The MAP Kinase Kinase Gene \textit{AbSte7} Regulates Multiple Aspects of \textit{Alternaria brassicicola} Pathogenesis

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(Received on July 5, 2018; Revised on November 15, 2018; Accepted on December 6, 2018)

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 \textit{AbSte7} gene, which encodes a mitogen-activated protein kinase kinase (MAPKK) in \textit{Alternaria brassicicola}, plays a central role in pathogenicity against host cabbage plants. In this research, we further characterized the role of \textit{AbSte7} in the pathogenicity of this fungus using \textit{ΔAbSte7} mutants.

Disruption of the \textit{AbSte7} gene of \textit{A. brassicicola} reduced accumulation of metabolites toxic to the host plant in liquid culture media. The \textit{ΔAbSte7} 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 \textit{AbSte7} gene also severely impaired fungal detoxification of reactive oxygen species. \textit{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 \textit{AbSte7} plays a pivotal role in \textit{A. brassicicola} during host infection by regulating multiple steps, and thus increasing pathogenicity and inhibiting host defenses.

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

**Handling Editor**: Sohn, Kee Hoon

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 \textit{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 \textit{S. cerevisiae} and other fungi in \textit{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 \textit{Magnaporthe grisea} (Xu et al., 1996), \textit{Pyrenophora teres} (Ruiz-Roldan et al., 2001), \textit{Bipolaris oryzae} (Moriwaki et al., 2007), \textit{Fusarium graminearum} (Jenczmionka et al., 2003), and \textit{Mycosphae-
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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 ΔAbSte7 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$_2$O$_2$, 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$_2$O$_2$ 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 ΔAbSte7 mutants according to Cho et al. (2015). Mycelial growth of *A. brassicicola* WT and ΔAbSte7 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 ΔAbSte7 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 ΔAbSte7 has reduced phytotoxicity.** ΔAbSte7 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 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).

**ΔAbSte7 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 ΔAbSte7, we
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 mutants decreased dramatically, where expression was only 0.012- and 0.016-folds, respectively, that of WT at 72 hpi (Fig. 2).

**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 | - |
| M1      | 68 ± 1.5 | 63 ± 3.1 | 52 ± 1.8 | 23% |
| M2      | 64 ± 2.7 | 66 ± 1.9 | 49 ± 2.5 | 22% |
| C1      | 85 ± 3.8 | 90 ± 3.5 | 86 ± 2.2 | - |

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

Fig. 2. Relative expression levels of the BHAb gene in ΔAbSte7 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 ΔΔCt method. The data are displayed as mean ± standard error for three independent experiments.

Fig. 3. Spectrophotometric analysis of H$_2$O$_2$ detoxification by A. brassicicola WT and ΔAbSte7 mutants. The absorbance at 240 nm was measured for a 30 mM H$_2$O$_2$ 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$_2$O$_2$. The data are presented as mean ± standard error for three independent experiments.
Disruption of the MAPKK *AbSte7* severely impairs detoxification of H$_2$O$_2$. H$_2$O$_2$ 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$_2$O$_2$ effectively. WT degraded 58.82% of the H$_2$O$_2$ after an incubation of 75 minutes, while M1 and M2 degraded 32.92 and 28.82% of the H$_2$O$_2$, respectively (Fig. 3), indicating that ROS detoxification was severely impaired in the *ΔAbSte7* mutants.

*AbSte7* is involved in regulation of CWDE activity. The growth rates of the *ΔAbSte7* 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 *ΔAbSte7* 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 *ΔAbSte7* 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 *ΔAbSte7* 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 *ΔAbSte7* mutants gradually increased but did not significantly differ between the strains (Fig. 5C). PME activity in the WT and *ΔAbSte7* 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 *ΔAbSte7* 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 *ΔAbSte7* mutants. It increased constantly from 12 to 72 hpi in WT. However, in the *ΔAbSte7* 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 *ΔAbSte7* mutants, at later infection stages with no differences observed at 12 hpi.
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 ΔAbSte7 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 ΔAbSte7 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

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**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 ΔAbSte7 M1 mutant strains. Bars represent standard errors of the mean. Means with an asterisk (*) indicate a significant difference at $P \leq 0.05$. 

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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 BHA*Ab* gene in the two Δ*AbSte7* mutants were severely down-regulated compared with those in WT strain during host-pathogen interaction. These results indicate that AbFus3-MAPK pathway, together with AbSlt2- 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 Δ*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 H$_2$O$_2$ detoxification by the Δ*AbSte7* and WT strains was analyzed, the Δ*AbSte7* M1 and M2 mutants degraded only 32.92 and 28.82%, respectively, of H$_2$O$_2$ 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 Δ*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 *xyn*11A, 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 Δ*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 Δ*AbSte7* mutant strains, the Δ*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 Δ*AbSte7*. The relationship between the reduced CWDE activity and pathogenicity defect in Δ*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 H$_2$O$_2$, 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*.

**Acknowledgments**

This work was supported by the Foundation of Shandong Province Modern Agricultural Technology System Innovation Team (No. SDAIT-25-07) and the Natural Science Foundation of China (Grant No. 31300130).

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