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Mutation of *AREA* affects growth, sporulation, nitrogen regulation, and pathogenicity in *Colletotrichum gloeosporioides*

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ABSTRACT

The GATA transcription factor AreA is a global nitrogen regulator that restricts the utilization of complex and poor nitrogen sources in the presence of good nitrogen sources in microorganisms. In this study, we report the biological function of an AreA homolog (the *CgareA* gene) in the fruit postharvest pathogen *Colletotrichum gloeosporioides*. Targeted gene deletion mutants of *areA* exhibited significant reductions in vegetative growth, increases in conidia production, and slight decreases in conidial germination rates. Quantitative RT-PCR (qRT-PCR) analysis revealed that the expression of AreA was highly induced under nitrogen-limiting conditions. Moreover, compared to wild-type and complemented strains, nitrogen metabolism-related genes were misregulated in Δ*areA* mutant strains. Pathogenicity assays indicated that the virulence of Δ*areA* mutant strains were affected by the nitrogen content, but not the carbon content, of fruit hosts. Taken together, our results indicate that *CgareA* plays a critical role in fungal development, conidia production, regulation of nitrogen metabolism and virulence in *Colletotrichum gloeosporioides*.

1. Introduction

Colletotrichum gloeosporioides is the causative agent of anthracnose, one of the important postharvest fruit diseases (Cannon et al., 2000; Hyde et al., 2009). Over 470 different host genera have been associated with this infection including various fruits (Hyde et al., 2009). Approximately 25% of the total production of fruits and vegetables is lost because of postharvest diseases in industrialized countries (Nunes, 2012). Due to its importance for fruit and vegetable production, great progress has been made in studying the fungal development, virulence and physical and biological control of this disease. However, efficient and safe strategies for disease control are still lacking. Studies of the infection mechanisms of fungal pathogens have been significant for the development of integrated control strategies for postharvest diseases.

Nitrogen starvation or limitation is generally encountered by hemibiotrophic fungal pathogens at the beginning of their infection cycle. During the infection cycle, the fungal pathogens sense and absorb nutrients from the host, and the regulation system must respond to the fluctuations of nutrient quality and quantity encountered throughout the host. To date, nitrogen and carbon nutrient utilization in filamentous fungi was considered to be modulated by nitrogen metabolite repression (NMR) and carbon catabolite repression (CCR) (Fernandez et al., 2012; Tanzer et al., 2003). The negative-acting transcription factor CreA functions in CCR to ensure the utilization of preferred carbon sources such as glucose prior to less preferred carbon sources (Dowzer and Kelly, 1991; Flipphi et al., 2003; Ruijter and Visser, 1997). NMR, activated by the positive-acting transcription factor AreA, is a regulatory strategy found in microorganisms that restricts the utilization of complex and secondary nitrogen sources in the presence of favored nitrogen sources, such as ammonium and L-glutamine (Horst et al., 2012; Wilson and Arst, 1998; Wong et al., 2008).

In plant fungal pathogens, functional studies of orthologous AreA/Nit2-encoding genes have been performed in the biotrophs *Cladosporium fulvum* and *Ustilago maydis*, and hemibiotrophs *Colletotrichum lindemuthianum*, *Colletotrichum coccodes*, *Magnaporthe oryzae*, *Fusarium fujikuroi* and *Fusarium graminearum*. In the tomato leaf mold pathogen *C. fulvum*, a strain containing a mutation in *Nrf1*, the AreA/Nit2 ortholog, was unable to utilize nitrogen sources except glutamine, ammonium, histidine and asparagine (Pérez-

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García et al., 2001). Further experiments suggested that deletion of *Nrf1* affects the colonization process on the host plant (Thomma et al., 2006). Moreover, recent research indicated that an inability to utilize gamma-aminobutyric acid (GABA) that accumulates during *C. fulvum* infection contributes to nutrient deficiency in *Nrf1*-deleted strains (Divon and Fluhr, 2007). A study in the basidiomycete *U. maydis* suggested that the AreA homolog Nit2 regulates nitrogen utilization and affects the signaling pathway mediating the dimorphic switch downstream of the bE/bW heterodimer (Horst et al., 2012). In the hemibiotroph *C. lindemuthianum*, deletion of the AreA/Nit2 ortholog Clnr1 did not cause anthracnose in the common bean. Additional analysis showed that the mutant strains can produce primary hyphae in the biotrophic phase, but is unable to produce secondary hyphae in the necrotrophic phase (Pellier et al., 2003). It has been suggested that instead of regulating nitrogen metabolism, Clnr1 is functional during the switch from the biotrophic to the necrotrophic phase. The AreA/Nit2 ortholog of the rice blast fungus *M. oryzae*, Nut1, is involved in growth, nitrogen utilization and metabolism of some amino acids, but is dispensable for pathogenicity (Fernandez and Wilson, 2012; Fernandez et al., 2012; Froeliger and Carpenter, 1996; Wilson et al., 2010). It has been suggested that Nut1 may be important for the regulation of nitrogen utilization in low nitrogen levels, implying that it has a critical role during early infection in the nitrogen-poor confines of the apoplast (Fernandez et al., 2014). In studies of the causal agent of *Fusarium* head blight on wheat and barley, *F. graminearum*, mutation of the AreA/Nit2 ortholog significantly affected vegetative growth, nitrogen metabolism, virulence, and deoxynivalenol production, a harmful mycotoxin to humans and animals (Hou et al., 2015). In gibberellin (GA)-producing fungus *F. fujikuroi* Δ*areA* mutant shows poor utilization of ammonium, glutamate and other nitrogen sources but not glutamine (Michielse et al., 2014). Moreover, GA biosynthesis strictly regulates by AreA, which is affected by interplay between AreB and AreA (Michielse et al., 2014). In addition, for the tomato pathogen *Colletotrichum coccodes*, mutation of AreA affects some nitrogen sources utilization, ammonia secretion under low pH and pathogenicity (Alkan et al., 2008). In general, AreA/Nit2 is a major nitrogen regulator involved in nitrogen metabolism in most plant fungal pathogens, but its roles in pathogenicity are complex and differ among species.

To determine the role of the *C. gloeosporioides* AreA homolog in growth and virulence, we generated *AREA* deletion mutants and complemented strains. The predicted AreA protein exhibits high amino acid sequence similarities with the AreA/Nit2 global nitrogen regulators in other filamentous fungi. Targeted gene deletion mutants are unable to use a wide array of nitrogen sources, indicating that AreA is the *C. gloeosporioides* major nitrogen regulator. Quantitative RT-PCR analysis revealed that nitrogen metabolism related-genes are misregulated in mutant strains, especially under nitrogen limitation conditions. Moreover, *AREA* mutant strains showed reduced growth and an apparent increase in conidia production. Pathogenicity assays showed that the virulence of AreA mutant strains is affected by nutrition condition of fruit hosts.

2. Materials and methods

2.1. Fungal isolate, media, and growth conditions

The WT strain Cg-14 isolate was obtained from an avocado (*Persea americana* cv. Fuerte) in Israel. All of the *C. gloeosporioides* strains were routinely grown on M_3S agar (Tu, 1985). M_3S liquid medium (primary medium) was also used for the initial growth of the fungus, prior to exposing it to different nutritional induction media. The M_3S medium contained the following reagents (per liter): 2.5 g $MgSO_4{\cdot}7H_2O$, 2.7 g KH_2PO_4, 1.0 g Bacto peptone, 1.0 g Bacto yeast extract, 10 g sucrose, and 250 mg chloramphenicol. The cultures were incubated at 24 °C in a shaking incubator at 150 rpm for 3 days and were harvested by filtration through a sterile funnel fitted with filter paper. The hyphal mat was washed twice with 40 ml of sterile distilled water. The washed mycelia were re-suspended in 40 ml of fresh minimal medium containing the following reagents (per liter): 4 g K_2HPO_4, 2 g $MgSO_4{\cdot}7H_2O$, 0.3 g $CaCl_2{\cdot}2H_2O$, 10 mg $FeCl_3$, and 1% glucose, with different fungal species or concentrations of nitrogen sources.

2.2. Fruit inoculation

All of the fruits that we used in this study were purchased from local fruit markets before inoculation. Eight microliters of spores (10^6 conidia ml^{-1}) were inoculated into a hole in a wounded fruit with a needle. Each treatment consisted of 4–5 inoculation spots on at least 10 different tomatoes, apples and plums. To inoculate avocado, a strip of peel tissue was removed and 0.5-cm-diameter disks of sterile filter paper containing 7 μl aliquots of the conidial suspension (10^6 conidia ml^{-1}) were place onto the fruit mesocarp. Two inoculation spots, longitudinally spaced on each side of 10 different avocado were inoculated. After inoculation, the fruits were incubated at 28 °C and 95% relative humidity in covered plastic containers containing wet paper towels until symptoms were observed. In all experiments, the average decay diameters ± standard error (SE) were reported. The inoculation experiments were repeated at least two times with similar results, and one representative result is presented.

2.3. Vector constructions and transformation

The *AREA* (TCONS_00002904, EQB57057.1) replacement construct, including the hygromycin marker gene and *AREA* flanking sequences, was made using the Gateway system as described previously (Shafran et al., 2008). Briefly, the 5′ flanking sequence (598 bp) and 3′ flanking sequence (502 bp) were amplified with the primer pairs (attB_AreA_5end and attB_AreA_3end) from *C. gloeosporioides* genomic DNA. The two PCR products were assembled together with the Hyg entry clone plasmid (Shafran et al., 2008) using the Multisite Gateway three-fragment vector construction kit. The *Not*I fragment was used to create the *AREA* knockout mutant. For the complementation construct, 4697 bp genomic DNA of the *AREA* gene including the 1181 bp native promoter and 376 bp terminator sequence was introduced into the pDESTphleo vector with the primer attB_AreA_comp (Table S3). Fungal transformation was performed by electroporation of germinated conidia as described previously (Robinson and Sharon, 1999). The primer pairs 5′F_end_Control with 925 and Hyg32 with 3′R_end_Control were used to confirm the correct integration in the genome of hygromycin resistant transformants for the knockout stains. The complemented strains were screened with phleomycin antibiotics and the expression level of *AREA* was confirmed with qRT-PCR.

2.4. RNA extraction and qRT-PCR analysis

RNA was extracted from 100 mg samples of frozen mycelia with the SV Total RNA isolation kit (Promega). The reverse transcription reaction was performed on 1 μg of total RNA with either the Verso cDNA Kit (Thermo Scientific, Lithuania, EU) or the Promega GoScript kit (Promega, Shanghai, China), and samples of cDNA were diluted 1:10 with ultrapure water. The quantitative real-time PCR was performed using either the StepOnePlus System (AB, applied biosystems) or the CFX96 Touch™ Real–Time PCR Detection System (Bio-rad). PCR amplification was performed with 3.4 μl of diluted cDNA template in a 10 μl reaction mixture con-