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The bZIP transcription factor PfZipA regulates secondary metabolism and oxidative stress response in the plant endophytic fungus *Pestalotiopsis fici*

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ABSTRACT

The bZIP transcription factors are conserved in all eukaryotes and play critical roles in organismal responses to environmental challenges. In filamentous fungi, several lines of evidence indicate that secondary metabolism (SM) is associated with oxidative stress mediated by bZIP proteins. Here we uncover a connection with a bZIP protein and oxidative stress induction of SM in the plant endophytic fungus *Pestalotiopsis fici*. A homology search of the *P. fici* genome with the bZIP protein RsmA, involved in SM and the oxidative stress response in *Aspergillus nidulans*, identified PfZipA. Deletion of *PfzipA* resulted in a strain that displayed resistant to the oxidative reagents tert-butylhydroperoxide (tBOOH), diamide, and menadione sodium bisulfite (MSB), but increased sensitivity to H_2O_2 as compared to wild type (WT). Secondary metabolite production presented a complex pattern dependent on PfzipA and oxidative reagents. Without oxidative treatment, the Δ*PfzipA* strain produced less isosulochrin and ficipyroneA than WT; addition of tBOOH further decreased production of iso-A82775C and pestaloficiol M in Δ*PfzipA*; diamide treatment resulted in equivalent production of isosulochrin and ficipyroneA in the two strains; MSB treatment further decreased production of RES1214-1 and iso-A82775C but increased pestaloficiol M production in the mutant; and H_2O_2 treatment resulted in enhanced production of isosulochrin, RES1214-1 and pestheic acid but decreased ficipyroneA and pestaloficiol M in Δ*PfzipA* compared to WT. Our results suggest that PfZipA regulation of SM is modified by oxidative stress pathways and provide insights into a possible role of PfZipA in mediating SM synthesis in the endophytic lifestyle of *P. fici*.

1. Introduction

The eukaryotic bZIP transcription factors play critical roles in organismal responses to the environment. These proteins are conserved transcriptional enhancers found in all eukaryotes and participate in many aspects of organismal development. A subset of bZIP proteins called Yap (yeast activator protein) transcription factors have been well defined in yeast (Rodrigues-Pousada et al., 2010). The function of Yap family bZIPs is associated with stress responses, most often with resistance to reactive oxygen species (ROS) and osmotic imbalances. In filamentous fungi, several orthologs of Yap-like bZIP proteins have been characterized since the finding of the first Yap protein in *Saccharomyces cerevisiae* (Moye-Rowley et al., 1989). Similar to the reported roles in yeast, these proteins typically are associated with resistance to ROS or antifungals. Several Yap-like bZIP proteins such as AtfA, NapA, Afyap1, Aoyap1, and Apyap1 have been characterized in *Aspergillus* spp. as responding to oxidative, osmotic, drug, nutrient and iron stress (Asano et al., 2007; Balazs et al., 2010; Hagiwara et al., 2008; Qiao et al., 2008; Reverberi et al., 2007, 2012; Roze et al., 2011). Yap-like bZIP proteins also play a role in the stress response in other fungal species. In *Neurospora crassa*, Yap-like proteins are involved in ROS and heavy metal resistance (Asano et al., 2007; Tian et al., 2011). MoAP1 in the rice blast fungus *Magnaporthe oryzae* mediates oxidative stress and is critical for pathogenicity (Guo et al., 2011; Tang et al., 2014). Furthermore,

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bZIP proteins in both oomycetes (Gamboa-Meléndez et al., 2013) (Ye et al., 2013) and the mushroom *Agaricus bisporus* (Navarro et al., 2014) are involved in a stress response. In the fungal symbiont *Epichloë festucae*, YapA was found to counter treatment with some but not all oxidative stress reagents (Cartwright and Scott, 2013).

More recently, studies have demonstrated both bZIP and oxidative stress regulation of secondary metabolite (SM) production in filamentous fungi (Hong et al., 2013; Montibus et al., 2013a, 2013b). A novel Yap-like bZIP, RsmA (restorer of secondary metabolism A), was found in a suppressor screen of an *Aspergillus nidulans* secondary metabolism mutant (Shaaban et al., 2010). Overexpression of *rsmA* was found to partially restore sterigmatocystin in SM mutants in *A. nidulans* (Shaaban et al., 2010). Mechanism of RsmA regulation on SM production has been linked to the responses to environmental challenges (Yin et al., 2012). Yap-like bZIPs have been associated with both positive and negative regulation of secondary metabolites. For example, the bZIPs AtfB and Apyap1 were reported to positively regulate aflatoxin in *Aspergillus parasiticus* (Roze et al., 2011). In the human pathogen *Aspergillus fumigatus*, RsmA positively regulates production of gliotoxin as well as its precursor cyclo (L-Phe-L-Ser) (Sekonyela et al., 2013). However, deletion of Aoyap1 and Apyap1 in *Aspergillus ochraceus* and *A. parasiticus* resulted in increases of ochratoxin and aflatoxin, respectively (Reverberi et al., 2007, 2012). MeaB was found to negatively regulate bikaverin in a nitrogen dependent manner in *Fusarium fujikuroi* (Wagner et al., 2010). In *Aspergillus flavus*, MeaB regulates SM and virulence (Amaike et al., 2013). BcAtf1 regulates phytotoxin production and controls various differentiation processes such as hyphal morphology and sexual and asexual differentiation in *Botrytis cinerea* (Temme et al., 2012). Fgap1 from *Fusarium graminearum* has been characterized to mediate oxidative stress response and trichothecene biosynthesis but not virulence (Montibus et al., 2013a, 2013b).

To date, there have been no reports on the identification of a bZIP protein in a plant endophytic fungus. Because of the unique ecological niches, plant-associated endophytic fungi such as *Pestalotiopsis fici* have been a rich resource for discovering novel bioactive molecules (Liu et al., 2008). Eighty-eight natural products including 70 novel compounds have been isolated from *P. fici* (Liu et al., 2009; Yang et al., 2012 and unpublished data). Due to the wealth of metabolites produced by *P. fici*, the genome was sequenced recently (Wang et al., 2015) and a genetic system was established for investigation of SM in this fungus (Xu et al., 2014).

Here, we characterized a RsmA homologue termed PfZipA in *P. fici*. We show that deletion of *PfzipA* results in resistant phenotypes to tBOOH, MSB and diamide but a sensitive phenotype to hydrogen peroxide. Furthermore, we find that treatment of the fungus with these oxidative stress reagents impacts SM in a PfZipA-dependent manner. In particular, production of the secondary metabolites isosulochrin, RES1214-1 and pestheic acid are significantly increased by H_2O_2 treatment of *ΔPfzipA* as compared to WT. Our finding suggests an important role of PfZipA in regulating SM synthesis in oxidative stress environments.

2. Materials and methods

2.1. Strains, media and culture conditions

P. fici CGMCC3.15140 and its transformants (Table 1) were grown at 25 °C on Potato Dextrose Agar (PDA) or Potato Dextrose Broth (PDB) with appropriate antibiotics as required.

Escherichia coli strain DH5α and *Agrobacterium tumefaciens* AGL-1 were propagated in LB medium with appropriate antibiotics for plasmid DNA. For DNA manipulation in yeast, *S. cerevisiae* strain BJ5464-NpgA (*MATα ura3-52 his3-Δ200 leu2-Δ1 trp1 pep4::HIS3 prb1Δ1.6R can1 GAL*) was used as the host and grown on Yeast Extract Peptone Dextrose Medium (YPD). After transformation, *S. cerevisiae* was selected on synthetic dextrose complete medium (SDCt) with appropriate supplements corresponding to the auxotrophic markers (Colot et al., 2006).

2.2. In silico identification of PfzipA and domain prediction

The sequence of RsmA from *A. nidulans* (accession no. XP_662166.1) was used as query to perform BLAST searches to find its homologue in *P. fici*. To predict the domain architecture, the proteins Yap3 (accession no. NP_011854.1) from *S. cerevisiae*; NapA (accession no. XP_680782) and AnRsmA (accession no. XP_662166) from *A. nidulans*, AfRsmA (accession no. XP_749389) from *A. fumigatus*, MoRsmA (accession no. XP_003721157.1) from *M. oryzae*, and GzbZIP020 (accession no. ESU14603) from *F. graminearum*, were performed manually using InterProScan on EBI web server (http://www.ebi.ac.uk/interpro/). Then, the domains were visualized using DOG (version 2.0) (Ren et al., 2009).

2.3. RNA extraction and reverse transcriptase PCR (RT-PCR) of PfzipA

P. fici was grown at 25 °C on PDA for 7 days. Then, mycelia were harvested and total RNA was extracted using TRIzol® Reagent (Life Technologies). Five μg of total RNA were reverse transcribed into first-strand cDNA using FastQuant RT Kit (with gDNase) (TIANGEN, China). For transcription assessment of *PfzipA*, the coding region of this gene was amplified using primer pairs 2800RT_F and 2800RT_R (Table 2). The expression of *Pfactin* (Table 2) was used as the internal control.

2.4. Creation of ΔPfzipA strain

The oligonucleotide sequences for PCR primers are given in Table 2. PCR amplification was carried out using the TransStart® FastPfu DNA polymerase (Transgene Biotech) on a T100™ Thermal cycler from Bio-Rad. For creation of deletion strains of *PfzipA* (PFICI_02800), two steps were carried out. Firstly, the deletion cassette of *PfzipA* was constructed via a yeast recombination strategy (Colot et al., 2006). Briefly, the marker gene hygromycin B was amplified by using pDHt-hyg (Table 2) as template. Around 1 kb fragments upstream and downstream of the target

Table 1
Fungal strains and plasmids used in this study.

Strain/plasmid	Description	Reference
Pestalotiopsis fici CGMCC3.15140	Wild type	Liu et al. (2008)
AGL-1	*A. tumefaciens* strain	Xu et al. (2014)
TYXW6.4	*ΔPfzipA*::hph	This study
pXW06	Yeast recombination vector	Yu et al. (2013)
pYXW13.1	*PfzipA* deletion cassette in pXW06	This study
pYXW23	*PfzipA* deletion cassette in pAG1-H3	This study

pXX = plasmid, TXX = original transformant.