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ROS and trehalose regulate sclerotial development in *Rhizoctonia solani* AG-1 IA

Chenjiaozi Wang, Lei Pi, Shaofeng Jiang, Mei Yang, Canwei Shu**, Erxun Zhou*

Guangdong Province Key Laboratory of Microbial Signals and Disease Control, Department of Plant Pathology, South China Agricultural University, Guangzhou, Guangdong, 510642, China

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ABSTRACT

Rhizoctonia solani AG-1 IA is the causal agent of rice sheath blight (RSB) and causes severe economic losses in rice-growing regions around the world. The sclerotia play an important role in the disease cycle of RSB. In this study, we report the effects of reactive oxygen species (ROS) and trehalose on the sclerotial development of *R. solani* AG-1 IA. Correlation was found between the level of ROS in *R. solani* AG-1 IA and sclerotial development. Moreover, we have shown the change of ROS-related enzymatic activities and oxidative burst occurs at the sclerotial initial stage. Six genes related to the ROS scavenging system were quantified in different sclerotial development stages by using quantitative RT-PCR technique, thereby confirming differential gene expression. Fluorescence microscopy analysis of ROS content in mycelia revealed that ROS were predominantly produced at the hyphal branches during the sclerotial initial stage. Furthermore, exogenous trehalose had a significant inhibitory effect on the activities of ROS-related enzymes and oxidative burst and led to a reduction in sclerotial dry weight. Taken together, the findings suggest that ROS has a promoting effect on the development of sclerotia, whereas trehalose serves as an inhibiting factor to sclerotial development in *R. solani* AG-1 IA.

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1. Introduction

Rice sheath blight (RSB), caused by *Rhizoctonia solani* Kühn AG-1 IA, is one of the most common and devastating diseases affecting rice production and causes great economic losses in rice-growing regions worldwide (Lee and Rush, 1983; Kimiharu et al., 2004; Linde et al., 2005; Tan et al., 2007; Khodayari et al., 2009; Zuo et al., 2014). *R. solani* is a complex species, consisting of at least 14 genetically isolated anastomosis groups (AGs) (Cubeta and Vilgalys, 1997; Strauss et al., 2000). Existing mainly as sclerotia in nature, *R. solani* can survive in soil for many years and is the primary source of infection in the disease cycle of RSB (Domingo et al., 2014; Feng et al., 2017; Moni et al., 2016). The sclerotium is a hard structure comprising a tangled mass of vegetative hyphae that adapt to various extreme environments (Wibberg et al., 2014). Elucidating the mechanism of sclerotia formation and development may lead to new ways of controlling RSB (Shrestha et al., 2016). In recent years, numerous studies on pathogenicity-related factors and the genetic structure of *R. solani* AG-1 IA have been conducted (Yang et al., 2012; Chen et al., 2017; Zhang et al., 2017), however, few studies have addressed the sclerotial development of *R. solani* AG-1 IA (Shu et al., 2015).

The sclerotial development of fungi is a complex process, which has captured the interest of many researchers (Georgiou et al., 2000, 2006; Calvo et al., 2004; Tran et al., 2014; Frisvad et al., 2014; Wu et al., 2014; Ordonez-Valencia et al., 2015). In 2006, Georgiou suggested that reactive oxygen species (ROS) induce sclerotial development in sclerotium-forming fungi. According to this important hypothesis, fungal cells are stable during the mycelial growth stage. While the concentration of intracellular oxygen remains comfortably low, super oxides cannot be generated. However, the transition period between growth and differentiation is unstable and it is at this stage, fungi produce hydroxyl radicals (▪OH), superoxide anion (O2▪$^-$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), as well as other ROS, which can neutralize or counteract the antioxidant effects of cells. This can lead to excessive accumulation of intracellular ROS, inducing the formation of sclerotia, which can resist the toxicity of ROS preserving the vitality of the fungi. Studies on sclerotial formation and

* Corresponding author.
** Corresponding author.
E-mail addresses: shucanwei@scau.edu.cn (C. Shu), exzhou@scau.edu.cn (E. Zhou).

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development of *R. solani* have mainly focused on the environmental factors such as light, temperature, air permeability, pH, nutrient content, and chemical compounds (Henis et al., 1965; Mukiibi, 1969; Chet and Henis, 1975; Willetts, 1978; Feng et al., 2017), but it has yet to be confirmed whether the development of sclerotia is induced by ROS in *R. solani* AG-1 IA.

Trehalose is a disaccharide with two glucose molecules linked together in an α, α-1,1-glycosidic linkage. As a nonreducing sugar, it is not easily hydrolyzed by acid, and the glycosidic bond is not cleaved by glucosidase (Chen and Haddad, 2004). Within cells, trehalose acts as a typical stress metabolite, and is synthesized in large quantities under stress and quickly degraded once the cell returns to its relaxed state (Antonio Cervantes-Chavez et al., 2016; Leite et al., 2016). Furthermore, trehalose has a protective effect on a variety of bioactive substances, which can be used to stabilize the biofilm, protein, and nucleotide molecules in cells. Trehalose can be directly cleaved by specific trehalases into two glucose molecules (Hottiger et al., 1987). Trehalose plays an important role in the pathogenicity of fungi. It has been reported that the absence of trehalose could affect pathogenicity of four pathogenic fungi: *Candida albicans* (Van Dijck et al., 2002), *Aspergillus fumigatus* (Puttikamonkul et al., 2010), *Magnaporthe grisea* (Wilson et al., 2007) and *Stagonospora nodorum* (Lowe et al., 2009). Trehalose is reported as a pathogenic inhibiting factor in many fungi. When *M. grisea* lost the ability of trehalose synthesis, the spores production decreased and pathogenicity increased (Foster et al., 2003). The thermal tolerability and pathogenicity of *A. fumigatus* was also affected by the synthesis of trehalose, the trehalose content significantly affect the cell wall's sensitivity to the stress conditions (Puttikamonkul et al., 2010). In fungi, trehalase reduces the content of trehalose via degradation, thereby weakening ROS scavenging. This inevitably leads to the excessive accumulation of ROS, which induce differentiation of the fungi (Jin et al., 2015). Therefore, studies on trehalose, trehalase and its inhibitor would aid in the development of new pesticides with high efficiency and low toxicity (Kono, 2002; Jin and Zheng, 2009). So far, little is known about the mechanism of sclerotial development and the regulation of antioxidant pathways by trehalose in *R. solani* AG-1 IA. Therefore, the objectives of this study are to: (1) verify whether ROS can induce sclerotial development in *R. solani* AG-1 IA; (2) investigate the production of trehalose in *R. solani* AG-1 IA in relation to oxidative stress levels and sclerotial development; and (3) explore the effects of exogenous trehalose on the sclerotial differentiation and oxidative stress levels of *R. solani* AG-1 IA.

2. Materials and methods

2.1. Chemicals

The chemicals used in this study were as follows: (1) hydrogen peroxide (H_2O_2, oxidant) (Sigma–Aldrich, St. Louis, MO, USA); (2) morin (antioxidant) (Sigma); (3) trehalose (Genview, League City, Texas, USA); (4) validamycin A (trehalase inhibitor) (Qianjiang Biochemical, Haining, Zhejiang, China). All chemicals mentioned above were of pure analytical grade.

2.2. Fungal strains, growth conditions, and developmental stages

Strain GD-118, a typical strain of RSB pathogen *R solani* AG-1 IA with strong virulence, was used in this study. The strain GD-118 has been extensively used in previous studies in our laboratory and other laboratories (Yang et al., 2012; Chen et al., 2015; Shu et al., 2015; Lu et al., 2016), and its whole genome was sequenced and released in 2013 (Zheng et al., 2013). In this study, the tested strain GD-118 was grown on the surface of a cellophane membrane (for the convenience of mycelia and sclerotia harvesting) superposed on a potato dextrose agar (PDA) plate (9 cm in diameter). Each PDA plate was inoculated with one mycelial disc (0.4 cm in diameter) cut from the periphery of 2-day-old culture. The inoculated PDA plates were incubated in an intelligent artificial climate incubator (ZRX-300ESW, Hangzhou, Zhejiang, China) with 12 h photoperiod at 28 ± 1 °C. After 36 h, the colony initially covered the Petri dish in the sclerotium-undifferentiated mycelial stage (MS), and thereafter the sclerotia appeared on the surface of PDA plate. Sclerotial formation occurred in two stages: (a) loosely bound, intertwined, white hyphal branches were formed by mycelia after 72 h, referred to as sclerotial initial (SI); (b) formation of the mature compact sclerotial mass saturated with melanin pigments was formed after 14 d of growth, called sclerotia mature (SM). Mycelia and sclerotia were harvested at 36 h (MS), 48 h, 60 h, 72 h (SI), 5 d, 7 d and 14 d (SM) after inoculation (Fig. 1). All harvested fungal materials (including mycelia and sclerotia) were frozen in liquid nitrogen and stored at −80 °C until further use.

2.3. Effects of exogenous H_2O_2 and morin on sclerotial differentiation

The effects of the oxidant H_2O_2 and antioxidant morin on the growth and differentiation of *R. solani* AG-1 IA were evaluated by the addition of H_2O_2 to PDB at the final concentrations of 0, 2, 3, 4 and 5 μmol L^{-1} (Zhou et al., 2011) and morin to PDA at the final concentrations of 0, 0.35, 0.4, 0.45, 0.47 mmol L^{-1} (Lu et al., 2016). *R. solani* AG-1 IA was cultured in PDB with or without H_2O_2 (CK) for 6 d, or on PDA with or without morin (CK) for 14 d. Then the dry weight (DW, which is referred to the weight of the sclerotia the water removed by placing fresh sclerotia into an 80 °C drying oven and heating until a stable weight is reached) of mature sclerotia, which had been cultured at an appropriate concentration so as not to affect the efficiency mycelial growth, was measured. Sclerotia DW was obtained by ovendrying samples at 80 °C for 2 h. Curves were constructed based on the amount of sclerotial DW present at different concentrations of H_2O_2 and morin. The degree of sclerotial differentiation is defined as the percentage by DW of mature sclerotia for various concentrations of H_2O_2 or morin. Sclerotial differentiation (SDIF) was defined as % sclerotia DW (in respect to 100 % SDIF of the control) per dish at a given scavenger concentration.

2.4. Effects of exogenous trehalose and validamycin A on sclerotial differentiation

The effects of trehalose and validamycin A on the growth and differentiation of *R. solani* AG-1 IA were evaluated by the addition of trehalose to PDA at the final concentrations of 0, 0.15, 0.30, 0.45 and 0.60 mol L^{-1} (Luo et al., 2008) and validamycin A to PDA at the final concentrations of 0, 10, 20, 30, 40 and 50 μg mL^{-1} (Shleeva et al., 2017). Sclerotia were harvested after 14 d. Identical experimental procedures and calculations to those in Section 2.3 were performed.

2.5. Effects of glucose on mycelial growth and sclerotial differentiation

As mentioned above, trehalose can be hydrolyzed (by trehalase) to 2 mol of glucose. Therefore, a parallel experiment was designed to observe whether glucose has the same effect with trehalose on the mycelial growth and sclerotial differentiation. For this purpose, glucose was added to PDA at the final concentrations of 0, 0.15, 0.30,

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