The MAP Kinase Kinase Gene *AbSte7* Regulates Multiple Aspects of *Alternaria brassicicola* Pathogenesis

Kai Lu¹, Min Zhang¹, Ran Yang¹, Min Zhang¹, Qinjun Guo¹, Kwang-Hyun Baek^{2*}, and Houjuan Xu^{1*}

¹Department of Plant Protection, Shandong Agricultural University, Tai'an 271018, China

²Department of Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 38541, Korea

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Mitogen-activated protein kinase (MAPK) cascades in fungi are ubiquitously conserved signaling pathways that regulate stress responses, vegetative growth, pathogenicity, and many other developmental processes. Previously, we reported that the *AbSte7* gene, which encodes a mitogen-activated protein kinase kinase (MAPKK) in *Alternaria brassicicola*, plays a central role in pathogenicity against host cabbage plants. In this research, we further characterized the role of *AbSte7* in the pathogenicity of this fungus using $\Delta AbSte7$ mutants. Disruption of the *AbSte7* gene of *A. brassicicola* reduced accumulation of metabolites toxic to the host plant in liquid culture media. The $\Delta AbSte7$ mutants could not efficiently detoxify cruciferous phytoalexin brassinin, possibly due to reduced expression of the brassinin hydrolase gene involved in detoxifying brassinin. Disruption of the *AbSte7* gene also severely impaired fungal detoxification of reactive oxygen species. *AbSte7* gene disruption reduced the enzymatic activity of cell wall-degrading enzymes, including cellulase, β -glucosidase, pectin methylesterase, polymethyl-galacturonase, and polygalacturonic acid transeliminase, during host plant infection. Altogether, the data strongly suggest the MAPKK gene *AbSte7* plays a pivotal role in *A. brassici-*

cola during host infection by regulating multiple steps, and thus increasing pathogenicity and inhibiting host defenses.

Keywords : *AbSte7*, *Alternaria brassicicola*, mitogen-activated protein kinase kinase (MAPKK), pathogenicity

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Mitogen-activated protein kinase (MAPK) cascades are ubiquitous signaling modules in fungi that regulate cellular responses to environmental changes (Pearson et al., 2001). In the budding yeast *Saccharomyces cerevisiae*, five MAPK signal transduction pathways have been identified and extensively studied. These pathways regulate mating, filamentous growth, high osmolarity responses, maintenance of cell wall integrity, and ascospore formation (Gustin et al., 1998; Widmann et al., 1999). Unlike *S. cerevisiae* and other fungi in Saccharomycetales, most filamentous fungi have only three major MAPK cascades, which are homologous to the yeast Fus3/Kss1, Slt2, and Hog1 pathways (Hamel et al., 2012; Román et al., 2007; Zhao et al., 2007).

Among these three MAPK pathways, the yeast Fus3/Kss1 homolog pathway has been the most extensively studied (Zhao et al., 2007). Many Fus3/Kss1 homologs characterized in phytopathogenic fungi control a wide variety of developmental processes but have a remarkably conserved role in regulating fungal pathogenesis (Xu, 2000; Zhao et al., 2007). Despite the crucial roles these factors play in fungal pathogenesis, aspects of infection regulated by Fus3/Kss1 homologs in phytopathogenic fungi are as diverse as the fungi themselves. For example, in *Magnaporthe grisea* (Xu et al., 1996), *Pyrenophora teres* (Ruiz-Roldan et al., 2001), *Bipolaris oryzae* (Moriwaki et al., 2007), *Fusarium graminearum* (Jenczmionka et al., 2003), and *Mycosphae-*

*Co-corresponding authors.

H. Xu

Phone) +86-538-824-1575, FAX) +86-538-824-1324

E-mail) xhjuan@sda.u.edu.cn

K.-H. Baek

Phone) +82-53-810-3029, FAX) +82-53-810-4769

E-mail) khbaek@ynu.ac.kr

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rella graminicola (Cousin et al., 2006), Fus3/Kss1 homologs mutants are completely nonpathogenic on intact host plants, while in *Cochliobolus heterostrophus* (Lev et al., 1999) and *F. graminearum* (Hou et al., 2002), these mutants only have partial inhibition of pathogenicity. Fus3/Kss1 homolog *P. teres* (Ruiz-Roldan et al., 2001) and *M. graminicola* mutants (Cousin et al., 2006) are also apathogenic on wounded host leaves. However, mutants of these homologs in *A. alternate* induce necrotic lesions indistinguishable from those produced by wild-type strains (Lin et al., 2010). These results suggest the Fus3/Kss1 homologs are involved in different infection steps in different fungi.

Alternaria brassicicola (Schwein.) Wiltshire is the causative agent of black spot disease in cruciferous vegetables. Although this disease has significantly reduced vegetable quantity and quality in many countries and become an important factor limiting cultivated *Brassica* species (Chen et al., 2005; Rop et al., 2009), the mechanisms of *A. brassicicola* pathogenesis are still not fully understood (Cho, 2015).

Recently, *AbSte7* (NCBI accession no. MH559277), which is the *A. brassicicola* equivalent to a MAPKK gene in the yeast *Fus3* pathway, was cloned and functionally characterized in our laboratory. *AbSte7* knockout mutants completely lost their ability to infect intact cabbage leaves (Xu et al., 2016), again demonstrating the conserved role of the *Fus3* pathway regulating fungal pathogenesis. In this study, we analyzed the regulation of infection by *AbSte7* and increased our understanding of the mechanisms of pathogenesis of *A. brassicicola*.

Materials and Methods

Strains, growth conditions, and DNA manipulation.

The fungal species *A. brassicicola abta14* was originally isolated from infected cabbage leaves (*Brassica oleracea*) in the providence of Shandong, China and was used as the wild-type (WT) strain in this study. Two $\Delta AbSte7$ mutant (M1 and M2) and the $\Delta AbSte7$ complement (C1) strains were generated in our lab as previously described (Xu et al., 2016). All strains were cultured on potato dextrose agar (PDA) at 25°C for growth and extraction of genomic DNA and RNA. All standard molecular techniques were performed according to the protocols described by Sambrook and Russell (2001).

Preparation of crude broth extract (CBE) and phytotoxicity assay. All strains were incubated in glucose yeast extract agar (1% glucose, 0.5% yeast extract, and 1% agar) media at 25°C for 5 days, then 50 agar plugs 5 mm in di-

ameter were cut from these plates and transferred to 200 ml of 20% V8 juice broth in 500 ml Erlenmeyer flasks. After being cultivated for 14 days at 25°C in the dark, the cultures were filtered using Whatman no. 1 filter paper (Whatman Co., England) to separate the broth from the mycelia. Crude broth extraction and phytotoxicity assays were conducted according to MacKinnon et al. (1999). The filtered broth was extracted with ethyl acetate and dried over NaSO₄ to acquire the CBE. Phytotoxicity assays were conducted with a 5% methanol solution of CBE on detached cabbage leaves.

Brassinin detoxification and *BHAb* gene expression in WT and $\Delta AbSte7$ mutants.

A. brassicicola WT strain *abta14* and $\Delta AbSte7$ mutants were grown on PDA plates at 25°C for 4 days. Brassinin detoxification by the strains was determined on PDA media supplemented with 0.1 and 0.2 mM brassinin. For the control, DMSO (1% v/v final concentration) was added instead of brassinin. Mycelial plugs (5 mm in diameter) from 4-day-old PDA plates of the strains were placed on the center of each plate supplemented with brassinin or DMSO. All the plates were incubated at 25°C and the colony diameters were measured at 6 days post-inoculation (dpi). All experiments were carried out in triplicate at least three times.

Expression of the brassinin hydrolase gene (*BHAb*, NCBI accession no. KC814178.1) was examined in the $\Delta AbSte7$ mutants and WT strain of *A. brassicicola* during plant infection using quantitative RT-PCR (qRT-PCR). The primer pair *BHAb-f* and *-r* (5'-CATCATGCACTGCTCGAACG-3'; 5'-GTGTTGCACGCTTTGGGTAG-3') were designed using the Primer Premier 6.0 program (PREMIER Biosoft International, Palo Alto, CA, USA). The primer pair for the housekeeping gene *Actin* (NCBI accession no. JQ671669) were also prepared as *Actin-f* and *-r* (5'-ATTGAGCACGGTGTCTCGTT-3'; 5'-TGGTGGAGAAGGTGTAACCG-3'). For RNA extraction, *A. brassicicola* WT and $\Delta AbSte7$ mutant strain mycelia were harvested from PDA plates cultured for 5 days at 25°C. Cabbage leaves were inoculated with the WT or mutant strain as described by Xu et al. (2016), then collected at 12, 24, 48, and 72 h after inoculation. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out with the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). The qRT-PCR reactions were performed with SYBR® Premix Ex Taq™ II (Takara, Japan).

H₂O₂ detoxification by *A. brassicicola* $\Delta AbSte7$. H₂O₂ detoxification was determined by measuring the decrease

in absorbance at 240 nm with a UV-vis spectrophotometer (TU-1950, Persee, China) (Chen et al., 2012). Five mycelial plugs (5 mm in diameter) of the *A. brassicicola* WT and $\Delta AbSte7$ mutants strains were picked from the periphery of mycelia on the PDA plates, transferred to 50-ml flasks containing 10 ml water and 30 mM H_2O_2 , and incubated at 25°C. For the control, a PDA agar plug lacking fungal hyphae was used to inoculate the flasks. The amount of H_2O_2 in the solution was monitored spectrophotometrically over time.

Enzymatic assays of cell wall-degrading enzymes. Assays evaluating colony growth in major carbon sources were conducted for $\Delta AbSte7$ mutants according to Cho et al. (2015). Mycelial growth of *A. brassicicola* WT and $\Delta AbSte7$ mutant strains was assessed in minimal medium supplemented with 1% citrus pectin (cat# P9135-100g, Sigma-Aldrich, St. Louis, MO, USA), 1% xylan (cat# X823251, Shanghai Macklin Biochemical Co., China), or 1% cellulose (cat# C804601, Shanghai Macklin Biochemical Co., China).

A series of cell wall-degrading enzymes (CWDEs), including polygalacturonase (PG), cellulase (Cx), β -glucosidase (β -G), polygalacturonic acid *trans*-eliminase (PGTE), pectin methyl-*trans*-eliminase (PMTE), and pectin methylesterase (PME), was detected in hosts infected with the WT and $\Delta AbSte7$ M1 mutant strains. At 12, 24, 48, and 72 hpi, the infected leaves were harvested for crude enzyme extraction and CWDEs were measured according to Yang et al. (2012). All treatments were replicated three times. The data was analyzed using SPSS 22.0 software (SPSS Inc., Chicago, USA). Statistically significant differences between strains were determined by Student's *t*-test ($P < 0.05$).

Results

CBE of $\Delta AbSte7$ has reduced phytotoxicity. $\Delta AbSte7$ mutants could not produce fully developed conidia but the secreted toxins of *A. brassicicola* had not been evaluated for the phytotoxicity, therefore, we examined the phytotoxicity of the CBE by inoculating it to the detached cabbage leaves. As shown in Fig. 1A, 5% methanol control (MA) caused needle lesion only at the site of inoculation, suggesting that the methanol solvent had no effect on the lesion development. Typical chlorotic lesions appeared on leaves inoculated with CBEs from the *A. brassicicola* WT and the complementation strain, which expanded along the vascular system and reached diameters of 10.2 and 9.6 mm at 6 dpi, respectively (Fig. 1B). Inoculation of leaves with

Fig. 1. Phytotoxicity assays of crude broth extracts from different strains. (A) Crude broth extracts from different *Alternaria brassicicola* strains were inoculated on detached cabbage leaves. WT: wild-type of *A. brassicicola*; M1 and M2: $\Delta AbSte7$ disruption mutants; C1: $\Delta AbSte7$ complementation strain; MA: 5% methanol control. (B) Lesion size following infection with different *A. brassicicola* strains was recorded at 5 days post-inoculation. Each column contains the mean $\pm$ standard error of three independent experiments with three replicates. The different letters in the column indicate significant differences as determined by Student's *t*-test ($P < 0.05$).

CBEs from the M1 and M2 strains also resulted in chlorosis at 3 dpi; however, the lesions expanded slowly, with diameters of 2.6 and 2.3 mm at 6 dpi, respectively (Fig. 1B).

$\Delta AbSte7$ has a reduced ability to detoxify brassinin.

Brassinin is an important phytoalexin in *Brassica* species and plays a significant role in the defense. To determine the deficient steps in the pathogenicity of $\Delta AbSte7$, we

Table 1. Effects of brassinin on the colony growth of *ΔAbSte7* mutants and wild-type strains of *Alternaria brassicicola* on potato dextrose agar plates

Strains	PDA	PDA+DMSO (0.05%)	PDA+Brassinin (0.1 mM)		PDA+Brassinin (0.2 mM)	
			Colony diameter (mm)	Growth inhibition rates (%)	Colony diameter (mm)	Growth inhibition rates (%)
WT	88 ± 3.2	86 ± 1.4	86 ± 4.3	-	84 ± 3.8	-
M1	68 ± 1.5	63 ± 3.1	52 ± 1.8	23%	33 ± 1.2	51%
M2	64 ± 2.7	66 ± 1.9	49 ± 2.5	22%	30 ± 2.2	53%
C1	85 ± 3.8	90 ± 3.5	86 ± 2.2	-	85 ± 2.4	-

WT: wild-type, M1 and M2: two *ΔAbSte7* mutants, C1: *ΔAbSte7* Complemented mutant.
Numbers represent mean ± standard error of three replicates.

examined the ability of *ΔAbSte7* to detoxify brassinin by comparing the colony growth rate on PDA and PDA supplemented with DMSO or brassinin. As shown in Table 1, DMSO used as the solvent for brassinin, had no effect on mycelial growth of *A. brassicicola* WT and *ΔAbSte7* mutant strains as their colony diameters were similar on PDA with or without DMSO.

However, the abilities of the *A. brassicicola* WT and *ΔAbSte7* mutant strains to detoxify brassinin were significantly different. The WT strain grew normally on PDA containing 0.1 or 0.2 mM brassinin with the colony diameter indistinguishable from that of WT strain grown on plain PDA. By contrast, the growth rates of the *ΔAbSte7* M1 and M2 mutants were remarkably reduced in the presence of

brassinin. When grown on 0.1 and 0.2 mM brassinin, the reductions in the colony diameters of M1 were 23 and 51% and M2 were 22 and 53%, respectively (Table 1).

We also examined *BHAb* gene expression level in mutants M1 and M2 during host-pathogen interactions. As shown in Fig. 2, *BHAb* was expressed during all stages of infection examined, but the expression levels in the *ΔAbSte7* mutant and WT strains were quite different. Expression in the *ΔAbSte7* M1 and M2 mutants was highest at 12 h post-inoculation (hpi), but was only approximately 0.4-fold that of WT. As the infection increased the expression level in the *ΔAbSte7* mutants decreased dramatically, where expression was only 0.012- and 0.016-folds, respectively, that of WT at 72 hpi (Fig. 2).

Fig. 2. Relative expression levels of the *BHAb* gene in *AbSte7* disruption mutants M1 and M2 at different time points during infection. The expression levels of *BHAb* in WT, M1, and M2 were compared to the expression levels of the gene encoding actin for normalization. Relative expression levels were calculated by dividing the expression levels of M1 or M2 by those of WT. The expression levels were calculated using the comparative $\Delta\Delta C_t$ method. The data are displayed as mean ± standard error for three independent experiments.

Fig. 3. Spectrophotometric analysis of H_2O_2 detoxification by *A. brassicicola* WT and *ΔAbSte7* mutants. The absorbance at 240 nm was measured for a 30 mM H_2O_2 solution following the addition of mycelia plugs of *A. brassicicola* WT or *ΔAbSte7* mutants M1 and M2 or agar plugs lacking mycelia (control) and incubating for 75 min. The *ΔAbSte7* mutants had a severely impaired ability to detoxify H_2O_2 . The data are presented as mean ± standard error for three independent experiments.

Disruption of the MAPKK *AbSte7* severely impairs detoxification of H_2O_2 . H_2O_2 detoxification of different strains over time was evaluated spectrophotometrically at an absorbance of 240 nm, the *AbSte7* disruption mutants M1 and M2 were found to be unable to detoxify H_2O_2 effectively. WT degraded 58.82% of the H_2O_2 after an incubation of 75 minutes, while M1 and M2 degraded 32.92 and 28.82% of the H_2O_2 , respectively (Fig. 3), indicating that ROS detoxification was severely impaired in the

$\Delta AbSte7$ mutants.

***AbSte7* is involved in regulation of CWDE activity.** The growth rates of the $\Delta AbSte7$ mutant and WT strains were measured in minimal medium supplemented with diverse carbon sources to explore carbohydrate utilization and hydrolytic enzyme production. As shown in Fig. 4A and B, the addition of one of three carbohydrates, especially cellulose, to minimal media reduced the growth rates of the WT strain. The $\Delta AbSte7$ mutants M1 and M2 grew more quickly on the minimum media (MM) supplemented with all three carbohydrates. Especially the colony diameters of M1 and M2 on MM plus with cellulose were 41.8 and 40.7 mm, respectively, while those of WT were only 4.3 mm (Fig. 4B). Furthermore, the aerial hyphae were more flourishing in minimal medium when supplemented with xylan and pectin. The growth rates of the complemented strain C1 in MM plates supplemented with all three carbohydrates were similar with that of WT strain. These results suggested the activities of several hydrolytic enzymes were negatively regulated by *AbSte7* during saprophytic growth under nutrient-poor conditions.

We then measured CWDE activity in the WT and $\Delta AbSte7$ mutants during host plant infection. As shown in Figure 5A, Cx activity increased from 12 to 24 hpi and then decreased in the WT strain. Cx activity in the M1 and M2 mutants declined progressively, but slightly, from 12 to 72 hpi and was significantly lower than that of WT during the entirety of the assay period except at 12 hpi (Fig. 5A). The $\Delta AbSte7$ mutants had lower β -G activity than WT for the entire assay period (Fig. 5B). The activity of β -G increased during early infection stages in WT by 48 hpi and in M1 by 24 hpi then declined rapidly.

PG activity in the WT and two $\Delta AbSte7$ mutants gradually increased but did not significantly differ between the strains (Fig. 5C). PME activity in the WT and $\Delta AbSte7$ mutants increased over 72 hpi with significantly higher levels of activity in WT than the mutants (Fig. 5D). The PGTE activity of the WT strain increased dramatically from 12 to 24 hpi and the growth rate was steady, but low, from 24 to 72 hpi. For the $\Delta AbSte7$ mutants M1 and M2, PGTE activity peaked at 48 hpi, then declined slightly to 72 hpi (Fig. 5E). PMTE activity differed between the WT and $\Delta AbSte7$ mutants. It increased constantly from 12 to 72 hpi in WT. However, in the $\Delta AbSte7$ mutants, PMTE activity increased from 12 to 24 hpi, but then remained approximately the same from 24 to 72 hpi (Fig. 5F). Both the PGTE and PMTE activities were higher in WT than the $\Delta AbSte7$ mutants, at later infection stages with no differences observed at 12 hpi.

Fig. 4. (A) Carbohydrate utilization by *A. brassicicola* WT and $\Delta AbSte7$ mutant strains. The $\Delta AbSte7$ mutant M1 and M2 and WT strains were grown on minimal media supplemented with the carbon sources xylan, cellulose, and pectin at 25°C for 7 d. (B) The carbohydrate utilization and hydrolytic enzyme production in $\Delta AbSte7$ and WT strains were determined by the growth rates in minimal medium supplemented with diverse carbon sources. The growth rates (D) were calculated as: $D = D1 - D2$. D1 and D2 were the colony diameter measured at 7 days after incubation and the diameter of the agar block for inoculation (8 mm), respectively. Each column represents the mean $\pm$ SE of the three independent experiments with three replicates.

Fig. 5. Enzymatic activity of cellulase (Cx, A), β-glucosidase (B), polygalacturonase (PG, C), pectin methylesterase (PME, D), polygalacturonic acid trans-eliminase (PGTE, E) and pectin methyl-trans-eliminase (PMTE, F) in infected cabbage leaves after inoculation with the *A. brassicicola* WT or $\Delta AbSte7$ M1 mutant strains. Bars represent standard errors of the mean. Means with an asterisk (*) indicate a significant difference at $P \leq 0.05$.

Discussion

The phytopathogenic *Alternaria* species usually produce host- and/or nonhost-specific toxins to damage plant tissues and facilitate colonization (Nishimura and Kohmoto, 1983; Thomma, 2003). However, the roles of *A. brassicicola* toxin (AB-toxin) on pathogenesis have not been completely characterized. AB-toxin, a host-specific protein toxin of *A. brassicicola*, is produced by germinating spores but not on culture media (Otani et al., 1998). AB-toxin is induced by recognition of host-derived oligosaccharides, but the encoding gene remains unknown and its specific function in pathogenesis remains unclear (Oka et al., 2005). Isolation of phytotoxic metabolites showed brassicicolin A is the

most selective phytotoxic metabolite produced in the liquid cultures of *A. brassicicola* (Pedras et al., 2009; Pedras and Park, 2015). Comparison of phytotoxicity of CBEs from $\Delta AbSte7$ mutant and WT strains indicates that disruption of the *AbSte7* gene reduced phytotoxic metabolite accumulation in culture media (Fig. 1). Because AB-toxin could not be produced in culture media, it was not responsible for the reduced phytotoxicity of the CBEs in the $\Delta AbSte7$ culture media. Instead, these results suggest that disruption of *Abste7* reduces levels of toxic metabolites, such as the phytotoxic metabolite brassicicolin A, in culture media. This reduction may be responsible, at least in part, for the reduced virulence of the M1 and M2 mutants.

Brassinin is an important phytoalexin produced by crucifers in response to the fungal attack and other forms of

stress (Pedras et al., 2010, 2012). Its antifungal activity is partly a result of its dithiocarbamate group, which has extensively been used in pesticides and fungicides (Pedras et al., 2012; Szolar, 2007). To overcome the phytoalexin, *A. brassicicola* produces brassinin hydrolase, which detoxifies brassinin into the intermediate metabolites N'-indolylmethanamine and N'-acetyl-3-indolylmethanamine (Pedras et al., 2011). This detoxification by *A. brassicicola* is highly detrimental to the host plant because brassinin is not only a crucifer phytoalexin, but also a biosynthetic precursor for several plant defense metabolites. Therefore, detoxification of brassinin by *A. brassicicola* is important for its pathogenicity. In this study, disruption of *AbSte7* reduced brassinin detoxification by *A. brassicicola*. On PDA containing 0.1 or 0.2 M brassinin, the WT strain grew normally, but *AbSte7* disruption mutants M1 and M2 significantly reduced the growth. BHAb, an inducible enzyme produced by *A. brassicicola*, catalyzes the detoxification of brassinin converting to 3-indolylmethanamine (Pedras and Minic, 2012). The expression levels of the *BHAb* gene in the two $\Delta AbSte7$ mutants were severely down-regulated compared with those in WT strain during host-pathogen interaction. These results indicate that Ab-Fus3-MAPK pathway, together with AbSlr2- and AbHog1-MAPK pathways (Joubert et al., 2011), was also involved in phytoalexin detoxification in *A. brassicicola*. The reduced phytoalexin detoxification capacity may be responsible for the reduced virulence of the $\Delta AbSte7$ mutants.

ROS play a major role in pathogen-plant interactions. Microbial infection results in the rapid and transient production of large amounts of ROS in hosts (Apel and Hirt, 2004). Therefore, the pathogen must be able to overcome oxidative stress in order to survive and successfully colonize the host. In the past few years, ROS detoxification has been demonstrated to be vital for the pathogenicity of many fungi (Lin et al., 2009, 2010, 2011; Molina and Kahmann, 2007; Yang and Chung, 2012). In *A. alternata*, signaling pathways involved in the response to oxidative stress are absolutely required for pathogenicity (Lin et al., 2009, 2010, 2011; Yang and Chung, 2012). There has been limited information regarding the role of ROS detoxification in the pathogenicity of *A. brassicicola*. When H₂O₂ detoxification by the $\Delta AbSte7$ and WT strains was analyzed, the $\Delta AbSte7$ M1 and M2 mutants degraded only 32.92 and 28.82%, respectively, of H₂O₂ after a 75-min incubation compared to 58.82% by WT. The reduced ROS detoxification may be responsible for the defects in pathogenicity displayed by the $\Delta AbSte7$ mutants.

CWDEs play important roles in the pathogenesis of necrotrophic fungi (Van Kan, 2006). Generally, these

necrotrophic fungi kill host cells directly or by inducing programmed cell death with toxins, and then decompose these cells using CWDEs to facilitate colonization and the release of carbohydrates for consumption (Choquer et al., 2007). The importance of CWDE genes in virulence has been demonstrated in many fungal species, including *Botrytis cinerea*, *Fusarium oxysporum*, and *Cochliobolus carbonum*. In *B. cinerea*, inactivation of *xyn11A*, a β -1, 4-xylanase gene, reduced lesion outgrowth by more than 70% (Brito et al., 2006). However, the role of CWDEs in the pathogenesis of *A. brassicicola* has not yet been determined. Due to functional redundancy, deletion of an individual gene encoding hydrolase results in little or no change in the pathogenicity of *A. brassicicola* (Cho, 2015). Therefore, we first analyzed carbohydrate utilization and then hydrolytic enzyme activity in the $\Delta AbSte7$ mutant. As shown in Fig. 4, *AbSte7* negatively regulated the activity of some hydrolytic enzymes during saprophytic growth under nutrient-poor conditions. When the activity of the CWDEs was measured in host tissues infected by the WT and $\Delta AbSte7$ mutant strains, the $\Delta AbSte7$ mutant was found to have less Cx, β -G, PME, PGTE, and PMTE activity than the WT during most infection stages, except PG, which had an activity similar to or higher than that of the WT (Fig. 5). Necrotrophic fungi usually produce toxins and CWDEs to kill host cells to acquire nutrients from dead tissues (Divon and Fluhr 2007). Therefore, the reduced CWDE activity may be responsible for the pathogenicity defect in $\Delta AbSte7$. The relationship between the reduced CWDE activity and pathogenicity defect in $\Delta AbSte7$ needs to be further analyzed in future work.

In summary, disruption of the *AbSte7* gene of *A. brassicicola* reduced the accumulation of toxic metabolites in culture media, detoxification of brassinin and H₂O₂, and the enzymatic activity of some CWDEs during host plant infection. Altogether, these data strongly suggest the MAPKK gene *AbSte7* plays a pivotal role in host infection by *A. brassicicola*.

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References

- Apel, K. and Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev.*

- Plant Biol.* 55:373-399.
- Brito, N., Espino, J. J. and González, C. 2006. The endo-beta-1,4-xylanase xyn11A is required for virulence in *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 19:25-32.
- Chen, L. H., Lin, C. H. and Chung, K. R. 2012. Roles for SKN7 response regulator in stress resistance, conidiation and virulence in the citrus pathogen *Alternaria alternata*. *Fungal Genet. Biol.* 49:802-813.
- Chen, L. Y., Price, T. V. and Silvapulle, M. J. 2005. Dark leaf spot (*alternaria brassicicola*) on Chinese cabbage: spatial patterns. *Aust. J. Agric. Res.* 56:699-714.
- Cho, Y. 2015. How the necrotrophic fungus *Alternaria brassicicola* kills plant cells remains an enigma. *Eukaryot. Cell* 14:335-344.
- Cho, Y., Jang, M., Srivastava, A., Jang, J.-H., Soung, N.-K., Ko, S.-K., Kang, D.-O., Ahn, J. S. and Kim, B. Y. 2015. A pectate lyase-coding gene abundantly expressed during early stages of infection is required for full virulence in *Alternaria brassicicola*. *PLoS One* 10:e0127140.
- Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J., Simon, A. and Viaud, M. 2007. Botrytis cinerea virulence factors: new insights into a necrotrophic and polyphageous pathogen. *FEMS Microbiol. Lett.* 277:1-10.
- Chung, K. R. 2012. Stress Response and Pathogenicity of the Necrotrophic Fungal Pathogen *Alternaria alternata*. *Scientifica* 2012:635431.
- Cousin, A., Mehrabi, R., Guilleroux, M., Dufresne, M., Van Der Lee, T., Waalwijk, C., Langin, T. and Kema, G. H. 2006. The MAP kinase-encoding gene MgFus3 of the non-appressorium phytopathogen *Mycosphaerella graminicola* is required for penetration and *in vitro* pycnidia formation. *Mol. Plant Pathol.* 7:269-278.
- Divon, H. H. and Fluhr, R. 2007. Nutrition acquisition strategies during fungal infection of plants. *FEMS Microbiol. Lett.* 266:65-74.
- Gustin, M. C., Albertyn, J., Alexander, M. and Davenport, K. 1998. MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 62:1264-1300.
- Hamel, L. P., Nicole, M. C., Duplessis, S. and Ellis, B. E. 2012. Mitogen-activated protein kinase signaling in plant-interacting fungi: distinct messages from conserved messengers. *Plant Cell* 24:1327-1351.
- Hou, Z., Xue, C., Peng, Y., Katan, T., Kistler, H. C. and Xu, J. R. 2002. A mitogen-activated protein kinase gene (MGV1) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Mol Plant-Microbe Interact.* 15:1119-1127.
- Jenczmionka, N. J., Maier, F. J., Lösch, A. P. and Schäfer, W. C. 2003. Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase gpmk1. *Curr. Genet.* 43:87-95.
- Joubert, A., Bataille-Simoneau, N., Campion, C., Guillemette, T., Hudhomme, P., Iacomini-Vasilescu, B., Leroy, T., Pochon, S., Poupard, P. and Simoneau, P. 2011. Cell wall integrity and high osmolarity glycerol pathways are required for adaptation of *Alternaria brassicicola* to cell wall stress caused by brassicaceous indolic phytoalexins. *Cell Microbiol.* 13:62-80.
- Lev, S., Sharon, A., Hadar, R., Ma, H. and Horwitz, B. A. 1999. A mitogen-activated protein kinase of the corn leaf pathogen *Cochliobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: diverse roles for mitogen-activated protein kinase homologs in foliar pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 96:13542-13547.
- Lin, C. H., Yang, S. L. and Chung, K. R. 2009. The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* in citrus. *Mol. Plant-Microbe Interact.* 22:942-952.
- Lin, C. H., Yang, S. L., Wang, N. Y. and Chung, K. R. 2010. The FUS3 MAPK signaling pathway of the citrus pathogen *Alternaria alternata* functions independently or cooperatively with the fungal redox-responsive AP1 regulator for diverse developmental, physiological and pathogenic processes. *Fungal Genet. Biol.* 47:381-391.
- Lin, C. H., Yang, S. L. and Chung, K. R. 2011. Cellular responses required for oxidative stress tolerance, colonization, and lesion formation by the necrotrophic fungus *Alternaria alternata* in citrus. *Curr. Microbiol.* 62:807-815.
- MacKinnon, S. L., Keifer, P. and Ayer, W. A. 1999. Components from the phytotoxic extract of *Alternaria brassicicola*, a black spot pathogen of canola. *Phytochemistry* 51:215-221.
- Molina, L. and Kahmann, R. 2007. An *Ustilago maydis* gene involved in H₂O₂ detoxification is required for virulence. *Plant Cell* 19:2293-2309.
- Moriwaki, A., Kihara, J., Mori, C. and Arase, S. 2007. A MAP kinase gene, BMK1, is required for conidiation and pathogenicity in the rice leaf spot pathogen *Bipolaris oryzae*. *Microbiol. Res.* 162:108-114.
- Nishimura, S. and Kohmoto, K. 1983. Host-specific toxins and chemical structures from *alternaria* species. *Annu. Rev. Phytopathol.* 21: 87-116.
- Oka, K., Akamatsu, H., Kodama, M., Nakajima, H., Kawada, T. and Otani, H. 2005. Host-specific AB-toxin production by germinating spores of *Alternaria brassicicola* is induced by a host-derived oligosaccharide. *Physiol. Mol. Plant Pathol.* 66:12-19.
- Otani, H., Kohnobe, A., Kodama, M. and Kohmoto, K. 1998. Involvement of host factors in the production of a protein host-specific toxin by *Alternaria brassicicola*. In: *Molecular Genetics of Host-Specific Toxins in Plant Disease*, eds. by K. Kohmoto and O. C. Yoder, pp. 63-69. Proceedings of the 3rd Tottori International Symposium on Host-Specific Toxins, Daisen, Tottori, Japan.
- Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K. and Cobb, M. H. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* 22:153-183.
- Pedras, M. S., Chumala, P. B., Jin, W., Islam, M. S. and Hauck,

- D. W. 2009. The phytopathogenic fungus *Alternaria brassicicola*: phytotoxin production and phytoalexin elicitation. *Phytochemistry* 70:394-402.
- Pedras, M. S. C. and Yaya, E. E. 2010. Phytoalexins from *Brassicaceae*: news from the front. *Phytochemistry* 71:1191-1197.
- Pedras, M. S. C., Yaya, E. E. and Glawischnig, E. 2011. The phytoalexins from cultivated and wild crucifers: chemistry and biology. *Nat. Prod. Rep.* 28:1381-1405.
- Pedras, M. S. C. and Minic, Z. 2012. Differential protein expression in response to the phytoalexin brassinin allows the identification of molecular targets in the phytopathogenic fungus *Alternaria brassicicola*. *Mol. Plant Pathol.* 13:483-493.
- Pedras, M. S. C., Minic, Z. and Hossain, S. 2012. Discovery of inhibitors and substrates of brassinin hydrolase: Probing selectivity with dithiocarbamate bioisosteres. *Bioorg. Med. Chem.* 20:225-233.
- Pedras, M. S. C. and Park, M. R. 2015. Metabolite diversity in the plant pathogen *Alternaria brassicicola*: factors affecting production of brassicicolin A, depudecin, phomapyrone A and other metabolites. *Mycologia* 107:1138-1150.
- Román, E., Arana, D. M., Nombela, C., Alonso-Monge, R. and Pla, J. 2007. MAP kinase pathways as regulators of fungal virulence. *Trends Microbiol.* 15:181-190.
- Rop, N. K., Kiprof, E. K. and Ochuodho, J. O. 2009. *Alternaria* species causing black spot disease of *Brassicas* in Kenya. *Afri. Crop Sci. Conf. Proc.* 9:635-640.
- Ruiz-Roldán, M. C., Maier, F. J. and Schäfer, W. 2001. PTK1, a mitogen-activated-protein kinase gene, is required for conidiation, appressorium formation, and pathogenicity of *Pyrenophora teres* on barley. *Mol. Plant-Microbe Interact.* 14:116-125.
- Sambrook, J. and Russel, D. 2001. Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, New York, USA. 2344 pp.
- Szolar, O. H. 2007. Environmental and pharmaceutical analysis of dithiocarbamates. *Anal. Chim. Acta* 582:191-200.
- Thomma, B. P. 2003. *Alternaria* spp.: from general saprophyte to specific parasite. *Mol. Plant Pathol.* 4:225-236.
- Van Kan, J. A. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* 11:247-253.
- Widmann, C., Gibson, S., Jarpe, M. B. and Johnson, G. L. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* 79:143-180.
- Xu, H., Zhang, Q., Cui, W., Zhang, X., Liu, W., Zhang, L., Islam, M. N., Baek, K.-H. and Wang, Y. 2016. *AbSte7*, a MAPKK gene of *Alternaria brassicicola*, is involved in conidiation, salt/oxidative stress, and pathogenicity. *J. Microbiol. Biotechnol.* 26:1311-1319.
- Xu, J. R. 2000. Map kinases in fungal pathogens. *Fungal Genet. Biol.* 31:137-152.
- Xu, J. R. and Hamer, J. E. 1996. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* 10:2696-2706.
- Yang, S. L. and Chung, K. R. 2012. The NADPH oxidase-mediated production of hydrogen peroxide (H₂O₂) and resistance to oxidative stress in the necrotrophic pathogen *Alternaria alternata* of citrus. *Mol. Plant Pathol.* 13:900-914.
- Yang, Z., Bi, Y., Li, Y., Kou, Z., Bao, G., Liu, C., Wang, Y. and Wang, D. 2012. Changes of cell wall degrading enzymes in potato tuber tissue slices infected by *Fusarium sulphureum*. *Sci. Agric. Sinica*. 45:127-134 (in Chinese).
- Zhao, X., Mehrabi, R. and Xu, J. R. 2007. Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryot. Cell* 6:1701-1714.