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Transcriptomic and biochemical analysis of highlighted induction of phenylpropanoid pathway metabolism of citrus fruit in response to salicylic acid, *Pichia membranaefaciens* and oligochitosan

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ABSTRACT

The effects of salicylic acid (SA), *Pichia membranaefaciens* and oligochitosan on induction of disease resistance in citrus fruit were investigated using transcriptomic and biochemical analysis in this study. The results of disease incidence and lesion diameter showed that application of exogenous elicitors SA (2.5 mmol L^{-1}), *P. membranaefaciens* (1×10^8 cells mL^{-1}) or oligochitosan (15 g L^{-1}) were all effective in inhibiting blue and green molds in citrus fruit. Transcript profiling analysis of citrus fruit peel tissues revealed more differentially expressed genes in phenylpropanoid pathway metabolism induced by the three elicitors compared with other biological pathways. Moreover, biochemical analysis demonstrated that the three elicitors effectively enhanced phenylpropanoid pathway-related enzyme activities and stimulated the synthesis of phenolic acids and their subsequent metabolite lignin. Therefore, global results indicated that the activation of the phenylpropanoid biosynthesis pathway plays an important role in the induction of resistance in citrus fruit by the three elicitors.

1. Introduction

Postharvest citrus fruit are subjected to a series of biotic and abiotic stresses that cause physiological and biochemical changes and eventually lead to fruit quality deterioration, nutrient loss, water loss and decay (Sheng et al., 2017). *Penicillium italicum* and *Penicillium digitatum*, which are the causal agents of blue mold and green mold, respectively, can cause tremendous losses to citrus fruit during storage and transportation (Zeng et al., 2010). Control of these fungi is mostly based on the use of chemical fungicides, such as imazalil, thiabendazole, pyrimethanil, prochloraz, and fludioxonil, which are used to control postharvest blue and green molds in citrus fruit (Hao et al., 2011). However, due to increasing concern about the potential harmful effects of fungicides in the environment and human health and the development of fungicide resistance in pathogens, researchers are seeking to find alternative means to control postharvest diseases (Palou et al., 2016; Ragsdale and Sisler, 1994). Application of biological, chemical, or physical agents known as resistance inducers or elicitors, which increase ecological security and safety for consumers, is an innovative approach to manage postharvest fruit decay (Landi et al., 2014; Romanazzi et al., 2016). These elicitors activate the natural phenomenon known as induced resistance with effects that are localized or, more often, systemic and that promote nonspecific resistance to pathogens (Walters et al., 2013).

SA, a simple natural plant phenolic compound, has been suggested to be an endogenous signal molecule for inducing a long lasting, broad spectrum and systemic immunity (systemic acquired resistance) in harvested fruit and vegetables (Garcia-Brugger et al., 2006). Recent studies have shown that exogenous application of SA to susceptible plants has universal effects on enhancing the disease resistance of postharvest fruit to fungal pathogens (Asghari and Aghdam, 2010; Babalar et al., 2007; Zhu et al., 2016). *Pichia membranaefaciens* is a well-known biocontrol yeast. Previous studies have reported that it can inhibit the growth of green and blue molds on citrus fruit and protect other fruit from fungal pathogen attack. In addition to competing for nutrients and space with pathogens, inducing resistance is another important strategy for reducing diseases in plants as well as harvested fruit (Chan and Tian, 2006; Luo et al., 2012; Wang et al., 2011). Except for SA and *P. membranaefaciens*, with regards to other biologically active substances, chitosan and its derivatives such as oligochitosan also function in disease response and defense action. Oligochitosan is easily soluble in water when compared with chitosan. It is prepared by the

Abbreviations: PAL, phenylalanine ammonialyase; POD, peroxidase; PPO, polyphenoloxidas; C4H, cinnamate 4-hydroxylase; 4CL, 4- coumarate: coenzyme A ligase; SA, salicylic acid; DEGs, differentially expressed genes

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enzymatic hydrolysis of deacetylated chitosan polymers, possesses versatile functional properties that are useful in the field of agriculture and has been considered a potential plant disease vaccine (Yin et al., 2010). In addition to the direct antifungal effect on the pathogen, oligochitosan can elicit multiple plant defensive reactions against various types of biotic and abiotic stresses in several plants (Deng et al., 2015). Many previous studies have analyzed the molecular and physiological bases of induced resistance of SA, *P. membranaefaciens* or oligochitosan at individual gene or enzyme activity levels. However, limited information related to the three elicitors is currently available, especially at a systemic biological level.

Secondary metabolites, especially the phenylpropanoid pathway, play an important role in plant defense mechanisms via direct toxic effects and the active and rapid deposition of barriers, such as lignin (Ballester et al., 2011; Bennett and Wallsgrove, 1994). The expression levels of phenylalanine ammonialyase (*PAL*), *trans*-cinnamate 4-monooxygenase (*C4H*) and 4-coumarate-CoA ligase (*4CL*), all of which are the key genes in this pathway, are associated with the synthesis of a wide range of plant defense compounds (Dixon and Paiva, 1995). The involvement of *PAL* gene expression and the synthesis of phenylpropanoid-derived compounds have been reported previously in the citrus fruit response to biocontrol agents and to the citrus postharvest pathogen, *P. digitatum* (González-Candelas et al., 2010; Lu et al., 2015). Moreover, our previous studies have also demonstrated that *P. membranaefaciens* and oligochitosan treatments could increase content of total phenolic compounds (Deng et al., 2015; Luo et al., 2012). However, there is also an important lack of knowledge about the effects of the three elicitors on the metabolic pathway of phenylpropanoids and its regulatory network in citrus fruit.

The objective of this study was to evaluate (a) the effect of *Pichia membranaefaciens*, SA and oligochitosan treatments against *P. italicum* and *P. digitatum* in citrus fruit; (b) the changes in the transcript pattern though RNA-Seq in citrus fruit induced by these elicitors; (c) the effect of these elicitors on phenylpropanoid pathway-related enzyme activities in citrus fruit; (d) the effect of these elicitors on phenylpropanoid pathway-related compounds in citrus fruit.

2. Materials and methods

2.1. Microorganisms and fruit materials

2.1.1. Fruit

Citrus fruit [*Citrus sinensis* (L.) Osbeck cv. Jincheng 447[#]] were harvested at commercial maturity from an orchard in Beibei District, Chongqing, China. After harvesting, the fruit were immediately transported to the laboratory and were selected based on uniform size, color, and absence of defects. The selected fruit were surface-disinfected with 2% (v/v) sodium hypochlorite for 2 min, washed with tap water, and air-dried at room temperature (20 °C) prior to use.

2.1.2. Antagonist

The *P. membranaefaciens* yeast strain was obtained from the Chinese General Microbiological Culture Collection Center, Beijing, China. *P. membranaefaciens* was prepared according to the method of Zhou et al. (2014). The yeast was resuspended in sterilized distilled water and adjusted to an initial concentration of 1×10^8 cells mL^{-1}.

2.1.3. Pathogen inoculum

P. italicum and *P. digitatum* were isolated from infected citrus fruit [*Citrus sinensis* (L.) Osbeck cv. Jincheng 447[#]] and identified by morphology and sequence of the internal transcribed spacer (ITS) rDNA region (Jeong et al., 2016). The pathogens were maintained on potato dextrose agar medium at 4 °C. Conidial suspensions of the pathogens were prepared by flooding the 5-day-old culture dishes and incubated on Petri dishes at 28 °C with sterile distilled water containing 0.1 g kg^{-1} Tween-80. The conidial suspension was adjusted to 1×10^5 spores mL^{-1} with sterile distilled water using a hemocytometer.

2.2. Elicitor treatment and sampling

In the current experiment, citrus fruit were wounded with a sterile borer at two points (4 mm deep × 4 mm wide) at the equator of each fruit. Each wound was inoculated with 30 μL of one of four following elicitors: (1) sterile distilled water as the control, (2) 2.5 mmol L^{-1} SA (purity of ≥99%, purchased from Sigma-Aldrich, St. Louis, MO, USA) solution, (3) *P. membranaefaciens* (1×10^8 cells mL^{-1}), or (4) 15 g L^{-1} oligochitosan (molecular mass: 1500–2000 Da, purchased from Jinan Haidebei Marine Bioengineering Co., Ltd., Shandong, China) solution. After air drying, fruit were directly packed in plastic bags and incubated at 20 °C and 85% to 90% relative humidity (RH). For RNA extraction and assaying enzyme and non-enzyme components related to disease resistance, peel samples were taken from the entire wounds, which was removed from 15 fruit (two wounds per fruit) using a cork borer (2 cm in diameter) at 0 (1 h after elicitor treatment), 12, 24, 48, 72, 96 and 120 h at 20 °C for each treatment. Each treatment had three replicates, with 15 fruits per replicate, and the experiment was conducted twice. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Efficacy of salicylic acid, P. membranaefaciens and oligochitosan on control of blue and green molds in fruit wounds

The citrus fruit were wounded and treated with different elicitors as previously described. After 24 h incubation at 20 °C, a 20 μL of 1×10^5 spores mL^{-1} suspension of *P. italicum* or *P. digitatum* was inoculated into each wound. After air drying, all fruit were separately packed in plastic bags and incubated at 20 °C and 85% to 90% RH. The disease incidence and lesion diameter of each fruit were measured according to the method of Zeng et al. (2006) and recorded daily. There were three replicates per treatment with 10 fruit per replicate, and the experiment was conducted twice.

2.4. Effects of salicylic acid, P. membranaefaciens and oligochitosan on induced disease resistance

Evaluation of induced disease resistance by different elicitors was performed according to the method of Droby et al. (2002). The citrus fruit were wounded and treated with different elicitors as previously described. To remove the direct effect between the elicitors and pathogens, after 24 h incubation at 20 °C, a second wound (4 mm deep × 4 mm wide) was made approximately 1 cm away from the initial wound and inoculated with of a spore suspension of *P. italicum* or *P. digitatum* (1×10^5 spores mL^{-1}). After air drying, all fruit were separately packed and stored as before. The measurement method and other details were the same as those in the experiment which the elicitors and pathogens were inoculated on the same wounds.

2.5. Measurement of phenylpropanoid metabolism related enzymes

For the phenylalanine ammonia-lyase (PAL) activity assay, 1.0 g of fresh citrus fruit peel sample was homogenized with 5.0 mL of ice-cold sodium borate buffer (100 mmol L^{-1}, pH 8.8) containing 5 mmol L^{-1} β-mercaptoethanol, 2 mmol L^{-1} ethylene diaminetetraacetic acid, and 4% (w/v) polyvinyl pyrrolidine. The homogenized sample was then thoroughly ground at 4 °C. The homogenate was centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernate was then collected for the enzymatic assay. PAL activity was determined according to the method described by Luo et al. (2012). The specific enzyme activity was expressed on fresh weight basis as units (U) g^{-1}. One unit of PAL activity was defined as the amount of enzyme that causes an increase of 0.01 in the absorbance at 290 nm in 1 h under specified conditions.