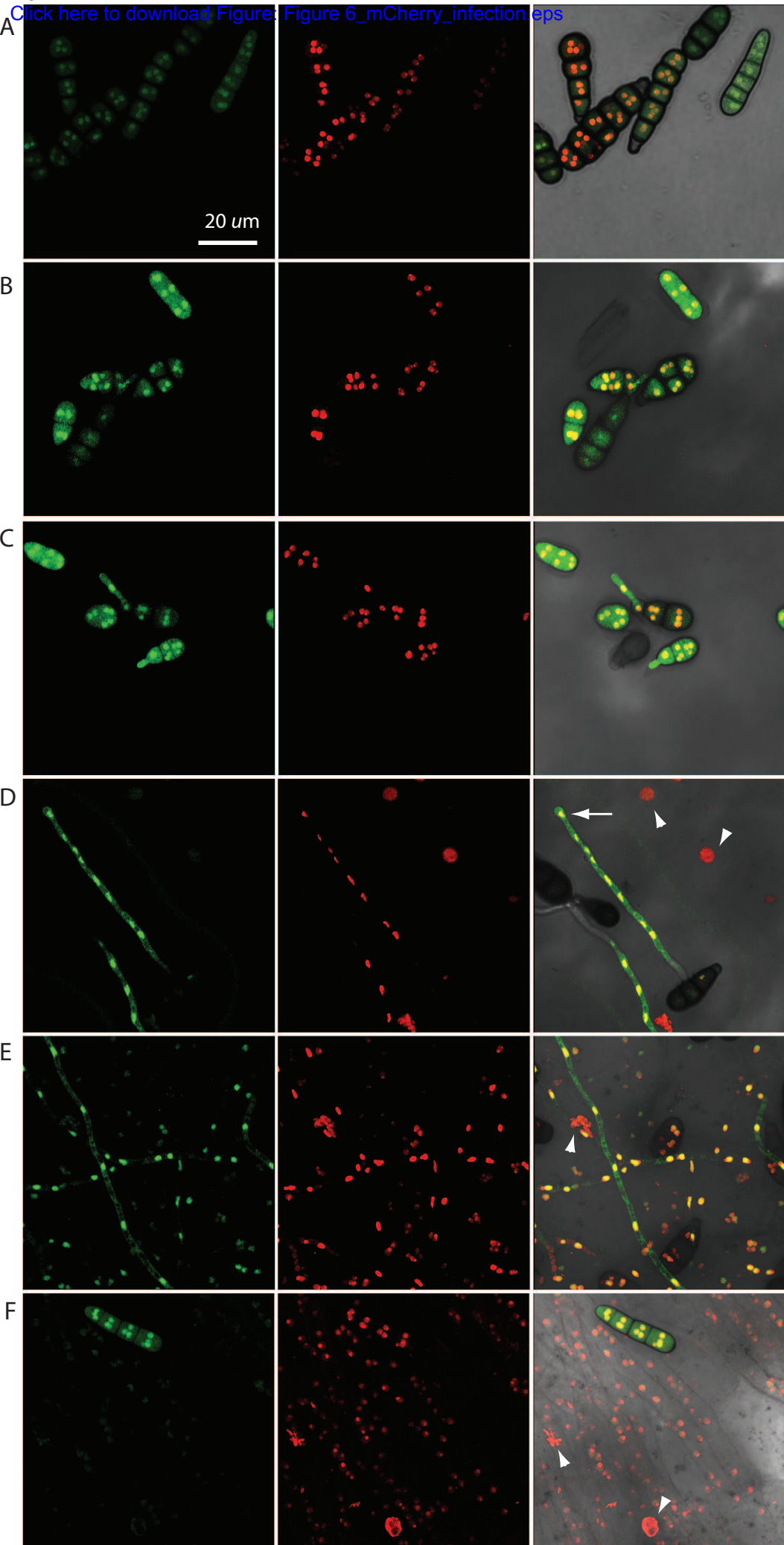


Figure 7  
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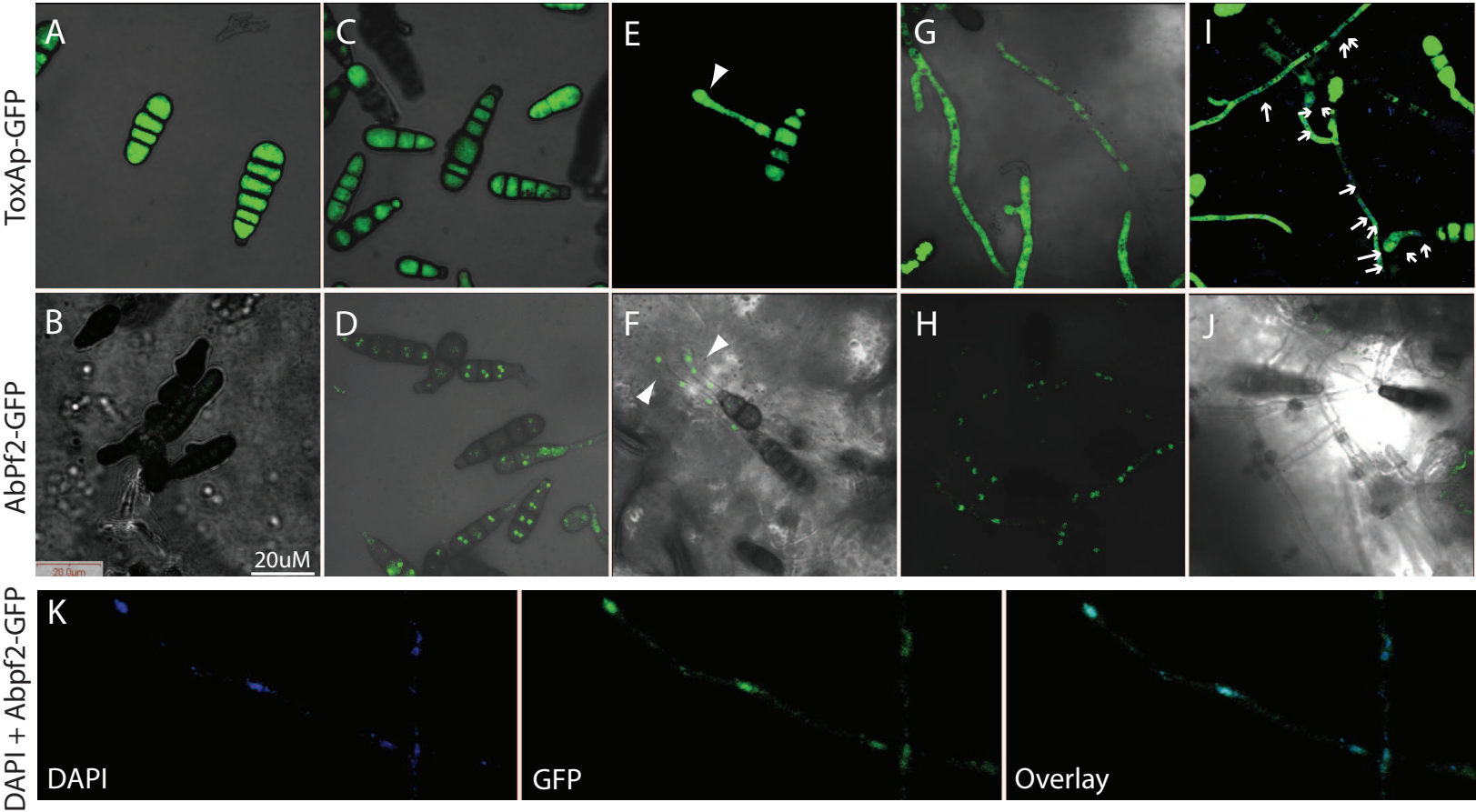
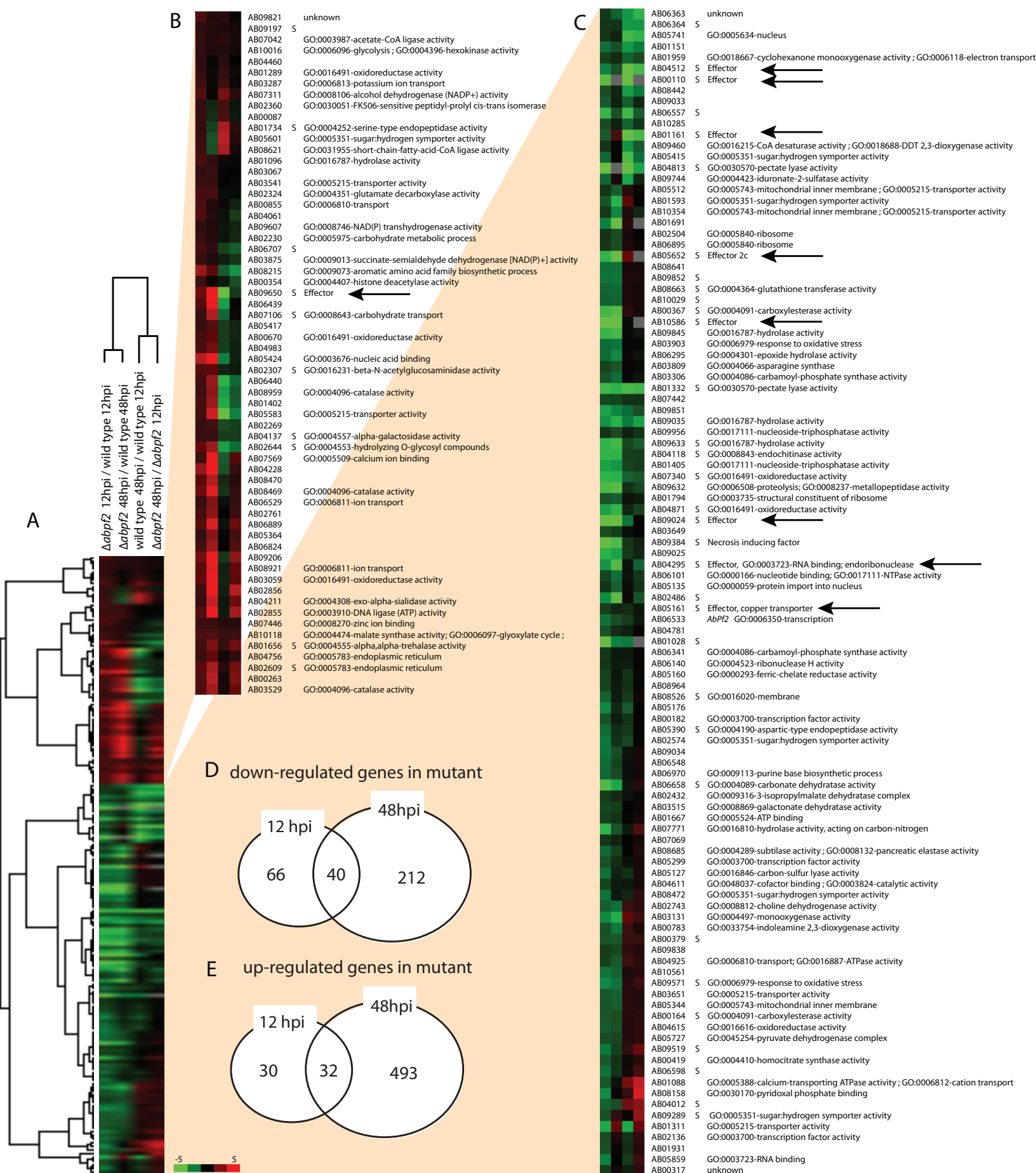


Figure 8  
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**Figure 9**  
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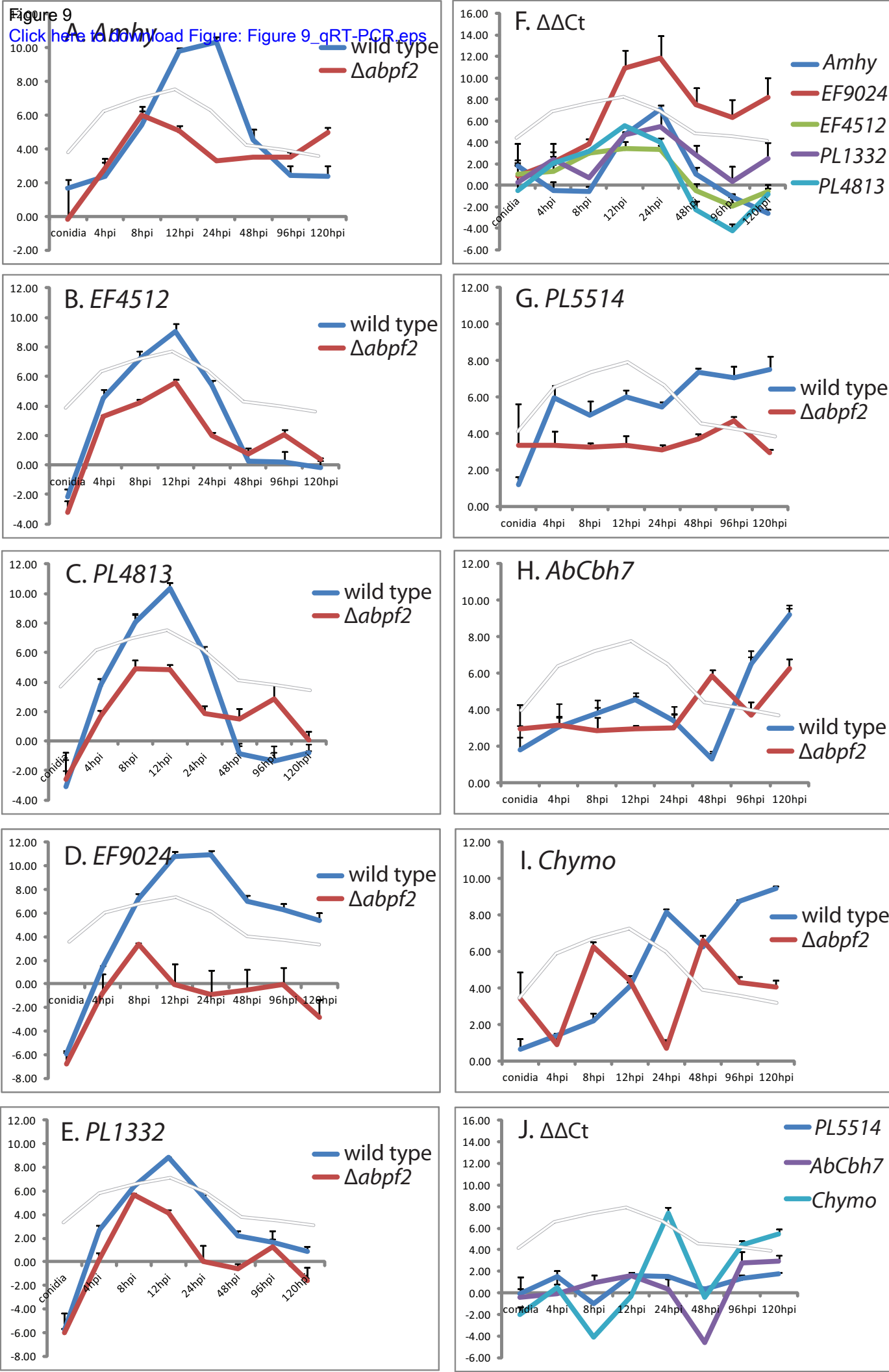


Figure 10  
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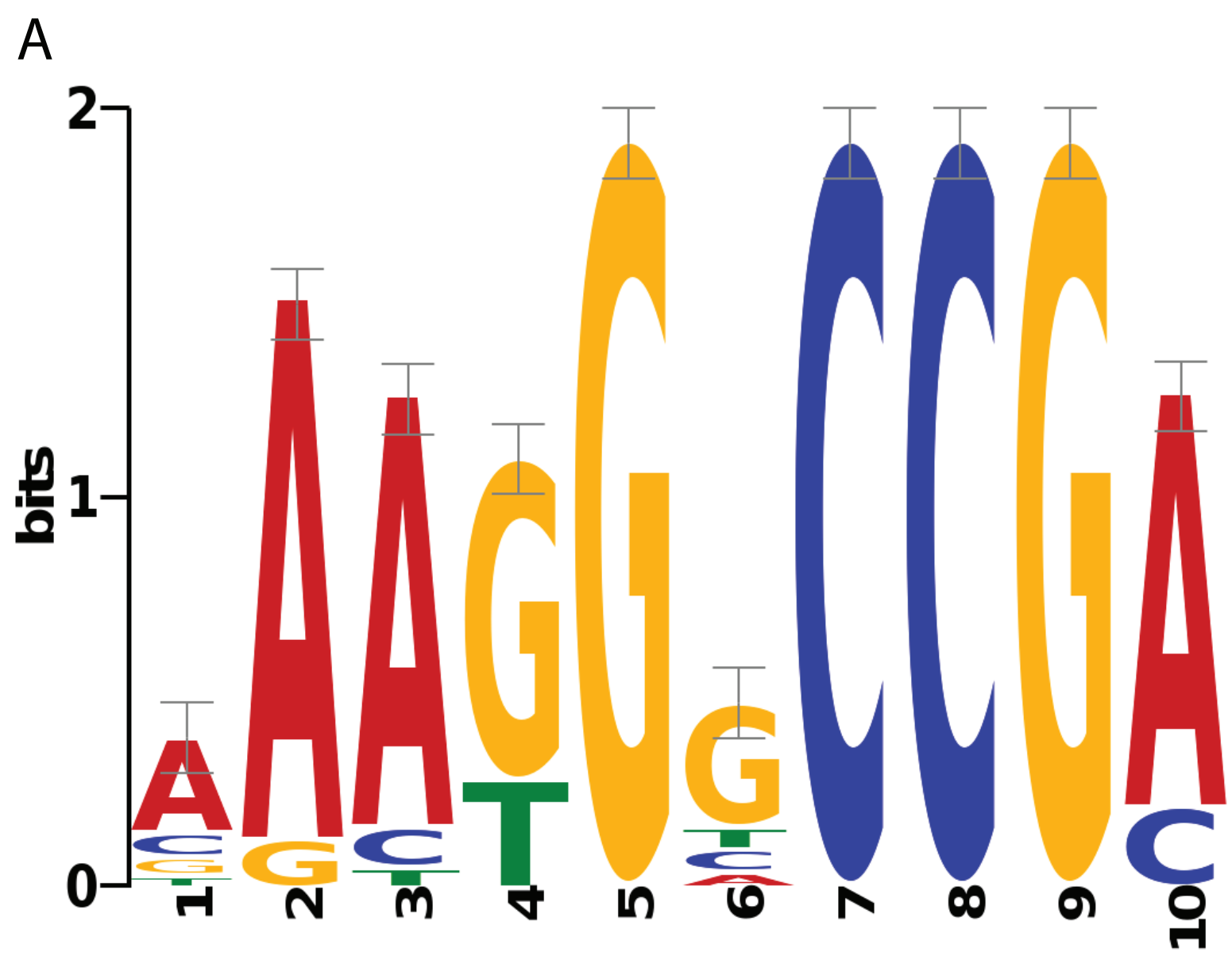


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