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Analysis of *Clonostachys rosea*-induced resistance to grey mould disease and identification of the key proteins induced in tomato fruit

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ABSTRACT

Tomato grey mould disease, which is caused by *Botrytis cinerea*, is a serious threat to tomato postharvest handling and storage. *Clonostachys rosea* is an effective antagonistic fungus to *B. cinerea* and can prevent this disease in tomatoes. To elucidate the mechanism of *C. rosea*-induced resistance in tomato, fruit were subjected to four treatments: spraying with *B. cinerea,* spraying with *C. rosea,* inoculation with *C. rosea* after spraying with *B. cinerea*, and inoculation with *B. cinerea* after spraying with *C. rosea*. Compared to the control (water), increases in indole acetic acid (IAA), salicylic acid (SA) and NO levels and enhanced phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) activities were observed, whereas catalase (CAT) activity and abscisic acid (ABA) levels decreased, particularly in the *B. cinerea*-plus-*C. rosea*- and *C. rosea*-plus-*B. cinerea*-treated fruit. We identified 22 proteins differentially expressed in the treated fruit compared to the control fruit using two-dimensional electrophoresis (2-DE) and mass spectrometry. ATP synthase CF1 alpha subunit was identified as an abundant protein in fruit sprayed with *C. rosea*. This study advances our understanding of *C. rosea* biocontrol mechanisms in tomato fruit.

1. Introduction

Tomato (*Solanum lycopersicum* (L.)) is grown worldwide due to the various uses and high nutritional value of its fruit. Cultivation of tomato plants occurs on all temperate and tropical continents, and these plants are likely the most preferred garden crop (Bergougnoux, 2014). A prominent focus of tomato breeding is the creation of cultivars that are resistant to biotic stresses inflicted by destructive pests and diseases as well as abiotic stresses (e.g., drought, salt, cold, heat), which can cause significant economic losses.

The pathogen *Botrytis cinerea* infects more than 200 plant species, including many economically important crops such as tomato, cucumber, eggplant, grape and strawberry. *B. cinerea* infects tomato tissues, particularly the fruit, via multiple strategies, including the secretion of cell wall-degrading enzymes, phytotoxic metabolites, and cell death elicitors (Huang et al., 2012). Ripening increases the susceptibility of tomato fruit to grey mould disease caused by *B. cinerea*, resulting in serious losses in tomato postharvest handling and storage.

Due to a lack of resistant tomato germplasm, no effective *B. cinerea*-resistant tomato cultivars have been bred to date. Chemical fungicides are the most widespread method for addressing grey mould disease. However, the indiscriminate use of chemical agents may result in problems such as the development of pathogen resistance in addition to toxic residues in fruit and potential risks to the environment and human and animal health. Consequently, over the long term, biological control agents offer more effective treatment compared to chemical fungicides and a promising alternative for managing *B. cinerea* both pre- and post-harvest (Nobre et al., 2005).

The mycoparasite *Clonostachys rosea* exhibits good potential for the suppression of fungal plant pathogens and can be used in the control of *B. cinerea* in tomato, strawberry and raspberry (Sutton et al., 1997; Mamarabadi et al., 2008). The antagonistic mechanisms of *C. rosea* are diverse and include hyperparasitism, competition, antibiosis, cell lysis and induced plant systemic resistance (Nobre et al., 2005). However, the mechanism of grey

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mould disease resistance induced by *C. rosea* in tomato fruit remains to be elucidated.

Plants have evolved a series of effective defence mechanisms against pathogen invasion that result in highly coordinated sequential changes at the cellular level, including altered levels of signalling molecules. The signalling molecules involved in plant defence mechanisms include phytohormones such as abscisic acid (ABA), zeatin (ZT), indole acetic acid (IAA), salicylic acid (SA), gibberellins (GAs), and reactive oxygen species (ROS), mainly nitric oxide (NO) and hydrogen peroxide (H_2O_2). These molecules trigger changes in the expression of defence genes, resulting in metabolic alterations that enhance plant defence responses (Shireen et al., 2015; Mai and Kinga, 2014). Phytohormones play crucial roles in regulating multiple physiological and developmental plant processes and cellular responses to biotic stresses (Sun et al., 2014), and as secondary messengers, H_2O_2 and NO are also involved in many physiological responses. Indeed, ROS, H_2O_2 and superoxide (O_2^-) are crucial components of plant stress responses to a variety of pathogens. In addition, ROS act as a trigger of the hypersensitive disease resistance response (HR) (Wojtaszek, 1997). These signalling molecules drive multiple different plant signal transduction pathways through complicated interactions that function independently.

Plant defence responses comprise a series of physiological and biochemical changes regulated by signal transduction networks (Dangl and Jones, 2001), and plants have evolved sophisticated mechanisms to respond to various stress resistance signals. Phytohormones are vital components of multiple signalling pathways and regulate plant defence responses synergistically or antagonistically, generally by regulating expression of resistance genes. Indeed, changes in the levels of ZT, IAA, ABA, SA and GA have been implicated in disease susceptibility or resistance.Defence enzymes play pivotal roles in host plant resistance against pathogen invasion, and reductions in disease severity in stressed plants have been attributed to changes in the activities of defence enzymes in several species (Binutu and Cordell, 2000; Marrs, 1996). For instance, catalase (CAT) is an important protective enzyme that maintains the balance of ROS to promote a plant's capacity to withstand environmental stresses. Phenylalanine ammonia lyase (PAL), which plays a significant role in the biosynthetic pathway of phytoalexins or phenolic compounds, is involved in the biosynthesis of SA. Polyphenol oxidase (PPO) is an enzyme involved in the oxidation of polyphenols into antimicrobial quinones.

The objective of the present study was to elucidate and utilize the mechanism of *C. rosea* as a biocontrol agent to control gray mould in tomato fruit. Tomato fruit were inoculated with *B. cinerea* and *C. rosea* to identify the mechanism by which changes in signalling molecules (ABA, ZT, IAA, SA, GA, O_2^-, NO, H_2O_2) and defence enzyme (CAT, PAL, PPO) activity induce resistance. Proteins differentially accumulating in mature fruit during the defence process were identified by two-dimensional electrophoresis (2-DE). Our study will facilitate elucidation of the mechanisms of *B. cinerea* biocontrol by *C. rosea* and identification of the crucial genes involved in *C. rosea*-induced resistance in an effort to further improve resistance to grey mould disease in tomato fruit.

2. Materials and methods

2.1. Plant material

The homozygous tomato variety 08016 was provided as a gift by the Tomato Research Institute of Northeast Agricultural University in China. The plants were grown from seeds in growth chambers at 25/22 °C (day/night), 75% relative humidity and a 16/8 h (day/night) photoperiod.

Mature and healthy red tomato fruit with similar size and color were used in this study. The fruit were free from visible wounds and diseases. Before each treatment, fruit were washed with water and then in 75% ethanol for 30 s, rinsed in sterile water and air dried.

2.2. Microbial culture

The *C. rosea* strain was isolated and maintained in our lab and propagated on potato dextrose agar (PDA) at 22 °C. The *B. cinerea* strain was isolated from a diseased tomato fruit and grown at 25 °C on PDA plates.

The spore suspensions of the strains were obtained from surface of the 7-day old cultures with 5 mL of sterile distilled water containing 0.0067 mol L^{-1} KH_2PO_4, 0.1 mol L^{-1} glucose, 0.1% Tween, pH 5, and then filtered through four layers of sterile cheesecloth to remove any adhering mycelia. Conidial suspensions were diluted to a concentration of 10^7 spores mL^{-1} with sterile water as described above.

2.3. Treatments and infection

Four treatments were established in this study: treatment *B. cinerea* (moistened fruit inoculated with *B. cinerea* suspension), treatment *C. rosea* (moistened fruit inoculated with *C. rosea* suspension), treatment *B. cinerea* plus *C. rosea* (moistened fruit inoculated first with *B. cinerea* suspension and then sprayed with *C. rosea* suspension 12 h later) and treatment *C. rosea* plus *B. cinerea* (moistened fruit sprayed first with *C. rosea* suspension and then inoculated with *B. cinerea* suspension 12 h later). Tomato fruit were through a suspension spraying process until the whole fruit were uniformly covered with tiny droplets (prepare about 40 mL suspension for each treatment). The control fruit were sprayed with sterile water as described above, which were labeled as treatment A. The treated samples were collected at different time points between 12 and 96 h. After treatment, the tomato fruit were incubated at 25 °C with a high level of relative humidity. Twenty fruit were used in each treatment in triplicate.

The disease incidence was calculated using the following formula: Disease incidence = the number of fruit diseased/the total of fruit × 100%. The degree of disease severity inoculation with *B. cinerea* comprised 0: no lesion; 1 = a few scattered lesions covering <2% of the fruit surface; 2 = extensive lesion covering >2% but <5% of the fruit surface; 3 = extensive lesion covering >5% but <25% of the fruit surface, and 4 = extensive lesions covering >25% of the fruit surface. The disease index for each treatment were calculated using the following formula:

$$\text{Disease index} = (\Sigma_{i=0}{}^{4}N_i \times i)/(4 \times \Sigma_{i=0}{}^{4}Ni) \times 100\%$$

Where i is the severity (0–4), 4 is the highest level, and Ni is the number of fruit with a severity of i.

2.4. Determination of ABA, ZT, IAA, SA and GA contents

The assays for measuring ABA, ZT, IAA and GA levels were described by Cui and Lin (2012). Tomato fruit samples were collected and cleaned with liquid water, and 0.6 g of fruit was ground into a powder in liquid nitrogen and extracted for 12 h with 20 mL of 80% cold aqueous methanol (<0 °C) in the dark at 4 °C. The extract was then centrifuged at 5,000 × *g* at 4 °C for 15 min. The supernatant was retained. The residue was extracted three times with fresh methanol. The total methanolic extract was collected by washing three times with 0.6% acetic acid followed by