International Journal of Food Microbiology 278 (2018) 1–10

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Characterization and overexpression of *RHO1* from *Cryptococcus laurentii* ZJU10 activates CWI signaling pathway on enhancing the inhibition of blue mold on pears

Ming Lin, Yangyang Zhang, Cui Sun, Yining Huang, Jiahui Zhang, Xiaodong Zheng, Ting Yu*

National Engineering Laboratory of Intelligent Food Technology and Equipment, Key Laboratory for Agro-Products Postharvest Handling of Ministry of Agriculture, Key Laboratory for Agro-Products Nutritional Evaluation of Ministry of Agriculture, Zhejiang Key Laboratory for Agro-Food Processing, Fuli Institute of Food Science, College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310058, People's Republic of China

ARTICLE INFO

Keywords:
Penicillium expansum
Cryptococcus laurentii
Postharvest biological control
RHO1
Induced resistance
Recombinant expression

ABSTRACT

Results from this study explored the inhibitory effect of *RHO1* gene (GenBank accession number KY859864) from the antagonistic yeast, *Cryptococcus laurentii* ZJU10, on the control of *Penicillium expansum* in pear fruit and its possible mechanism involved. The *RHO1* gene was successfully cloned and overexpressed in *Saccharomyces cerevisiae*. Sequence analysis showed high similarity with Rho family proteins, implying a primary role of Rho1 in the cell wall integrity (CWI) signaling pathway. Gene expression of *RHO1* and other five CWI-related genes (including Pkc1, Rlm1, Fks1, Fks2 and Chs3) were significant up-regulated in the treatment of SC/Rho1-induced strain (*Saccharomyces cerevisiae* transformed with *RHO1* and induced by galactose). Meanwhile, SC/Rho1-induced treatment reduced about 61.5% of disease incidence and almost 5-times lower lesion diameter compared to the control. In addition, the growth of transformed strains was slightly lower in contrast to the wild *Saccharomyces cerevisiae* and the induction of fruit resistance was significantly enhanced, which was tightly linked with triggering stronger host defensive responses by priming activation. This is the first study that Rho1 has a potential function of suppressing fungal disease in harvested fruit by activating CWI signaling pathway and indicates an alternative strategy for postharvest disease management.

1. Introduction

Penicillium expansum is one of major pathogens of pear fruit worldwide (Sanchez et al., 2016), which causes considerable losses during transportation and postharvest storage period (Banani et al., 2014; Sharma et al., 2009). Besides the economic aspects, metabolite secreted by *P. expansum* leads the potential risk of human's health and environment, which has attracted more and more public attention. Moreover, persistent misuse of synthetic fungicides has caused increasing resistance of pathogens. Hence, the need to develop alternative control method has become increased (Nunes, 2012; Palou et al., 2016).

Cryptococcus laurentii has been shown great efficacy on controlling postharvest disease of various fruit, including anthracnose on mangos (Ocampo-Suarez et al., 2017), *P. expansum* on peaches (Zhang et al., 2017) and on apples (Jiangkuo et al., 2017). An extensiveness of modes of action have been proposed as mechanisms for biocontrol, such as competition for nutrients and space (Yu and Lee, 2015), secretion of lytic enzymes (Banani et al., 2015), induction of resistance (Lu et al., 2013), mycoparasitism and antibiosis (Pretorius et al., 2015; Zhong, 2012). Most of the works are focused on secretion of lytic enzymes and competition for nutrients and space, however, a clear understanding of molecular mechanism (including induction of resistance) is still difficult due to the lack of genetic background of antagonistic yeast and methods. Transcriptomic analysis by high-throughput sequencing technology makes it possible to study gene expression changes and provides an insight on the molecular mechanism, so as to accurately define and help improve the effectiveness of biological control.

The cell wall is the first barrier for fungal cells to connect with the outside world and has been demonstrated involved in reducing disease resistance in pear fruit (Sun et al., 2018). Rho1 is an essential protein that controls CWI signaling pathway which receives inputs from the cell surface and regulates activation of protein kinase C (Pkc1), organization of actin cytoskeleton, activity of β-1,3-glucan synthase (GS) and polarized secretion (Levin, 2005, 2011). Meanwhile, Delgado et al. (2016) demonstrated that Rho1 accumulation provides a complementary means to combat resistance to antifungal proteins. Nevertheless, few studies have been reported in the role of antagonistic yeast Rho1 in inhibition of fungal disease of harvested fruit.

* Corresponding author.
E-mail address: yuting@zju.edu.cn (T. Yu).

https://doi.org/10.1016/j.ijfoodmicro.2018.04.022
Received 24 August 2017; Received in revised form 5 April 2018; Accepted 13 April 2018
Available online 19 April 2018
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Table 1
Primers for CWI pathway genes used in this study.

Gene	GenBank accession	Forward primer (5′-3′)	Reverse primer (5′-3′)
RHO1	KY859864	AGGTCGACGGCAAGAAAG	CGAGTCAATGGCGAAACA
Pkc1	NM_001178345.2	TGGTGGTTCTTCTCTCGCT	TGATGTATTTTCGCCTTGC
Rlm1	NM_001183903.1	TATCCTTTCGGAAGTGGGT	CTGGGTTTGTCTTTGAGCT
Fks1	NM_001182231.1	TCGTTTTCAATCCACATCAG	ACGTGCCCTAGACATCCTC
Fks2	NM_001181161.3	GATGTTGATTGGCGTTGTT	ATTCCCTTGTCGGTTGAGT
Chs3	NM_001178371.1	GCAGACTTCTACGAAACGG	TCTCACCACAAAGACCCAT
Actin	NM_001179927.1	AATTGTCCGTGACATCAAGG	CGGCAGATTCCAAACCC

The objective of this study was to assess *RHO1* gene from *C. laurentii* ZJU10 overexpressed in *S. cerevisiae* on control of blue mold decay caused by *P. expansum* in pear fruit during the postharvest storage and the potential mechanism involved.

2. Materials and methods

2.1. Fruit, microorganisms and pathogen

Pear fruit (*Pyrus pyrifolia* Nakai, Shuijing) was harvested at commercial maturity in Hebei Province, China and sorted based on ripeness, uniform size and absence of any apparent injuries or infections. Prior to treatments, pears were disinfected in 0.1% (v/v) sodium hypochlorite solution for 1 min, then rinsed with tap completely and air-dried at room temperature (25 °C).

Isolation of *C. laurentii* ZJU10 was obtained from the surface of pears and cultured on nutrient yeast dextrose agar (NYDA) media at 28 °C for 48 h (Zeng et al., 2015). *Saccharomyces cerevisiae* was purchased from Institute of Microbiology, Chinese Academy of Sciences (Beijing, China) and cultured in yeast extract peptone dextrose (YEPD) medium.

The pathogen *P. expansum* was obtained from decayed pear fruit and examined morphology by microscope, then maintained on potato dextrose agar (PDA). The yeast cells and *P. expansum* spore concentration were adjusted to the desired concentration with sterile distilled water by a hemocytometer (Zeng et al., 2015).

2.2. Plasmid and molecular kits

The *Escherichia coli* strain DH5α used as host for plasmid was from Tiangen Biotech (Beijing, China). The vector pYES6/CT was purchased from Invitrogen (Life Technologies, Carlsbad USA). Kits for PCR reaction was from Vazyme Biotech (Nanjing, China) and enzymes were obtained from TaKaRa Biotechnology (Dalian, China). The PCR purification and plasmid extraction were purchased from Corning Life Science (Wujiang, China). Moreover, One Step Cloning Kit was from Vazyme Biotech (Nanjing, China).

2.3. Expression of RHO1 in S. cerevisiae

2.3.1. Clone of RHO1 gene and construction

To study the *RHO1* sequence, we constructed cDNA library from *C. laurentii* ZJU10 and obtained 30,374 Unigenes clusters. Among these Unigenes clusters, one suspected sequence of *RHO1* was found. Primers for amplifying the Open Reading Frame (ORF) encoding *RHO1* were designed as URS + *RHO1*-F: 5′–CTTGGTACCGAGCTCGGATCCATGTC CGGCGAAATCAGACG-3′ and DRS + *RHO1*-R: 5′-GAAGGGCCCTCTAG ACTCGAGGAGAACAACACAGCCGCCCT-3′. Genomic DNA was extracted from *C. laurentii* ZJU10 according to the molecular cloning procedures (TaKaRa Biotech. Dalian, China). PCR reaction program was 95 °C for 30 s at an initial step, followed by 35 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min, with final extension 72 °C for 5 min. The purified PCR fragment was ligated into the linearized pYES6/CT vector in-frame to the GAL1 promoter. Insertion of the coding fragment was confirmed by DNA sequencing at TSINGKE Biological Technology (Hangzhou, China).

2.3.2. Transformation and selection of recombinant strains

The recombinant plasmid pYES6/CT-Rho1 was transformed into the competent *S. cerevisiae* cells by electroporation according to Bio-Rad manufacture instruction. The original plasmid (pYES6/CT) was also transformed into *S. cerevisiae* for negative control. As plasmids carried the blasticidin resistance gene (bsd), the positive transformants were selected through YEPD medium containing blasticidin S. To identify whether the *RHO1* gene had been transferred into *S. cerevisiae*, PCR amplification and sequencing were operated. Primers used for the reaction, GAL1-F: 5′-AATATACCTCTATACTTTAACGTC-3′ and CYC1-R: 5′-GCGTGAATGTAAGCGTGAC-3′ were synthesized according to the manufacturer. *S. cerevisiae* that transformed with pYES6/CT and pYES6/CT-Rho1 were designated as SC/pYES6 and SC/Rho1, respectively.

2.3.3. Analysis of RHO1 expression in S. cerevisiae

The transformed strain cultured in the medium containing galactose was designated as SC/Rho1-induced, while SC/Rho1 grown with glucose served as a control. Transcription would be induced by adding galactose instead of glucose as a carbon source (Giniger et al., 1985). The RT-qPCR was performed to quantify the expression of *RHO1* at different time course (2, 4, 8 h after galactose induction). As Rho1 is a key factor of the CWI signaling pathway, other genes involved in pathway (Pkc1, Rlm1, Fks1, Fks2 and Chs3) were also examined for support of activating. The primers used for this study were as Table 1. Relative quantifications were performed as Banani et al. (2015) with some modification. Each treatment included three biological replicates.

To characterize the Rho1 protein synthesized by recombinant strains, western-blotting was performed. Rho1 protein synthesized in the cells was obtained by Yeast Protein Extraction Reagent (TaKaRa Biotech. Dalian, China) and then subjected to SDS-PAGE. The monoclonal anti-V5 was taken as primary antibody and HRP-labeled Goat Anti-Mouse IgG (H + L) as secondary antibody. Results were examined by FR-1800 Luminescent and fluorescent biological image analysis system (Furi-tech, Shanghai, China).

2.4. Biocontrol of recombinant strains against P. expansum on pear fruit

Pear fruit was sterilized and gently wounded with a sterilized punch in the equatorial region (5 mm in diameter and 5 mm deep). Each wound was added 50 μL of yeast cell suspensions (10^7 cells/mL) and sterile distilled water as control. Then aliquots of 30 μL *P. expansum* spore suspensions at 10^4 conidia/mL were inoculated into the wound after 2 h. Pear fruit were placed in plastic trays and covered with preservative film after air-dried, then maintained in relative high humidity (90%) at 25 °C in the dark.

The antifungal activity of recombinant strains against *P. expansum* was assessed following the method of Ren et al. (2012) with modifications. Each of the infected pears was monitored daily after 48 h. For each treatment, twenty-seven pears were selected as a unit and each treatment included three replicates.

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