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bZIP transcription factor CgAP1 is essential for oxidative stress tolerance and full virulence of the poplar anthracnose fungus *Colletotrichum gloeosporioides*

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ABSTRACT

Yeast AP1 transcription factor is a regulator of oxidative stress response. Here, we report the identification and characterization of CgAP1, an ortholog of YAP1 in poplar anthracnose fungus *Colletotrichum gloeosporioides*. The expression of *CgAP1* was highly induced by reactive oxygen species. *CgAP1* deletion mutants displayed enhanced sensitivity to oxidative stress compared with the wild-type strain, and their poplar leaf virulence was obviously reduced. However, the mutants exhibited no obvious defects in aerial hyphal growth, conidia production, and appressoria formation. CgAP1::eGFP fusion protein localized to the nucleus after TBH (*tert*-Butyl hydroperoxide) treatment, suggesting that CgAP1 functions as a redox sensor in *C. gloeosporioides*. In addition, CgAP1 prevented the accumulation of ROS during early stages of biotrophic growth. CgAP1 also acted as a positive regulator of several ROS-related genes (i.e., *Glr1*, *Hyr1*, and *Cyt1*) involved in the antioxidative response. These results highlight the key regulatory role of CgAP1 transcription factor in oxidative stress response and provide insights into the function of ROS detoxification in virulence of *C. gloeosporioides*.

1. Introduction

To successfully colonize and reproduce in host plants, pathogens need to overcome innate plant defenses (Chi et al., 2009). Plants have two types of defense mechanisms against an attack by pathogenic microbes: a general one against a variety of microorganisms, and a specialized one targeting specific pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). The general defense mechanism is known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). One of the major and earliest responses of plant PTI is a rapid accumulation of reactive oxygen species (ROS) at the site of infection (Apostol et al., 1989). This defense mechanism comprises the so-called oxidative burst, which contributes to ROS production, primarily superoxide (O_2^-) and hydrogen peroxide (H_2O_2), at the site of the attempted invasion (Apel and Hirt, 2004). ROS cause molecular damage, such as DNA mutations, lipid peroxidation, and protein oxidation, eventually leading to cell death of the organism (Beckman and Ames, 1998).

ROS sensitivity of a fungal pathogen is predominantly determined by the effectiveness of its own ROS detoxification abilities. To survive under aerobic conditions, fungi detoxification systems must effectively scavenge ROS, maintain reduced redox states within subcellular microenvironments, and repair ROS-triggered damage (Miller and Britigan, 1997; Mittler, 2002; Thammavongs et al., 2008). ROS play a crucial role in plant defenses. Accordingly, successful pathogens have adapted counter-defense mechanisms against plant ROS-mediated resistance to facilitate pathogenicity. Using enzymatic and non-enzymatic antioxidant mechanisms, pathogens can detoxify ROS and successfully invade their hosts (Apel and Hirt, 2004; Cessna et al., 2000). One of the key regulators mediating the oxidative stress response is the Activating Protein 1 (AP1) class of basic leucine zipper (bZIP) transcription factors (Cartwright and Scott, 2013; Jamieson, 1998; Toone and Jones, 1999). AP1, a bZIP transcription factor in yeast and filamentous fungi, is a transcriptional activator produced in response to oxidative stress (Reverberi et al., 2008; Toone and Jones, 1998; Wu and Moye-Rowley, 1994).

Saccharomyces cerevisiae YAP1 is one of the most important determinants of yeast's response to oxidative stress as Yap1 is responsible for transcriptional activation of various genes involved in ROS detoxification (Toone et al., 2001). YAP1 mutations give rise to fungi that are sensitive to a variety of oxidizing agents, drugs, and xenobiotic compounds (Alarco and Raymond, 1999). Subsequently, Yap1 homologs were identified and characterized in several fungal pathogens, and share the function in oxidative stress

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tolerance, but differ in pathogenicity. Yap1-mediated ROS detoxification is an essential virulence determinant in *Alternaria alternata*, *Candida albicans*, *Magnaporthe oryzae*, and *Ustilago maydis*, but is not involved in virulence of *Aspergillus fumigatus*, *Cochliobulus heterostrophus*, *Cryptococcus neoformans*, or *Fusarium graminearum* (Alarco and Raymond, 1999; Guo et al., 2011; Lessing et al., 2007; Lev et al., 2005; Lin et al., 2009; Molina and Kahmann, 2007; Montibus et al., 2013; Paul et al., 2015).

Colletotrichum gloeosporioides (teleomorph: *Glomerella cingulata*) is one of the most ubiquitous fungal species worldwide and infects ca. 200 plant species, including crops, vegetables, weeds, and trees (Cannon et al., 2008; Farr et al., 1989). This fungus is a hemibiotrophic phytopathogen responsible for anthracnose diseases in a number of economically important plants in the temperate, tropical, and subtropical regions throughout the world (Bailey and Jeger, 1992). *C. gloeosporioides* is also a causal agent of poplar anthracnose, one of the most devastating diseases of the genus *Populus*.

Worldwide, poplar comprises an important woody biomass and is a potential bioenergy tree species on account of its high growth capacity and adaptability to various climates and soil types, as well as various uses of its wood (Ahuja, 1988; Bradshaw et al., 1996). In particular, it is a good source of fuel, pulpwood, and solid wood products (Ostry and McNabb, 1985). However, rapid development of *Populus* plantations led to an increase in poplar diseases (Xiang and Zhu, 2000; Yu et al., 2004). The species is susceptible to microbial pathogens, particularly pathogenic fungi, which results in considerable losses in poplar productivity (Cellerino, 1999). Specifically, the poplar anthracnose disease caused by *C. gloeosporioides* is very common in northeast China, causing substantial economic losses (Li et al., 2012). All these factors are increasing the demand for effective control of poplar anthracnose that would be based on the understanding of the molecular mechanisms of *Populus-C. gloeosporioides* interactions. Therefore, there is a need for effective disease management strategies based on *Populus* and *C. gloeosporioides* as a model system of host tree-fungal pathogen interactions (Narusaka et al., 2012).

The AP1-mediated ROS detoxification pathway is well characterized in other fungi but is less understood in *C. gloeosporioides*. In this study, we addressed the role of *C. gloeosporioides* YAP1 homolog, *CgAP1*. We show that CgAP1 is a major regulator of oxidative stress response in this fungus and we establish its relevance during fungal infection of plants. Our results reveal a key role of CgAP1 in the oxidative stress response and virulence of the poplar anthracnose fungus *C. gloeosporioides*.

2. Materials and methods

2.1. Fungal strains, culture conditions, and stress treatments

C. gloeosporioides strain BDL-3, isolated from *Populus* × *euramericana* Neva in Beijing, China, was used as the wild-type strain throughout this work. Both BDL-3 and its derivatives were cultured on PDA medium (200 g potato, 20 g glucose, 20 g agar, per 1 L of water) or in CM medium (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, 0.01% vitamins, per 1 L of water, pH 6.5) for 3–15 days at 28 °C to assess the growth and colony characteristics. Fungal mycelia were harvested from liquid CM and used for genomic DNA and RNA extractions. The strains were grown on PDA for conidiation assays. Stress tests were performed on CM plates for 5 days, with NaCl (1 M), Sorbitol (2 M), Congo Red (200 μg/mL), or H_2O_2 (10 mM). Each plate was inoculated with a 5 mm-diameter mycelial plug cut from the edge of a 3-day-old colony grown on PDA. All assays were performed in triplicate, in three independent experiments.

2.2. Bioinformatics analysis

The complete sequence of *CgAP1* was downloaded from the *C. gloeosporioides* genome database (http://genome.jgi.doe.gov/Gloci1/Gloci1.home.html). *CgAP1* homologs in other fungal species were identified by the blastp search at the National Center for Biotechnology Information. Multiple sequence alignments were conducted with the ClustalX 2.0 program (Larkin et al., 2007) using full-length protein sequences. The phylogenetic tree was constructed by MEGA 6.0 with full-length protein sequences and neighbor-joining with 1000 bootstrap replications (Tamura et al., 2013).

2.3. Targeted gene disruption and complementation

CgAP1 gene deletion mutants were generated using the split-marker gene replacement strategy (Goswami, 2012). Two flanking sequences of *CgAP1* (each approximately 1.2 kb) were amplified using primers AP-5Ffor and AP-5Frev, for the 5′-flanking sequence, and primers AP-3Ffor and AP-3Frev, for the 3′-flanking sequence, and these flanking sequences had approximately 20 bp overlaps with the respective 5′- and 3′-terminal sequences of the hygromycin resistance cassette (*HPH*). *HPH* was amplified with primers HPH-for and HPH-rev from the pRF-HU vector as template (provided by Fungal Genetics Stock Center, (FGSC)). The resulting 5′ and 3′ flanking sequences were fused to the hygromycin resistance cassette by overlap-PCR. The recombinant fragments were verified by sequencing and used directly for protoplast transformation. Transformants were selected on media supplemented with 300 μg/mL hygromycin. The DNA fragment for complementing *CgAP1* deletion strains, containing the entire *CgAP1* coding region, its native promoter, and terminator regions, was PCR-amplified using primers AP-Cfor and AP-Crev, and inserted into modified pBC-phleo vector conferring phleomycin resistance (provided by FGSC). Genomic DNA was extracted. Polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR) and Southern blotting analysis were performed to identify the mutations. Monoconidia were isolated from all fungal transformations and purified.

2.4. Gene expression analysis

Total RNA was extracted with TRIzol reagent (Invitrogen) and purified with the PureLink RNA Mini Kit (Ambion), in accordance with the manufacturers' instructions. Total RNA was incubated with DNase I (TaKaRa) at 37 °C for 30–60 min to remove contaminating DNA, before reverse transcription of mRNA. mRNA was transcribed into cDNA with Oligo-DT and SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) reactions were performed with SuperReal Premix Plus (TIANGEN, China), employing SYBR Green dye and the ABI 7500 real-time PCR system (Applied Biosystems, USA). Quantification of β-tublin2 gene expression was performed as an internal reference. Each gene was assayed independently and in triplicate. Relative expression ratios were calculated by the ΔΔCT method (Livak and Schmittgen, 2001). ROS-induced *CgAP1* expression and the expression of *CgAP1*-modulated genes under ROS stress were assessed in cultures grown in liquid CM for 3 days and treated with 100 μM *tert*-Butyl hydroperoxide (TBH, Sigma-Aldrich) for 1 h. The control treatment was distilled water. All primers used in this study are listed in Table 1.

2.5. Conidiation, appressoria formation, and agar diffusion tests

For the conidiation assay, 5 mm-diameter mycelial plugs taken from the edge of a 3-day-old colony were inoculated onto PDA plates and grown at 28 °C for 7 days. Spores were collected from PDA by pouring 5 mL of sterile water onto each dish and filtering the resulting