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

Xueyan Ren [a,b], Qingjun Kong [b], Huili Wang [a], Ting Yu [a], Wen-Wen Zhou [a,*], Xiaodong Zheng [a,*]

[a] School of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310058, Zhejiang, China
[b] Department of Life Science, Shihezi University, Shihezi 832003, Xinjiang, China

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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).

* Corresponding authors. Tel.: +86 57188982398; fax: +86 57188982861.
E-mail addresses: zww2003@gmail.com (W.-W. Zhou), xdzheng@zju.edu.cn (X. Zheng).

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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 5×10^4 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).

P. pastoris strain GS115/pPIC (GS115 transformed with pPIC9k) was used as a negative control.

2.5. In vitro arthroconidium germination assay

Yeast cultures of 3 days were centrifuged at 7000*g* for 10 min, resuspended in sterile distilled water, and then were crushed with acidic glass beads three times. The samples were centrifuged and resuspended twice to remove culture media. The concentration of cells in the suspensions was counted with a haemocytometer, and then diluted to a final concentration as required with sterile distilled water, according to the method of Liu et al. (2009). The effect of recombinant strains GS115/CEC on arthroconidium germination was tested in potato dextrose broth (PDB). Aliquots of 100 μl of GS115/CEC suspensions at 1×10^8 cells/ml were added to a 10 ml glass tube containing 5 ml PDB. At the same time, aliquots of 100 μl of *G. citri-aurantii* at 1×10^7 arthroconidia/ml were added to each tube and 100 μl GS115/pPIC 1×10^8 cells/ml and H_2O were used as negative controls. After 10 h of incubation at 26 °C on a rotary shaker (200 rpm), the samples were examined with a BH-2 light microscope (Olympus, Japan). Then, at least 200 arthroconidia per replicate were observed microscopically to determine germination rate.

2.6. Growth of recombinant strains in citrus fruit wounds

To determine the suitability of recombinant strains GS115/CEC for biocontrol tests on citrus fruit, the growth of recombinant strains GS115/CEC and nontransformed strains GS115 in citrus fruit tissue were evaluated. The amounts of strains GS115/CEC and GS115 were determined as described previously. Two yeast cultures were inoculated to achieve 1×10^5 cells/ml and shaken at 200 rpm at 28 °C. Citrus fruits were wounded with a cylindrical tool (about 5 mm in diameter and 3 mm deep), and then injected with 25 μl of the yeast suspension. Fruits were placed on trays in plastic boxes at 24 °C. The samples were removed at different times (0, 12, 24, 36, 48, 72 and 96 h) after inoculation using a bigger sterile cork-borer (10 mm diameter × 10 mm deep) and ground with a mortar and pestle in 10 ml of sterile distilled water. A haemocytometer was used to count GS115/CEC and GS115 yeast cells. There were three replicates of six citrus fruits for each treatment, and the experiment was conducted twice.

2.7. Biological control tests on citrus fruit

Yeast cultures of 3 days were harvested, crushed and counted as described previously. Citrus fruits were placed on trays in plastic boxes, wounded as described above, and the wounds inoculated with 50 μl of an aqueous suspension of the transformed yeast GS115/CEC and GS115/pPIC at 1×10^8 cells/ml or sterile distilled water, respectively. Four hours later, the wounds were inoculated with 20 μl of the conidial suspension of *G. citri-aurantii* at 5×10^4 spores/ml. Each treatment was kept in a polyethylene-lined plastic box to maintain high relative humidity (about 95%) at 25 °C for 5 days (Wang et al., 2011). The disease incidence (the percentage of wounds infected with sour rot and diameter of lesion) was examined after storage. Fruit with no infection was not counted for lesion size measurements. Each treatment was recorded three times with 20 fruits per replicate, and the experiment was repeated twice.

2.8. Statistical analyses

All data were analysed using SAS software Ver. 8.0 (SAS Institute, Cary, NC). Mean separations were performed by one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Differences at $P < 0.05$ were considered as significant. Data of the population level of GS115/CEC and GS115 yeast (cells per wound) were log-transformed to increase the homogeneity of variances.

3. Results

3.1. In vitro inhibition effects assay of cecropin A

In order to study the fungicidal activity of cecropin A on *G. citri-aurantii* spores, MTT method was used to confirm the inhibitory rate of peptides of different concentrations to *G. citri-aurantii* spores. Results showed the obvious dose–response relationship between the inhibitory rate and doses of peptides cecropin A. Fig. 1 showed that the IC_{50} of this drug was determined as about 10 mM on these spores, and when the concentrations of peptides was 40 mM, its inhibitory rate can reach 80.69%.

3.2. Construction of recombinant P. pastoris and expression of cecropin A

Due to effective antimicrobial effects of cecropin A on *G. citri-aurantii* spores, the corresponding cecropin A cDNA was cloned. According to the reported sequence of cecropin, a 125 bp DNA product was synthesized, which was ligased into pPIC9k vector, resulting in the recombinant vector pPIC/CEC. Then the pPIC9k and pPIC/CEC plasmids were transformed into *P. pastoris*, GS115 stain to obtain recombinant strain GS115/pPIC and GS115/CEC. After incubation for 3–4 days in selection medium, the Mut^+ phenotypes grew normally on both MMH and MDH, whereas the Mut^s phenotype grew very slowly on MMH plates and the high Zeocin™-resistant positive transformants were selected on MDH plates. The transformants were confirmed by PCR amplification from genomic DNA using the primers specific for pPIC/CEC (data not shown). We investigated the expression of recombinant strain GS115/CEC in shake flasks. Tricine–SDS–PAGE revealed that the molecular weight of expressed cecropin A in recombinant strain GS115/CEC was about 5 kDa, which was consistent with the theoretical molecular weight (4.95 kDa) (Fig. 2).

3.3. Inhibition of arthroconidium germination

In order to determine whether the recombinant strain GS115/CEC can influence arthroconidium germination, the result was observed by microscopy after 8 h incubation. Fig. 3 showed the effect of recombinant strain GS115/CEC on arthroconidium germination.

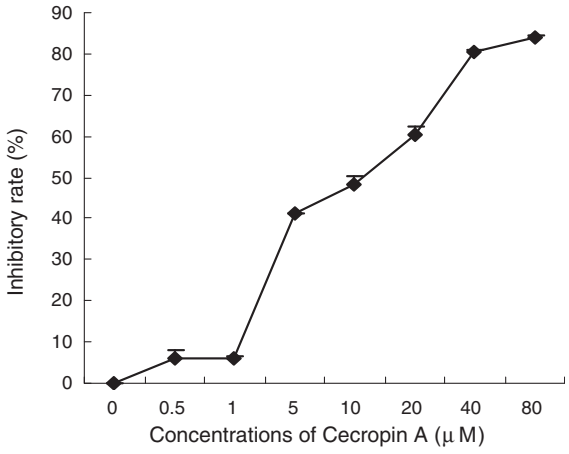

Fig. 1. The inhibitory rate of different concentrations of cecropin A to *G. citri-aurantii* spores. Error bars represent standard error from the mean.

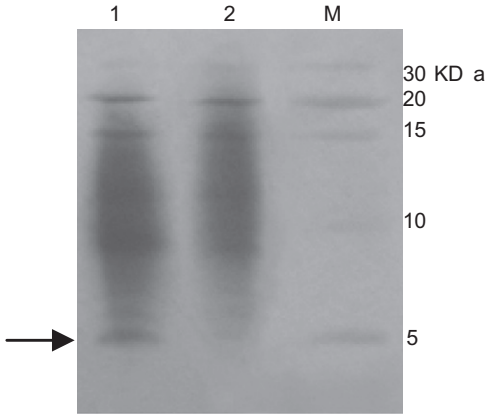

Fig. 2. Tricine–SDS–PAGE analysis of media from yeast cultures expressing secreted cecropin A. Culture medium was taken and concentrated by 100% TCA after 72 h culture induced by methanol, separated by 16% Tricine–SDS–PAGE and silver stained. Lane 1, sample from recombinant strain GS115/CEC after 3 days of methanol induction; lane 2, sample from *P. pastoris* strain GS115/pPIC; and lane M, low range protein molecular weight marker.

The suspensions of recombinant strain GS115/CEC strongly influenced the arthroconidium germination of *G. citri-aurantii*. When the spore germination of control (GS115/pPIC and sterile distilled water) was 50% and 34%, respectively, the spore germination treated by recombinant strain GS115/CEC was only 12%. It dropped 76% compared with sterile distilled water control group and 64.7% compared with GS115/pPIC control group.

3.4. Growth of recombinant strains in citrus fruit wounds

In the wounds of citrus fruits, there is no big difference in cell growth between recombinant strain GS115/CEC and GS115 (Fig. 4). The amount of two yeast strains increased rapidly and reached approximately fourfold of log values during the first 48 h after incubation, and the population of the two yeasts decreased slightly and gradually stabilized after this.

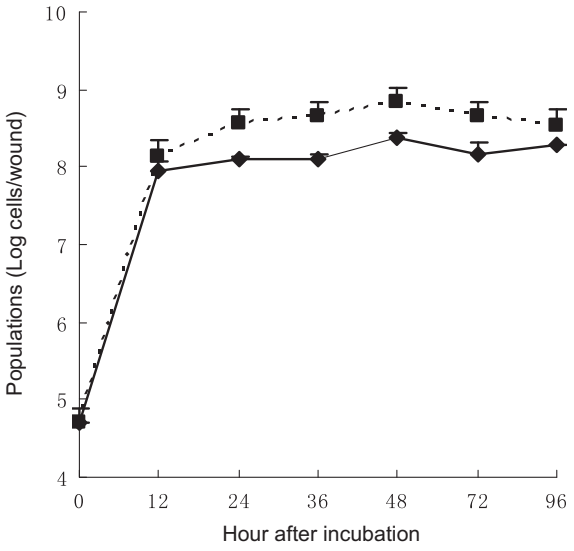

Fig. 4. Population dynamics of *P. pastoris* strains GS115 and recombinant strain GS115/CEC in wounds of citrus fruits. The fruit were wound-inoculated with both strains and stored on trays in plastic boxes at 24 °C. Error bars represent standard errors of three replications. (-■-) GS115/CEC; (-◆-) GS115.

3.5. Biocontrol tests

As shown in Fig. 5, the application of transformed yeast strains was effective in control of sour rot incidence by more than 52.7%,

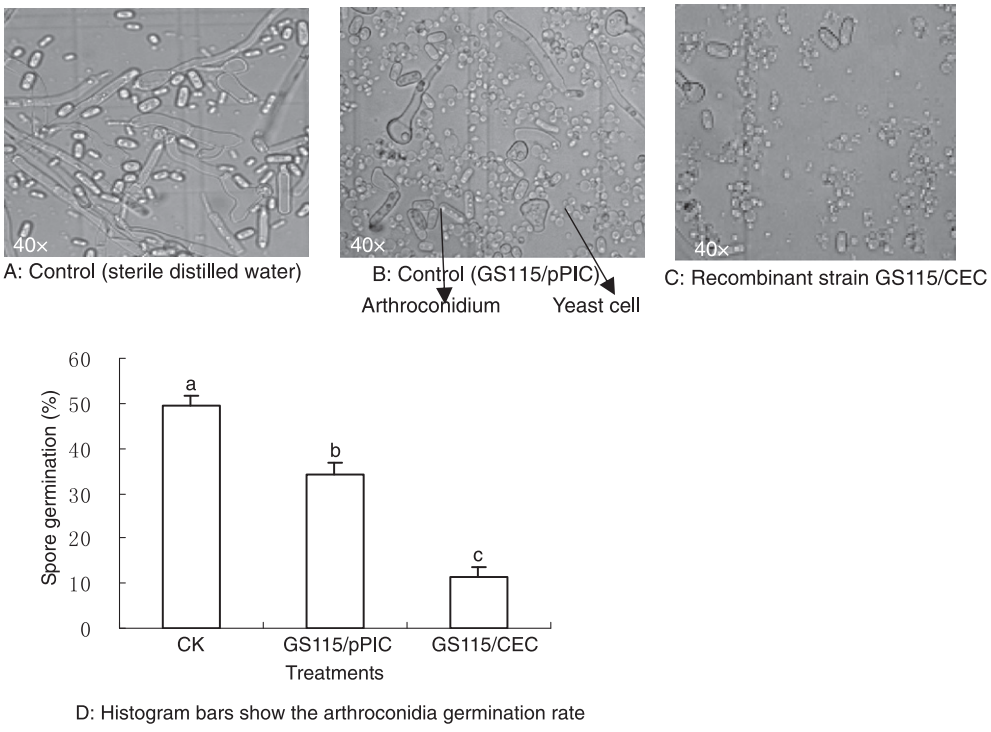

Fig. 3. Light electron micrographs of *G. cirti-aurantii* mycelium and arthroconidia treated with recombinant strain GS115/CEC or control. (A) Displayed light micrographs of arthroconidia germination, cultured in PDB and measured after 8 h incubation in controlled sterile distilled water, chambers maintained at 26 °C. (B) Incubation in control GS115/pPIC. (C) Incubation in recombinant strain GS115/CEC. (D) Effects of recombinant strain GS115/CEC on arthroconidia germination of *G. citri-aurantii*, Germination rate were measured after 10 h incubation at 26 °C in PDB. Significant differences ($P < 0.05$) between means were indicated by different letters above histogram bars.

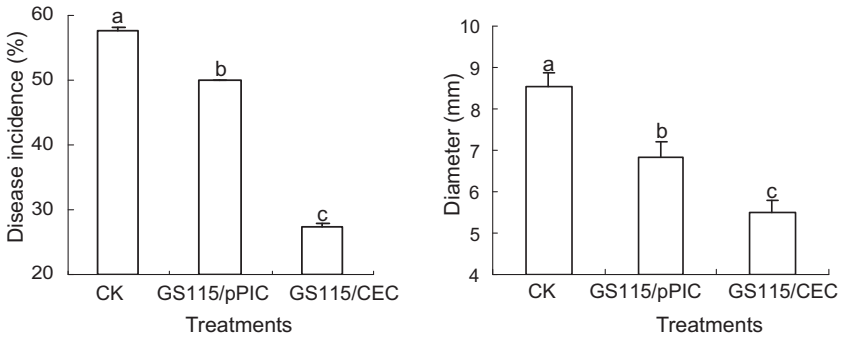

Fig. 5. Inhibition of *G. citri-aurantii* on artificially inoculated and wounded citrus fruits by recombinant strain GS115/CEC. Significant differences ($P < 0.05$) between means were indicated by different letters above histogram bars.

sour rot incidence on both control citrus was 57.69% and 50%, whereas the incidence in GS115/CEC-treated citrus was only 27.31%. Lesion diameter with GS115/CEC treatment was 5.48 mm and the diameters of controls were 8.55 and 6.82 mm, respectively.

4. Discussion

The postharvest diseases of fruits caused by fungal pathogens generally start as a result of wound infections or latent infections generated in the field (Prusky, 2003). Sour rot decay of citrus fruits is not controlled with the currently registered fungicides imazalil and thiabendazole, and is partially controlled with sodium *o*-phenylphenate (Wild, Rippon, & Nicholls, 1976). However, use of sodium *o*-phenylphenate was limited due to risk of fruit scalding (Droby et al., 1998). Sour rot could be partially prevented by sanitation control and low-temperature storage but chilling injury and temperature fluctuation during transport and marketing were still major bottlenecks (Mercier & Smilanick, 2005). Therefore, exploration of effective methods controlling sour rot has become very important.

Biological control has in recent years emerged as one of the most promising alternatives to synthetic fungicides (Quaglia, Ederli, Pasqualini, & Zazzerini, 2011). However, the inhibitory effect of a single antagonist is not very good compared with fungicides, as no single method of chemical control is as consistently effective as fungicides on inhibiting postharvest diseases of fruit; promising alternatives, such as improving antagonists through genetic manipulation, need to be evaluated. Genetic manipulation offers tremendous potential for improving biocontrol. Result from our experiments indicated that an integrative method using recombinant strains expressing cecropin A antifungal peptide is a successful strategy to inhibit postharvest decay of citrus fruits.

Furthermore, like the bacterial expression system, the yeast expression system also shows several advantages: growth is fast, inexpensive, yeast cells are eukaryotes, having the machinery for post-translational modifications and so on (Cereghino & Cregg, 2000), and even very toxic protein can be produced in large scale in this system. Recently, several antibacterial peptides have been expressed in *P. pastoris* (Li et al., 2005; Somboonwiwat et al., 2005). In the present study, the methylotrophic yeast, *P. pastoris* was used as the host for the expression of recombinant GS115/CEC. Tricine-SDS–PAGE revealed that the cecropin mature peptide was successfully secreted into the culture supernatants.

The *in vitro* inhibition effects of cecropin A on *G. citri-aurantii* spores were confirmed by MTT assay. These studies have demonstrated that low concentrations of cecropin A (10 μM) can inhibit about 50% of spores. Results revealed that cecropin A has a strong inhibition effect on *G. citri-aurantii* spores at micromolar concentrations. Similar anti-fungal activities have been reported for cecropins (Kim et al., 2010; Xu et al., 2007). Jin et al. (2006) reported that a housefly cecropin gene has high activity in inhibiting the growth of five fungi *in vitro* (*Pyricularia oryzae*, *Botrytis cinerea*, *Penicillium crustosum*, *Valsa mali* and *Fusarium oxysporum*), and fungal growth inhibition was associated with the permeating of their membranes after treatment with recombinant strains expressing cecropin A antifungal peptide. With these as the prerequisite, the recombinant strains GS115/CEC was used as a new antagonist to inhibit decay development caused by *G. citri-aurantii* in citrus fruits. The results of the present study indicated that recombinant strain GS115/CEC suspension obviously inhibited the growth of *G. citri-aurantii*.

Biocontrol tests on citrus fruits against sour rot decay revealed evident differences in the effectiveness and consistency among recombinant strain and controls (GS115/pPIC and sterile water). The disease incidence of inoculated citrus fruit was reduced about 52.7% compared to the control (sterile water). With respect to the population growth of GS115 strain, expressing cecropin A gene did not affect the growth of the *P. pastoris* yeast in wounds of citrus fruit.

On the other hand, the results from this study also showed that *P. pastoris* GS115 transformed with the vector significantly inhibited the growth of *G. citri-aurantii in vitro* and reduced decay, indicating the potential of this yeast for biocontrol. Similar results were found in some other previous reports, because other *Pichia* sp. have been reported to control various diseases. For example, *B. cinerea* on strawberries in the field (Guetsky, Elad, Shtienberg, & Dinoor, 2002), *Pichia guillermondi* controlled postharvest decays on citrus fruit (Lahlali et al., 2011) and *Pichai. membranefaciens* controlled gray mould of grapevine (Santos & Marquinq, 2004) and postharvest disease caused by *B. cinerea* in tomato fruit (Zong et al., 2010), while *P. anomala* could control spoilage in long-term storage of moist feed grain (Druvefors, Jonsson, Boysen, & Schnurer, 2002).

Developing new biocontrol agents or improving antagonists through genetic manipulation against postharvest fruit diseases and using them as a delivery system for various biocontrol traits isolated from other microorganisms or other foreign genes responsible for antifungal activity have been suggested in the past (Janisiewicz et al., 2008). Efficient control of the fruit wound-invading pathogens was considered to be a prerequisite for a potential microbe candidate. Jones and Prusky (2002) used this approach to express an antifungal peptide in *Saccharomyces* as a biocontrol agent by cloning a cecropin-based gene. This genetically modified yeast expressed the cloned gene and controlled postharvest decay caused by *Colletotrichum coccodes* on tomatoes. Janisiewicz et al. (2008) expressed Psd1 antifungal peptide, a plant defense, with *P. pastoris* recombinant strains to inhibit blue mould decay of apple caused by *Penicillium expansum*. Wisniewski et al. (2005) cloned and expressed in *P. pastoris* a defensin gene isolated originally from

peach bark that had antifungal activity against *B. cinerea* and *P. expansum in vitro*, the major postharvest pathogens of various fruits. The biocontrol of fruit decay by this transformant was not reported.

It is well-known that the most appealing feature of antibacterial peptides is that they rarely induce drug resistance (Jin et al., 2006), which has become a serious problem with conventional antibiotics. Therefore, antimicrobial peptides have become one of the most promising candidates for a new group of antibiotics. The use of antagonists producing antibacterial peptides on consumable products, apart from other regulatory issues, will have to overcome the hurdles of potential allergic reactions and mammalian toxicity before approval can be anticipated. Antimicrobial peptides, cecropins, have been reported to display antibacterial activity towards Gram-positive, Gram-negative bacterial and fungi and to be virtually non-toxic to human cells (Kim et al., 2010). All these cases confirm the validity of this approach in improving biological control of fruit decays. The *Pichia* system has an additional appeal because this yeast has been approved as a dietary supplement to feed animals [58 FR 59170, November 8, 1993; 21 CFR Chapter 1 (4-1-02 Ed.) Section 573.750], and moreover, on the safety test, the results of acute toxicity to mice (food standard) also confirmed the dried powder of the recombinant strain GS115/CEC is safe (data not show). Optimization expression of cecropin A under a variety of conditions (e.g. fruit wounds, storage conditions, etc.) and some strategies to improve foreign protein expression in *P. pastoris* appears to be the next major challenge in determining the commercial potential of this system.

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