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Expression of the *LePR5* gene from cherry tomato fruit induced by *Cryptococcus laurentii* and the analysis of LePR5 protein antifungal activity

Jun Guo[a,b,c], Xin Zhao[d], Huili Wang[b,c], Ting Yu[b,c], Ying Miao[e,*], Xiaodong Zheng[b,c,**]

[a] Key Laboratory of Food Nutrition and Safety, Ministry of Education, Tianjin Key Laboratory of Food Nutrition and Safety, Tianjin University of Science and Technology, Tianjin 300457, People's Republic of China
[b] Zhejiang Key Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou, 310058, People's Republic of China
[c] Fuli institute of Food Science, Zhejiang University, Hangzhou 310058, People's Republic of China
[d] Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou 310058, People's Republic of China
[e] The Center for Molecular Cell and Systems Biology, College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China

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ABSTRACT

The biocontrol yeast *Cryptococcus laurentii* could induce disease resistance of cherry tomato fruit and significantly reduce black rot incidence. Previous research reported that *LePR5* was up-regulated when postharvest cherry tomato fruit were treated by biocontrol yeast *C. laurentii* for 24 h. LePR5, a defense related protein from cherry tomato fruit, belongs to the thaumatin-like proteins. The amino acid sequence and predicted protein model of LePR5 have all the reported structural elements that are presumed necessary for activity resistant to pathogens. Using real-time PCR analysis this study showed that *LePR5* transcript levels were increased significantly after inoculation with *Alternaria alternata* and *C. laurentii*. The *LePR5* expression level induced by *A. alternata* was significantly higher than by *C. laurentii* after 48 h. Furthermore, LePR5 protein was produced and purified by using an *Escherichia coli* expression system and its refolded protein showed antifungal activity to *A. alternata* in vitro and in vivo. Finally, LePR5 protein treatment apparently controlled the disease incidence of black rot infection caused by *A. alternata* in cherry tomato fruit in a dose-dependant manner. These results suggest that LePR5 plays a role in the defense system of cherry tomatoes and is involved in the biocontrol mechanism involving yeast by inducing a resistance protein against pathogens in postharvest fruit.

1. Introduction

Cherry tomato (*Lycopersicon esculentum*) fruit is popular and is consumed on a worldwide scale. The loss of cherry tomato fruit due to *Alternaria alternata*, a saprophytic pathogen of tomato, is significant (Troncoso-Rojas et al., 2005; Feng and Zheng, 2007). However, the use of microbial inoculants for biocontrol is a promising solution for a sustainable, environmentally friendly agriculture (Berg, 2009; Lugtenberg et al., 2013) and biological control with antagonistic yeasts has been considered to be an effective method to control postharvest diseases of fruits (Sharma et al., 2009).

Cryptococcus laurentii was selected as a biological antagonist to protect fruits against a number of postharvest pathogens (Filonow, 1998; Meng et al., 2010; Yu et al., 2008). The activation of host defenses is one of the mechanisms of biocontrol activity in antagonistic yeasts (Yu et al., 2008; Droby et al., 2009; Sharma et al., 2009).

Plants may defend themselves by activating a variety of defense responses against pathogen infections. Pathogen-related (PR) proteins in plants, a major factor against fungal pathogens, are induced in the response to pathogen invasion and related stress factors (Van Loon et al., 1994; Ferreira et al., 2007). These proteins are classified into different families on the basis of sequence, serological relationship, and biological function (Van Loon et al., 1994, 2006).

* Corresponding author at: Fujian Agriculture and Forestry University, No. 15 Shangxiadian Road, Fuzhou 350002, People's Republic of China.
** Corresponding author at: Zhejiang University, No. 866 Yuhangtang Road, Hangzhou 310058, People's Republic of China. Fax: +86 571 88982191.
E-mail addresses: ymiao2013@hotmail.com (Y. Miao), xdzheng@zju.edu.cn (X. Zheng).

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PR5 family proteins are also called thaumatin-like proteins (TLPs) for their sequence similarity with thaumatin, a sweet-tasting protein found in the fruit of *Thaumatococcus danielli* (Wel and Loeve, 1972). PR5 proteins are widespread in the plant kingdom and the expression of *PR5* genes are induced by biotic and abiotic stress factors (Liu et al., 2010; Van Loon et al., 2006). To date, several PR5 proteins have shown in vitro antifungal activity, including Zeamatin from *Zea mays* (Roberts and Selitrenniko, 1990), PR5d from *Nicotiana tabacum* cv. Samsun NN (Koiwa et al., 1997) and RlemTLP from *Citrus jambhiri* Lush (Kim et al., 2009). Overexpression of *PR5* proteins in plants shows enhanced resistance to pathogens (Fu et al., 2005; Mackintosh et al., 2007; Velazhahan and Muthukrishnan, 2003), however, the exact function of PR5 proteins has not yet been explored clearly.

In the present report, the efficiency of *C. laurentii* in suppressing black rot caused by *A. alternata* was studied. In previous research in this laboratory, Affymetrix Tomato Genechip microarrays were used to evaluate changes in gene expression in response to *C. laurentii* in cherry tomato fruit. In one study, the *LePR5* gene was significantly up-regulated (Jiang et al., 2009) and in the other study *LePR5* gene was cloned (Ren et al., 2011). In the present study, to analyze the role of *LePR5* gene of cherry tomato fruit in induced defense response to *C. laurentii*, the expression patterns of the *LePR5* transcript were studied when the cherry tomato fruit were inoculated with *C. laurentii* and *A. alternata*, respectively. LePR5 recombinant protein was obtained using an *Escherichia coli* expression system. The antifungal activity of LePR5 recombinant protein on *A. alternata* was further investigated in order to better understand the role of LePR5 protein in the defense mechanisms of cherry tomato fruit and its potential application in genetic engineering.

2. Materials and methods

2.1. Microorganisms

The antagonistic yeast *C. laurentii* (Kufferath) Skinner (CGMCC no. 3590) was isolated from the surface of pear fruit and identified by VITEK 32 Automicrobic System (bioMerieux Company, Marcy l'Etoile, France) (Yu et al., 2007). The yeast was then grown on nutrient yeast dextrose agar (NYDA: 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar in 1 L of distilled water) and incubated at 28 °C for 48 h. The yeast cells were then inoculated into a nutrient yeast dextrose broth (NYDB) and kept in a rotary shaker with 3.33 s^{-1} at 28 °C for 24 h. The cells were harvested by centrifuging at 1500 × *g* for 10 min and washed twice in sterile distilled water to remove the growth medium. The cell pellets were re-suspended in the sterile distilled water, counted on a hemocytometer and then adjusted to the desired concentrations.

The fungal pathogen *A. alternata* (CGMCC no. 3.4578) was obtained from IMCAS (Institute of Microbiology, Chinese Academy of Sciences, China) and was maintained on potato dextrose agar (PDA: the extract from 200 g boiled potato with 20 g glucose and 20 g agar in 1 L distilled water) at 4 °C, and fresh cultures were grown on PDA plates at 25 °C before use. Conidia were rubbed off from the agar surface of a seven-day-old culture of *A. alternata* with a sterile inoculating loop and then suspended in sterile distilled water. Spore concentration was determined by microscopic counting with a hemocytometer, and then adjusted as required with sterile distilled water (Wang et al., 2008).

2.2. Cherry tomato fruit

Cherry tomato fruit (*L. esculentum* Mill. cv. Miny Tomato) that were not injured or infected were collected at MG4 (mature green) stage and selected for uniformity of size from tomato plants grown under greenhouse conditions. Cherry tomato plants were grown in a growth chamber at 26 °C under 150 μmol m^{-2} s^{-1} illumination with a 14 h photoperiod. Fruit were surface-disinfected with 0.1% sodium hypochlorite for 2 min, then the fruit were rinsed with tap water and air-dried at room temperature in plastic crates.

2.3. Induction of disease resistance to A. alternata by C. laurentii

Cherry tomato fruit were randomly assigned to 2 groups and a single wound (5 mm diameter and ~3 mm deep) was punched in each fruit using a sterile borer. There were three replicates of 20 fruit in each group. In the first fruit group, 15 μL of suspensions of 1×10^8 cells/mL *C. laurentii* was inoculated in the wound. In the second fruit group, 15 μL of sterile distilled water was inoculated as a control. After being incubated at 20 °C for 48 h, a second wound (5 mm diameter and ~3 mm deep) was made approximately 5 mm beneath the initial wound in each fruit and inoculated with 15 μL *A. alternata* (1×10^4 cells/mL). The disease incidence was recorded daily at 20 °C. This experiment was repeated twice.

2.4. Reverse transcription quantitative real-time PCR (RT-qPCR) analysis for the LePR5 mRNA expression level

Cherry tomato fruit were divided into 3 groups and a single wound (5 mm diameter and ~3 mm deep) was punched in each fruit using a sterile borer. The fruit of the three groups were inoculated with 15 μL (1) sterile distilled water as control, (2) 1×10^8 cells/mL *C. laurentii*, (3) 1×10^4 cells/mL *A. alternata*, respectively. The samples were then stored at 20 °C and sampled at 0, 12, 24, 36 and 48 h after treatment by the method described by Jiang et al. (2009).

Total RNA was extracted from the cherry tomato fruit tissue with Trizol reagent (Invitrogen, Carlsbad, California, USA) by the method described by Ren et al. (2011). The quality of RNA samples was checked on 1.0% (w/v) agarose/formaldehyde gels. The concentration of total RNA was determined using a NanoDrop1000 spectrophotometer (Thermo scientific, Wilmington, USA). The cDNA was synthesized using a PrimeScript RT reagent Kit with gDNA Eraser according to the manufacturer's instruction (TaKaRa, Dalian, China).

RT-qPCR was performed to evaluate the expression of the *LePR5* gene (GenBank accession number: GU184125). The primers used for *LePR5* were *LePR5* F: 5′-TGAATGCCCTGGTTCGCT-3′ and *LePR5* R: 5′-CATCGGGACATCTTTGTTTGA-3′. The amplification product was 140 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (GenBank accession number: U97257) was chosen as a reference gene. The primers of *GAPDH* were *GAPDH* F: 5′-CAACGAGAATGAATACAAGCCA-3′ and *GAPDH* R: 5′-GAGACCCT-CAACAATGCCAA-3′. The amplification product was 118 bp. The selected primers were tested by PCR. RT-qPCR was performed using an SYBR® Premix Ex-Taq™ II Kit (TaKaRa; Dalian, China). The reaction consisted of 2.0 μL cDNA and 1.0 μL of each gene-specific primer (10 μmoL/L) in a final volume of 25 μL. An ABI PRISM 7500 (Applied Biosystems, Shanghai, China) was used for the following thermal cycles: a single cycle at 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. A total of 5 data sets were obtained for each treatment at each time point for further analysis. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product. For relative quantification, the transcript levels were calculated as the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001). The *LePR5* gene expression of two groups treated by *C. laurentii* and *A. alternata* was expressed as fold-changes in relation to the control group at each time point, significant differences greater than 2.0-fold up were shown between transcript abundance in inoculated and control groups.