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Rhodosporidium paludigenum induced resistance in Ponkan mandarin against *Penicillium digitatum* requires ethylene-dependent signaling pathway

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ABSTRACT

This work determined if the ethylene dependent signal pathway was required for antagonist-mediated fruit defense mechanisms through investigation of disease resistance against *Penicillium digitatum* in Ponkan mandarin induced by 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, and *Rhodosporidium paludigenum*. Blocking ethylene perception with 1-MCP resulted in an increase in *ACS1*, *ACS2* and *ACO* expression, and consequently an increase in ethylene production during mechanical wounding and resistance induction. The expression of the ethylene receptors *ETR1*, *ETR2* and *ETR4* as well as ethylene response factor (*ERF*) were observed with similar responses to yeast and 1-MCP stimuli, with *ETR3* mRNA accumulation being the most sensitive to yeast application while *ERS1* was the least sensitive. When applied at concentrations greater than 500 nL L^{-1}, 1-MCP pre-fumigation significantly reduced the fruit's natural protection and *R. paludigenum* induced disease resistance to *Penicillium* decay, indicating that ethylene perception was involved in inducting disease resistance. Moreover, expression of the defensive genes *CHI*, β-1,3-glucanase, *PAL* and *CIN* up-regulated by yeast was inhibited to different degrees by the 1-MCP pre-treatment. This study provides evidence that the biocontrol yeast *R. paludigenum* increased disease resistance in Ponkan mandarin against *P. digitatum* infection due to ethylene and signaling pathway dependent mechanisms.

1. Introduction

In natural environments, fruit frequently has to cope with various biotic stresses, such as bacteria, viruses, fungi and pests. Because of attack by a wide range of pathogens, fruit have developed a range of defense mechanisms that when stimulated can be rapidly activated, with reduction in damage levels (Prusky et al., 2013). However, as the physiology of the fruit changes during maturation and senescence, inhibitors of fungal growth decrease and the fruit becomes more susceptible to postharvest pathogens, especially to necrotrophs (Castoria and Wright, 2010). Many researchers have studied the enhancement of the natural defensive capacity of fruit by developing various treatments (Venditti et al., 2005; Liu et al., 2010; Ballester et al., 2011). In particular, biocontrol using yeast was found to be effective when activating inducible resistance responses in fruit tissues, and proved to be superior to chemical or physical resistance activators due to its additional biological control effects (Droby et al., 2009; Liu et al., 2013).

Information on the mechanisms by which antagonists suppress disease beyond inhibition are still incomplete, despite high throughput technologies having been extensively used to explore the mechanisms underlying the fruit-antagonist interaction. A significant amount of evidence has been generated that points to ethylene playing an important role in promoting host resistance in citrus fruit (Ballester et al., 2011; Hershkovitz et al., 2012). Traditionally, ethylene has been studied in the context of fruit ripening and senescence, and more recently in abiotic and biotic stress responses. Wounding and pathogen attack in the fruit tissues triggers a burst of ethylene production in most cases (Hase et al., 2006; Tatsuki, 2010). It is particularly interesting that when peel disks or whole fruit were exposed to *Penicillium digitatum*, exogenous ethylene resistance was induced (Droby and Chalutz, 1994). Many disease resistance responses have been linked to being dependent on ethylene in postharvest apples (Akagi et al., 2011),

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tomatoes (Blanco-Ulate et al., 2013) and oranges (Mehta et al., 2007; Gonzalez-Candelas et al., 2010). However, at this time, no unequivocal evidence has been reported on the direct involvement of ethylene in biocontrol yeast-induced resistance in fruit, and whether or not this hormone mediates specific fruit responses to antagonistic yeast induction is still unclear.

Ethylene in plant tissues is synthesized from S-adenosyl-L-methionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC) by two enzymes: ACC synthase (ACS), which catalyses the conversion of SAM into ACC, and ACC oxidase (ACO), which converts ACC into ethylene. In all the species examined, the two enzymes, ACS and ACO, which are encoded by multigene families, are regarded as the limiting factor in ethylene biosynthesis (Yang and Hoffman, 1984). The members of the *ACS* or *ACO* gene families are differentially expressed during plant development or in response to biotic and abiotic stress stimuli (Broekaert et al., 2006). In Fortune mandarin (*C. Clementina* × *C. reticulata*), two *ACS* and one *ACO* cDNAs have been isolated by RT-PCR from flavedo tissue, which shows a co-ordinated temporal and spatially distinct up-regulation in the burst of ethylene production upon chilling injury or pathogen infection (Zacarías et al., 2003; Marcos et al., 2005). In addition, as in *Arabidopsis*, a small family of receptors, such as *ETR1*, mediate the ethylene responses in citrus fruit. Full-length nucleotide sequences of four ethylene receptors (*CsETR1*, Cs*ETR2*, *CsETR3* and *CsERS1*) have been cloned from *C. sinensis* 'Valencia' by John-Karuppiah and Burns (2010). Likewise, these signaling receptors work as negative regulators in ethylene-dependent pathways, which actively repress the ethylene response in the absence of the hormone and are inactivated by ethylene binding (Hua and Meyerowitz, 1998). However, the responses observed in the receptors (*CsETR1*, *CsETR2*, *CsETR3* and *CsERS1*) are different due to different cultivars exhibiting different responses when exposed to ethylene.

Our previous studies indicated that the beneficial yeast *Rhodosporidium paludigenum* Fell & Tallman could effectively improve disease resistance in citrus fruit against *P. digitatum* (Lu et al., 2013a,b; unpublished results). These studies suggested that in the induction of host resistance, ethylene plays an important role, although its involvement is still speculative. 1-Methylcyclopropene (1-MCP) has the potential to be used in researching the ethylene-signaling dependence response, since it acts by binding to the ethylene receptor and thereby blocking ethylene binding sites (Tassoni et al., 2006). To determine which parts of the ethylene signal-transduction pathway are required for *R. paludigenum*-mediated defense mechanisms, we analyzed the ability of Ponkan mandarin to express defensive responses when ethylene perception was blocked by 1-MCP. In addition, over a range of different resistance induction stages in the host, the expression of genes related to the expression of ethylene biosynthesis, perception and host protection were analyzed.

2. Materials and methods

2.1. Fruit and microorganisms

Ponkan mandarins (*Citrus reticulata* Blanco cv. Ponkan) were obtained from an experimental orchard located in Chun'an City (Zhejiang Province, China). Shortly after harvest, mature fruit of similar size and color were surface sterilized and dried as previously described by Lu et al. (2013a).

The yeast antagonist *R. paludigenum* (IMI 394084, CABI Bioscience Identification Services, UK) was originally isolated from the south East China Sea, and cultured in 250 mL flasks containing nutrient yeast dextrose broth (NYDB) (Wang et al., 2008). After incubation in a gyratory shaker at 28 °C for 36 h, yeast cells were collected by centrifugation at 50 s^{-1} for 15 min, washed twice and resuspended with sterile distilled water.

The pathogen *P. digitatum* (Pers.: Fr.) Sacc. was isolated from decayed mandarin fruit and cultured on potato-dextrose agar (PDA) plates at 25 °C in the dark. Conidia were flooded from 1-week-old plates, and resuspended in sterile distilled water containing 0.05% Tween 20. The number of yeast cells and fungal spore concentration were determined with a hemocytometer.

2.2. Fruit 1-MCP treatment and inoculation

Fruit were fumigated with either air (control) or 1-MCP gas at concentrations of 50 nL L^{-1}, 500 nL L^{-1} and 5000 nL L^{-1} (Jiacheng biotechnology Ltd., Lanzhou, China) prior to inoculation. Active 1-MCP was released by dissolving the appropriate amount of the compound in 1 mL of 1% potassium hydroxide solution, which was immediately placed inside the containers. Treatments were carried out at 25 °C for 18 h in sealed 25 L glass jars (12 fruit per jar).

Using the method of Lu et al. (2013a), the biocontrol activity of the antagonistic yeast *R. paludigenum* was evaluated. After the gas treatments, the fruit were wounded twice at the blossom ends, and each wound was inoculated with 50 μL of either sterile distilled water or a cell suspension of *R. paludigenum* at 1×10^8 cells mL^{-1}. After incubation at 25 °C for 48 h, a second wound (2 mm deep and 5 mm diameter) was made approximately 6 mm away from the initial wound and inoculated with 30 μL of a spore suspension of *P. digitatum* (5×10^4 spores mL^{-1}). The fruit were stored in enclosed plastic trays to maintain high RH (90–95%) at 25 °C. After incubation for 60 h, the percentage of infected wounds and the lesion diameters were recorded. There were three replicates per treatment with twelve fruit samples per replicate, and the experiment was conducted twice with similar results.

2.3. Ethylene production

Ethylene production from fruit pre-treated with different gases was determined at 0, 24 and 48 h after inoculation with *R. paludigenum*, and three replicates of 27 fruit per treatment were weighed and sealed in a 2.09 L container at 25 °C. They were incubated for 2 h and then 1 mL of the head space gas from each jar was removed and injected into a gas chromatograph (GC; GC-2014C, Shimadzu Co., Ltd., Kyoto, Japan) equipped with a 2000 × 3 mm stainless-steel column of aluminum oxide to determine the ethylene production. The temperatures of the column, injector and flame ionization detector (FID) were 85 °C, 140 °C and 150 °C, respectively. N_2 (0.1 MPa) was used as the carrier gas, and the results were expressed as nL g^{-1} h^{-1}.

2.4. Growth of microorganisms in 1-MCP pre-treated surface wound

For the yeast population dynamics assays, fruit samples were taken as described by Zheng et al. (2007) at different time intervals (0, 24, 48, 72 and 96 h) after inoculation and peel tissue was removed using a cork borer. The resulting cylinders of excised tissue (2 mm deep × 1 cm wide) from three fruit were placed in a mortar with 15 mL of sterile distilled water and ground with a pestle. Then, serial tenfold dilutions were made and sprayed on to nutrient yeast dextrose agar (NYDA, containing 20 g agar in 1 L of NYDB) glass plates. The number of yeast colonies was determined after incubation at 25 °C for 2 days.

For the germination rate assays of *P. digitatum* spores, 30 μL of the spore suspension of *P. digitatum* (5×10^7 spores mL^{-1}) was added into the surface wounds on fruit treated with different gases and stored as above. Then the spores of *P. digitatum* were washed out from the wounds with sterile distilled water and counted using a hemacytometer. The number of germinated spores was expressed