Fungal Genetics and Biology 75 (2015) 46–55

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Function of small GTPase Rho3 in regulating growth, conidiation and virulence of *Botrytis cinerea*

Bang An [a,b], Boqiang Li [a], Guozheng Qin [a], Shiping Tian [a,*]

[a] Key Laboratory of Plant Resources, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 5 November 2014
Accepted 8 January 2015
Available online 24 January 2015

Keywords:
Rho3
Botrytis cinerea
Virulence
ROS
Mitochondria

ABSTRACT

Small GTPases of the Rho family play an important role in regulating biological processes in fungi. In this study, we mainly investigated the biological functions of Rho3 in *Botrytis cinerea*, and found that deletion of the *rho3* from *B. cinerea* significantly suppressed vegetative growth and conidiation, reduced appressorium formation and decreased virulence. Microscopy analysis revealed that the distance between septa was increased in the Δrho3 mutant. In addition, mitochondria were suggested to be the main sources of intracellular reactive oxygen species (ROS) in *B. cinerea* based on dual staining with 2′,7′-dichlorodihydrofluorescein diacetate and MitoTracker orange. The Δrho3 mutant showed less accumulation of ROS in the hyphae tips compared to the WT strain of *B. cinerea*. These results provide the novel evidence to ascertain the function of small GTPase Rho3 in regulating growth, conidiation and virulence of *B. cinerea*.

1. Introduction

Small GTP-binding proteins (GTPase) are monomeric G proteins which function as molecular switches to regulate biological processes. These proteins are grouped into five subfamilies: Ras, Rho (Ras homologue), Arf/Sar, Ran and Rab/Ypt (Takai et al., 2001). Among these groups, proteins of the RAS and RHO families are the best characterized in fungi. The proteins of RAS are involved in growth, differentiation, cell cycle, and cell polarity (Harris, 2011; Schumacher et al., 2008; Virag et al., 2007). The Rho superfamily contains three kinds of GTPase named Rac, Cdc42, and Rho, which regulate a wide spectrum of cellular functions (Kwon et al., 2011). Minz-Dub et al. (2013) found that RAC protein was involved in the growth, differentiation and the virulence by regulating the actin localization and nuclear division in *Botrytis cinerea*. Rho3, one monomeric GTPase of the Rho superfamily, is found only in fungi. Rho3 was first isolated in *Sacchoromyces cerevisiae*, and subsequently shown to modulate vesicle delivery, cell growth, cell polarity, cell division, exocytosis, and vesicle secretion in yeast (Adamo et al., 1999; Nakano et al., 2002; Wu et al., 2010). Rho3 have specific functions in different filamentous fungi. In *Trichoderma reesei*, the Rho3 mainly participates in the protein secretion (Vasara et al., 2001). In *Ashbya gossypii*, Rho3 could directly activate formin-driven actin cable nucleation through its interaction with Boi1/2p, and thereby ensuring polarized tip growth (Knechtle et al., 2006). Dünkler and Wendland (2007) found that the Rho3 was required for the polarized growth and cell separation in *Candida albicans*. Zheng et al. (2007) reported the Rho3 was dispensable for polarized hyphae growth, but required for the appressorium development and virulence in *Magnaporthe oryzae*.

In the early events of plant–microbe interactions, there is a "respiratory burst" occurring in plants, which could produce rapid and transient of reactive oxygen species (ROS) for killing invading microorganisms (O'Brien et al., 2012). Despite of its toxic damage to macromolecules and membranes, ROS also act as an important secondary messenger to regulate both differentiation and defense in multi-cellular organisms (Aguirre et al., 2005). It has been shown recently that fungi could also produce ROS during development and interaction with plant host (Egan et al., 2007; Takemoto et al., 2011). And ROS are continuously produced as byproducts of various metabolic pathways localized in different cellular compartments in fungi (Heller and Tudzynski, 2011). Besides, filamentous fungi possess the functional NADPH oxidases (Nox), which could produce ROS using electrons derived from intracellular NADPH (Lara-Ortíz et al., 2003). These ROS function as second signals to regulate the growth, differentiation and virulence in fungi. In *Aspergillus nidulans*, ROS is important for regulation of apical dominance of hyphae tips (Semighini and Harris, 2008). ROS is also involved in conidiation in *Septoria tritici* (Shetty et al., 2007) and mutualistic interaction between fungus *Epichloë festucae* and perennial ryegrass (Tanaka et al., 2008). Egan et al. (2007) reported

* Corresponding author at: Institute of Botany, Chinese Academy of Sciences, Xiangshan Nanxincun 20, Haidian District, Beijing 100093, China. Fax: +86 10 8259 4675.
E-mail address: tsp@ibcas.ac.cn (S. Tian).

http://dx.doi.org/10.1016/j.fgb.2015.01.007
1087-1845/

that *M. oryzae* could produce ROS which is associated with development of appressorium; and scavenging of the ROS significant impaired appressorium development.

Small GTPase of the Rho subfamily play important role in regulating the ROS generation by Nox in animal, plant, and fungi (Bedard and Krause, 2007; Fluhr, 2009; Tanaka et al., 2008; Takemoto et al., 2011). But in *B. cinerea*, deletion of Nox genes did not impair the ROS production in hyphae, indicating that there are alternative sources of ROS (Segmüller et al., 2008). Chiarugi et al. (2003) found that Rho subfamily could mediate ROS production by Nox independent mechanisms, which is essential for the cell adhesion. ROS produced by respiratory chain has been proved to be as signaling molecules to regulate communication between the mitochondria and the cytosol (Nemoto et al., 2000). However, it is not clear whether there are other sources of ROS production mediated by Rho subfamily in filamentous fungi. *B. cinerea* Pers. Fr. is an airborne plant pathogen with a necrotrophic lifestyle, attacking over 200 crop species worldwide and causing serious decay in mature or senescent tissues of dicotyledonous hosts (Elad et al., 2004), particularly in harvested fruits and vegetables with high market value (Qin et al., 2010). The pathogen is difficult to control as a result of its genetic plasticity. There is an extensive ROS generation in *B. cinerea*, which could induce a significant oxidative burst and even hypersensitive response (HR) in its host plant (Williamson et al., 2007).

The objectives of this study were (i) to identify the function of small GTPase Rho3 in regulating growth, differentiation and virulence; (ii) to investigate the relationship between Rho3 and ROS generation; (iii) to determine the possible mode of Rho3 in regulating ROS generation in *B. cinerea*.

2. Materials and methods

2.1. Fungal strains and culture conditions

B. cinerea Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) B05.10 was used as recipient strain for the transformation experiments and as a WT control. *B. cinerea* strains were grown on potato dextrose agar (PDA) at 22 °C. For DNA isolation and RNA isolation, mycelium was grown for 2 days at 22 °C in 300 ml Erlenmeyer flasks with 100 ml of a Yeast Nitrogen Base (YNB) (BD, USA) medium.

2.2. Standard molecular methods

Fungal genomic DNA and total RNA were isolated using a Plant Genomic DNA Kit and a TRNzal-A+ Reagent (TIANGEN Biotech, Beijing, China). Plasmid DNA was isolated using a plasmid DNA preparation kit (TIANGEN Biotech, Beijing, China).

2.3. Vector constructions

Vector pLOB7 (Patel et al., 2008) was used to construct the replacement vector. A replacement vector for *rho3* was designed

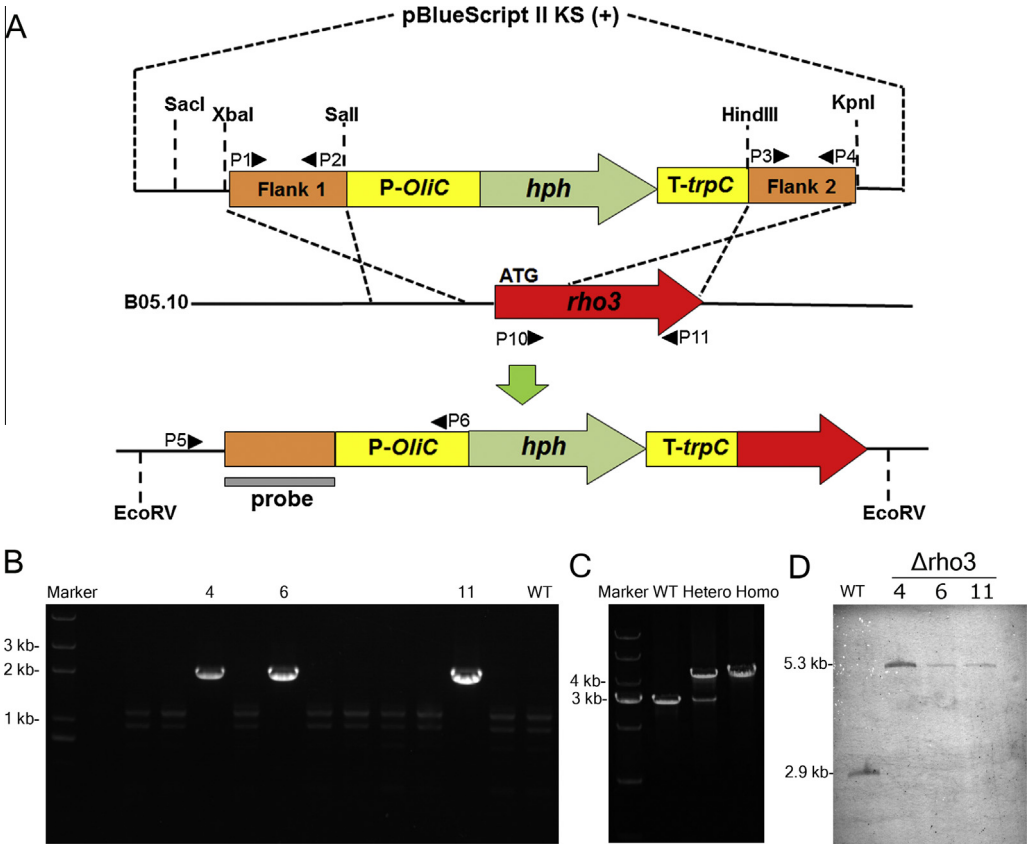

Fig. 1. Replacement strategy for the *B. cinerea rho3*. (A) Deletion of the *B. cinerea rho3* by the *SacI/KpnI* excised replacement fragment via homologous recombination. Flanking regions are indicated as black bars and restriction enzyme sites are marked in black letters. Primer binding sites for diagnostic PCR are indicated as small black triangles. The large red arrows indicate the 5′–3′ orientation of the gene. The genomic region used as a probe for Southern analysis is represented by a white bar. (B) PCR analysis for homologous integration of the replacement fragments of the 5′flanking region. Three transformants showed the correct diagnostic fragments were named as Δrho3–4, Δrho3–6 and Δrho3–11, respectively. (C) PCR analysis for purified single conidia colonies. The "Hetero" stands for the un-purified transformants; the "Homo" stands for the purified transformants. (D) Southern analysis of selected transformants. DNA of transformants was digested with *EcoRV*, separated on an agarose gel, blotted and radioactively probed with the 5′ flank. The WT *EcoRV* digested fragment is 2.4 kb smaller than the fragment of the Δrho3 mutant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)