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# Biocontrol of fungal decay of citrus fruit by *Pichia pastoris* recombinant strains expressing cecropin A

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ABSTRACT

Cecropin A gene was cloned into the expression vector pPIC9k and was successfully expressed in methylotrophic yeast, *Pichia pastoris* GS115. The yeast had effective antimicrobial activity on *Geotrichum citri-aurantii* spores by the thiazolyl blue (MTT) assay. There was no large growth difference between non-transformed strain GS115 and recombinant strain GS115/CEC in citrus fruits wounds. Yeast transformants could significantly inhibit growth of germinated *G. citri-aurantii* spores and inhibited decay development caused by *G. citri-aurantii* in citrus fruits compared to the yeast strain GS115/pPIC. This study demonstrates the potential of expression of an antifungal peptide in yeast for enhancing suppression of postharvest diseases and represents a new approach for the biological control of postharvest diseases.



## 1. Introduction

It has been reported that citrus sour rot caused by *Geotrichum citri-aurantii* is an important postharvest disease of citrus fruit worldwide (Lahlali, Hamadi, Guilli, & Jijakli, 2011; Liu et al., 2009). It can cause tremendous losses during periods of high rainfall. In China, most postharvest diseases are controlled by chemical fungicides, which bring some problems in human safety and environment protection (Droby, 2006). Biological control of postharvest decay (BCPD) with antagonistic yeasts is a promising strategy for postharvest disease control (Janisiewicz et al., 2008) and is currently used to control various decays in citrus, pome fruits, stone fruits, avocado, seed potatoes, and sweet potatoes (Stockwell & Stack, 2007; Wang et al., 2011). However, the effectiveness of general yeast on postharvest decay control is not comparable to fungicides under changeable postharvest environments (Wang et al., 2011). The future expansion of antagonistic yeast usage will largely depend on improving its effectiveness under an increased range of conditions and expanding its activity spectrum to new commodities and new diseases. This may be achieved by discovering new antagonists (Mercier & Jimenez, 2004), by combining antagonists with other alternatives to synthetic fungicides (e.g. GRAS substances or physical treatments; Droby, 2006; Obagwu & Korsten, 2002; Wang et al., 2011; Zong, Liu, Li, Qin, & Tian, 2010), by combining antagonists with different mechanisms of biocontrol (Conway, Leverentz, Janisiewicz, Saftner, & Camp, 2005), or by improving antagonists using genetic manipulation. Genetic manipulation shows tremendous potential for improving BCPD. For example, antagonists can be manipulated to over-express mechanisms of biocontrol, or foreign genes can be transferred to antagonists to increase their tolerance to environmental stresses or to produce antifungal substances (Jones & Prusky, 2002; Wisniewski et al., 2005). In this way, it may be feasible to convert microorganisms that can colonize fruit but do not exhibit antagonistic activity into biocontrol agents (Jones & Prusky, 2002).

Antimicrobial peptides are an integral component of the innate immune system. They can counteract outer membrane pathogens, such as bacteria, fungi, viruses, protozoa and so on (Kim et al., 2010). Insects produce a variety of antimicrobial peptides that play a crucial role in protecting them from invading microorganisms. Insect antibacterial peptides are classified into five major groups: cecropins, insect defensins, glycine-rich peptides, proline-rich peptides, and lysozymes (Kim et al., 2010). The molecular size of cecropins which are considered as the most potent antibacterial peptides is about 3500–4000 Da. Cecropins have a strong basic amino (N)-terminal part and a long hydrophobic carboxyl (C)-terminal stretch interrupted by a hinge region composed of a Gly-Pro sequence (Steiner, Hultmark, Engstrom, Bennich, & Boman, 1981). Cecropins were first isolated from *Hyalophora cecropia* (Hultmark, Engstrom, Bennich, Kapur, & Boman, 1982; Hultmark, Steiner, Rasmuson, & Boman, 1980), which have a broad spectrum activity against Gram-positive, Gram-negative bacteria and fungi and they act by destroying the ionic balance of the bacterial membrane by the formation of ionic pores (Hakan, Andreu, & Merrifield, 1988).

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The most attractive feature of antibacterial peptides is that they rarely induce drug resistance (Jin, Xu, Zhang, & Gu, 2006), which has become a serious problem with conventional antibiotics. Therefore, antimicrobial peptides have emerged from a new class of antibiotics as one of the most promising candidates.

For years, the heterologous expression system of the yeast *P. pastoris* has been successfully used for the production of a variety of proteins from different sources (Cereghino & Cregg, 2000; Janisiewicz et al., 2008). The yeast expression system offers many advantages. The yeast growth is fast, low cost and as eukaryotes, they have the machinery for post-translational modifications (Cereghino & Cregg, 2000). Recently, many antibacterial peptides have been expressed in *P. pastoris*, including *Pisum sativum* defensin1 (Janisiewicz et al., 2008), penaeidin (Li et al., 2005), and antilipopolysaccharide factor (Somboonwiwat et al., 2005). The main research fields of these recombinant strains are mostly in medicine, animal feed and so on, and rarely in biology control.

In an effort to improve the control of postharvest decay by biological means and to study the potential of recombinant strains expressing cecropin A for further characterization of this peptide in inhibiting postharvest decay of citrus fruits caused by *G. citri-aurantii*, as a precondition, the inhibition effects of this peptide on *G. citri-aurantii* spores *in vitro* was evaluated by MTT method, and then the corresponding cecropin A cDNA was cloned and expressed in the methylotrophic yeast *P. pastoris* (GS115). In this paper, the transformation of *P. pastoris* strain GS115 with pPIC9k/CEC and its use as a biocontrol agent for the control of citrus fruit decay caused by *G. citri-aurantii* was reported.

Through this study, we developed a new approach to control postharvest pathogens by expressing an antimicrobial peptide in *P. pastoris*. We chose the citrus fruit fungal pathogen *G. citri-aurantii* as a target to demonstrate the potential of bioengineered yeast in disease control.

## 2. Materials and methods

### 2.1. Materials

TA cloning vector pGEM-T, T4 DNA ligase, and Taq DNA polymerase were purchased from Dalian TaKaRa Biotechnology Company (Dalian, China). *P. pastoris* GS115, expression plasmid pPIC9K and *Escherichia coli* DH5α were purchased from Invitrogen (Carlsbad, CA) and were used for routine plasmid amplification. All restriction enzymes and low range protein markers were purchased from MBI Fermentas (Thurmo Fisher, Waltham, MA).

Citrus fruit cultivar '*Satsuma mandarin*' used in this paper was harvested at commercial maturity from Chun'an (Zhejiang province, China). Fresh fruits were surface-sterilized by incubation for 5 min in sodium hypochlorite (0.1%) solution, washed with distilled water, and then air dried prior to wounding.

### 2.2. Medium, fungi and culture

The yeast medium components of YPDS, MMH, MDH, BMGY and BMMY are as referred to Jin et al. (2006).

*G. citri-aurantii* was isolated from decayed *Satsuma mandarin* citrus fruit. The fungus was incubated on potato dextrose agar (PDA) plates at 4 °C (Liu et al., 2009). Arthroconidium suspension was rubbed from the medium surface with 5 ml of sterile distilled water. An arthroconidium suspension was determined by a haemocytometer and adjusted to the needed concentration.

### 2.3. In vitro inhibition effects assay

Antimicrobial effects on *G. citri-aurantii* spores were assayed using the thiazolyl blue (MTT) method. In MTT assay, the arthroconidium suspensions (100 μl) at a density of $5 \times 10^4$ spores/ml were plated in 96-well microtitre plates and incubated for 24 h at 28 °C. The peptides cecropin A (Sigma, Munich, Germany) solution at different concentrations (0.5, 1, 5, 10, 20, 40 and 80 μM) were added to each well and the well without peptides as control, then further incubated for 24 h under the same conditions. Then 20 μl of the MTT (Sigma) solution were added to each well and incubated for 4 h. During this period, living cells produce blue insoluble formazan from the yellow soluble MTT. The reaction was stopped by addition of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) (100 μl/well) and the contents of the wells were spontaneously dissolved during 2–3 min. Absorbance of each well was measured spectrophotometrically at 570 nm using an ELISA plate reader (Awareness Technology, Palm City, FL). All the tests were performed in triplicates.

The inhibitory rate of different concentrations peptides was calculated by the formula: $R = ((A_{control} - A_{treated})/A_{control}) \times 100\%$, where "*A*" and "*R*" represent absorbance and inhibitory rate, respectively. Dose–response curves were generated and the half maximal inhibitory concentration ($IC_{50}$) values of the peptides were defined as the concentration of compound required for inhibiting conidia proliferation by 50%.

### 2.4. Expression of cecropin A in P. pastoris (GS115)

#### 2.4.1. Clone of cecropin A and construction

According to the sequence of amino acids of mature peptides cecropin A reported in GeneBank (AAA29185), a cDNA fragment encoding the mature peptide was synthesized. To express the native N-terminus of cecropin A, an *Xho*I restriction site was introduced, at the same time, the *Not*I restriction site and 6× His-tag sequence was introduced along with a stop codon at the C-terminus. The cDNA fragment, which encodes the mature peptide of cecropin A was digested with *Xho*I and *Not*I enzymes and the digested fragment was ligated into the *Xho*I/*Not*I-digested pPIC9k in-frame to the α-factor secretion signal and down stream of the alcohol oxidase 1 (AOX1) promoter. This recombinant plasmid (pPIC/CEC) was transformed into competent *E. coli* DH5α and the insert was sequenced to ensure that the coding sequence of cecropin was correct and in-frame with the α-factor secretion signal.

#### 2.4.2. Transformation of P. pastoris and selection of transformants

The expression plasmids were digested by *Sal*I, and the plasmids were purified by agarose gel electrophoresis. The linearized plasmids were transformed into the competent *P. pastoris* GS115 ($his^-Mut^+$) cells by electroporation according to the manufacturer's instructions (Invitrogen). The transformants were selected according to the method of Jin et al. (2006). Then, the positive $Mut^s$ phenotype strains were obtained and the inserts were verified by PCR using genomic DNA as a template and CEC-up (5′-CTCGAGAAGTGGAAGTTGTTTA-3′) and CEC-down (5′-GCGGCCGCCTTAGCAATTTGA-3′) as primers. The positive $Mut^s$ strains named as GS115/CEC were used for suspension culture.

#### 2.4.3. Expression of cecropin A in P. pastoris

The highest level secreting *P. pastoris* clone (GS115/CEC) was cultured in 50 ml of BMGY medium for approximately 18 h at 30 °C with constant shaking. When these cultures reached an $OD_{600}$ nm of about 2.0–6.0, cells were centrifuged and the cell mass was resuspended in 100 ml of BMMY medium to induce expression of the recombinant proteins. The culture was supplemented daily with 0.5% methanol. Nine hundred microlitres of the expression medium were taken and concentrated by 100% TCA after 72 h culture and analysed for expression of recombinant proteins by Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Tricine–SDS–PAGE).