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Recombinant expression of antimicrobial peptides in *Pichia pastoris*: A strategy to inhibit the *Penicillium expansum* in pears

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ABSTRACT

The alteration of safety-secured yeast is a crucial step before scale applications. Based on previous studies, *Pichia pastoris* showed great potential and value to improve its biocontrol ability. The original sequences of antimicrobial peptide *Ac*-AMP2 and MiAMP1 were optimized according to the preference of *Pichia pastoris* and ligated into pPICZαA plasmid which is emerged as a high-performance vector for transformation and expression. The results of RT-qPCR and Western blotting could imply that pPICZαA/*Ac*-AMP2 and pPICZαA/MiAMP1 were successfully overexpressed in *Pichia pastoris* GS115. The peptide concentration of GS115/*Ac*-AMP2 reached a maximum value of 210 mg L^{-1} at 60 h while GS115/MiAMP1 was 220 mg L^{-1} at 96 h. The biocontrol experiment indicated that the recombinant strain GS115/*Ac*-AMP2 and GS115/MiAMP1 could highly suppress the pathogen *Penicillium expansum* in vivo, which was respectively 42 % (GS115/*Ac*-AMP2) and 29.2 % (GS115/MiAMP1) of incidence disease lower than the sterile distilled water treatment. In the case of the experimental results considered, the modified GS115/*Ac*-AMP2 and GS115/MiAMP1 might be promising biological agent in postharvest applications.

1. Introduction

The squander of fruit caused by postharvest diseases during storage and transportation is considered as a wastage of resources (Droby et al., 2016). Numberous surveys have indicated that *Penicillium expansum* accounted for a crucial part of the main infectious pathogen (Sánchez et al., 2016; Youssef and Hussien, 2020). The patulin (4-hydroxy-4H-furo [3,2c] pyran, 2[6 H]-one), which is toxic to animals, causes intestinal injuries, and is shown to be mutagenic accumulate in mold growth (Andersen et al., 2004). Certain universally known limitations restrict the promotion of chemical agents, including of its negative impacts on both human and environment (Bazioli et al., 2019). In contrast, biological fungicides show great potential to be the alternative for synthetic fungicides (Dukare et al., 2019; Youssef et al., 2019).

Biocontrol technology has gradually attracted people's attention and been widely recognized due to its safety, non-toxicity and environmental friendliness. Some possible mechanisms may contribute to the reduction of disease rate: competition for nutrients and space, antibiosis through antibiotic production, mycoparasitism, production of cell wall lytic enzymes, and induction of host resistance (Di Francesco et al., 2016; El-Ghaouth, 1997; Sharma et al., 2009).

As one of the most widely used eukaryotic expression systems, *Pichia pastoris* is genetically stable, produces no toxins, has less secreted proteins, and can correctly translate, process and modify heterologous genes (Banani et al., 2014; Cereghino and Cregg, 2000). It has been widely used as one of the most successful protein expression systems since 1987, when hepatitis B surface antigen was successfully expressed in the *Pichia Pastoris* (Cregg et al., 1987).

Antimicrobial peptide is one kind of small-molecule polypeptides encoded by specific genes of organisms, which have high-efficiency spectrum killing ability against microbes, as well as the strong inhibitory effects on fungi, viruses and protozoa (Mensa et al., 2011; Zhang et al., 2008). Antimicrobial peptides have broad application prospects in the prevention of fruit and vegetable diseases as well (Lachhab et al., 2015). Studies have elaborated that the peptides extracted from *Bacillus subtilis* medium could effectively inhibit grape spoilage (Zhang et al., 2018). Published work also demonstrated that CgPep33 antimicrobial peptide was effective in inhibiting the germination of *Botrytis cinerea* in strawberries (Liu et al., 2007).

Ac-AMP2 is derived from the seeds of *Amaranthus caudatus* while

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MiAMP1 is emanated from *Macadamia integrifolia*. Seven pathogenic fungi could be inhibited by *Ac*-AMP2, including *Alternaria brassicola*, *Ascochyta pisi*, *Botrytis cinera*, *Colletotrichum lindemuthianum*, *Fusarium culmorum*, *Trichoderma hamatum* and *Verticillium dahliae* (Broekaert et al., 1992). Marcus et al. (1997) performed experiments to investigate that MiAMP1 was amenable to the bacteriostatic tests against *Alternariu heliarzthi* and *Botrytis cinerea*. Both of the above antimicrobial peptides have been evaluated to be innoxious based on the human cell hemolysis assay, which proved applicable for their utilization and commercialization (Broekaert et al., 1992; Marcus et al., 1997).

The present study aimed to evaluate the biocontrol efficacy of yeasts that can overexpress antimicrobial peptides mentioned above. At the same time, contributed to explore the feasibility of the application of modified yeast.

2. Materials and methods

2.1. Microorganisms and culture condition

Pichia pastoris strain GS115 was purchased from Invitrogen (Carlsbad, USA), generated under non-selective condition in yeast extract peptone dextrose medium (YEPD) or selective condition in YEPD with 100 mg L^{-1} of *Zeocin*.

Escherichia coli strain DH5α, as a host of expression vectors, was obtained from Vazyme Biotech (Nanjing, China) and cultured in low-salt Luria-Bertani (LB) or in low-salt LB with 25 mg L^{-1} *Zeocin* under selective condition. The plasmids used in this study included pPICZαA (Invitrogen) and pUC-SP (Sangon Biotech, Shanghai, China).

Penicillium expansum was preserved in the laboratory and grew on potato dextrose agar medium (PDA).

2.2. Cloning of the target gene

To ensure the natural activity of the N-terminal, as well as and the efficient expression of the sequence, a Kex2 restriction site (AAAAGA) was added in front of the target gene. The *Ac*-AMP2 and MiAMP1 sequences were modified according to the codon preference of *P.pastoris* GS115. *Xho*I and *Not*I were added to two sides of the optimized sequences as restriction endonuclease sites. Sangon Biotech were entrusted to insert the final gene sequences into pUC-SP vectors.

2.3. Construction of the expression vector

Both pPICZαA and the pUC-SP, containing the optimized gene, were digested with *Xho*I and *Not*I. The products were constructed on pPICZαA plasmid by T4 ligase after purification. The constructed vectors were transformed into DH5α, and then cultured on low-salt LB +*Zeocin* plates (25 mg L^{-1} *Zeocin*) overnight at 37 °C. Single colonies were picked for identification through polymerase chain reaction (PCR), while 5′ AOX1: 5′ -GACTGGTTCCAATTGACAAGC-3′ and 3′AOX1: 5′-GCAAATGGCATTCTGACATCC-3′ were primers. A successful recombinant expression vector was constructed and named pPICZαA/*Ac*-AMP2 or pPICZαA/MiAMP1.

2.4. Transformation and selection of recombination strains

The extracted pPICZαA/*Ac*-AMP2, pPICZαA/MiAMP1 and pPICZαA were digested with *Sac*I. 10 μL of each linear vectors were then transformed into 80 μL competent *P. pastoris* through electroporation by Bio-Rad MicroPulser, respectively. The *P. pastoris* were cultured on YEPD +*Zeocin* plates (100 mg L^{-1} *Zeocin*) at 28 °C after 1 h static culture with 1 mL sorbitol, verified by PCR (same condition with the PCR in 2.3) as well. The successful recombinant transformants were named GS115/*Ac*-AMP2, GS115/MiAMP1 and GS115/pPICZαA.

2.5. Expression of antimicrobial peptides in P. pastoris

The verified recombinant strains GS115/*Ac*-AMP2, GS115/MiAMP1 and GS115/pPICZαA were cultivated in buffered glycerol-complex medium (BMGY) at 30 °C, 250 rmp overnight. The yeasts were collected by centrifugation and resuspended in buffered methanol-complex medium (BMMY) to OD (600 nm) = 1.0 for inducible expression. Methanol was added to the final concentration of 1 % (v/v) every 24 h for 120 h. Samples were taken every 12 h to determine the expression level of antimicrobial peptides.

The supernatant was obtained after the centrifugation, tested for protein concentration using the Braford assay. At the same time, sample the obtained supernatant by trichloroacetic acid (TCA)-acetone precipitation method. 10 μL of the sample was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE). The gels were then transfected and shaken slowly in western blocking solution at room temperature for 1 h. After blocking, the primary antibody and the secondary antibody were incubated, so that the protein could been detected.

On the other hand, the RNA of samples was extracted using RNAiso Plus (TaKaRa, Dalian, China) and reverse transcribed to cDNA using PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). The expression level of the antimicrobial peptide genes was quantitatively detected through real-time quantitative polymerase chain reaction (RT-qPCR) which regarded the obtained cDNA as the template and analyzed using $2^{-\Delta\Delta CT}$ method. Specific primers are presented in Table 1.

2.6. In vitro experiment of antimicrobial peptides against P. expansum

The supernatant was collected after the expression of each recombinant yeast. 2 mL of uniform 50 % BeyoGold™ His-tag Purification Resin was centrifuged (1000 ×*g*) for 30 s at 4 °C to discard the storage solution. Added a column volume of non-denaturing lysis buffer to balance the gel. Antimicrobial peptide containing supernatant was added in a ratio of 1:8 (v/v) of the gel to the supernatant after centrifugation, and shaken slowly for 1 h on a 4 °C decoloring table. The supernatant and Ni-NTA mixture was washed through the affinity chromatography column for 3 times. The column was then washed 5 times by adding 2 column volumes of non-denaturing wash. After that, the antimicrobial peptide was eluted for 10 times with 1 column volume of eluate each time. The obtained purified antimicrobial peptide sample was also assayed according to the method mentioned in 2.5.

The *P. expansum*, which had been cultured on PDA for two generations until sporulation was carried out, was scrapped by aseptic

Table 1
Primers used for qPCR of yeasts nucleotide sequences.

Gene	Accession number	Primer Sequence(5′-3′)	Product (bp)
Actin	NM_001179927.1	Forward: AATTGTCCGTGACATCAAGG Reverse: CGGCAGATTCCAAACCC	197
Ac-AMP2	EF066732.1	Forward: GGGAGTTGGATAATGCG Reverse: CTTGATGGTCTACGGTCG	119
MiAMP1	Y10903.1	Forward: CTCTTACACCGGTCAAACA Reverse: GGGCAGAACTCCCAAA	83

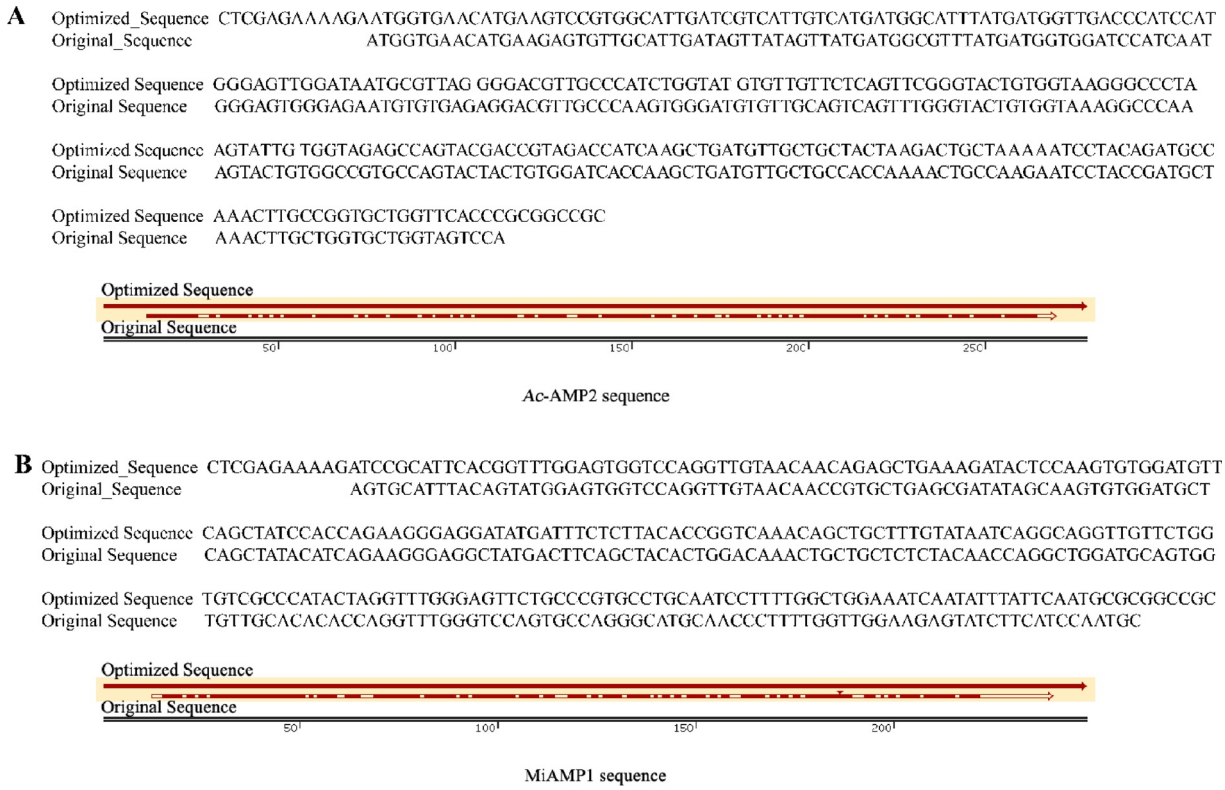

Fig. 1. Alignment of *Ac*-AMP2 and MiAMP1 on optimized sequence and original sequence.

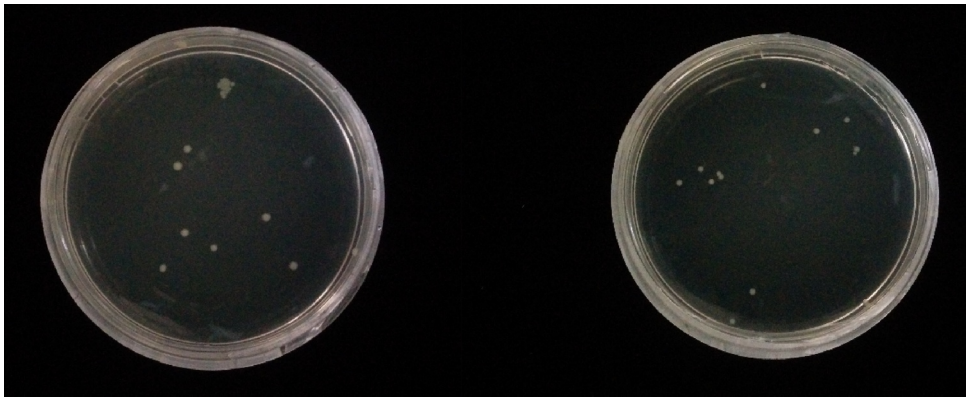

Fig. 2. Screening results of recombinant pPICZαA/*Ac*-AMP2 (left) and pPICZαA/MiAMP1 (right) on selected plates.

inoculating loop to acquire the spore suspension (1×10^7 spores L^{-1}). It was added to the PDB medium which had added purified antimicrobial peptides to final concentrations of 0, 25, 50, 75 and 100 mg L^{-1}, respectively. After incubation for 12 h at 25 °C (200 rpm), the germination of 200 random spores was observed under the microscope. The detraction effect of the antimicrobial peptide was represented by the average germination rate (%) of the spores. Three replicates were used for each treatment, and each experiment was repeated twice.

2.7. Biocontrol test of antimicrobial peptides

The experimental pears (*Pyrus bretschneideri* Nakai. cultivar Shuijing) were picked without obvious damage nor disease. Pears were soaked in 0.1 % sodium hypochlorite for 2 min before the experiment, then rinsed with water, dried naturally. In the equatorial region of each pear, the wound with a diameter of 5 mm and a depth of 2 mm were made by a sterilized puncher. 50 μL of sterile water (control), 25 mg L^{-1}, 50 mg L^{-1}, 75 mg L^{-1} and 100 mg L^{-1} of *Ac*-AMP2 and MiAMP1 were added to the wounds respectively. After drying for 2 h, each wound was inoculated with 30 μL of *P. expansum* (1×10^7 spores L^{-1}). Nine pears were selected as a treatment in a box, and stored at a thermostatic chamber of 25 °C. Observed and recorded the disease incidence of fruit every day. The direct inhibition of different antimicrobial peptides against *P. expansum* in pears were evaluated by mean lesion diameter (mm) and average incidence (%). Nine pears for each replicate, three replicates for each treatment, and each experiment was repeated twice.

2.8. Biocontrol test of recombination strains

Both of the recombinant strains (GS115/*Ac*-AMP2 and GS115/MiAMP1) and the control strains (GS115/pPICZαA and GS115) were activated on YEPD for two generations before they were inoculated to BMMY. The yeasts were finally reached 1×10^{10} cells L^{-1} after being washed twice with sterile water.

The pretreatment and the wound manufacturing method of pears

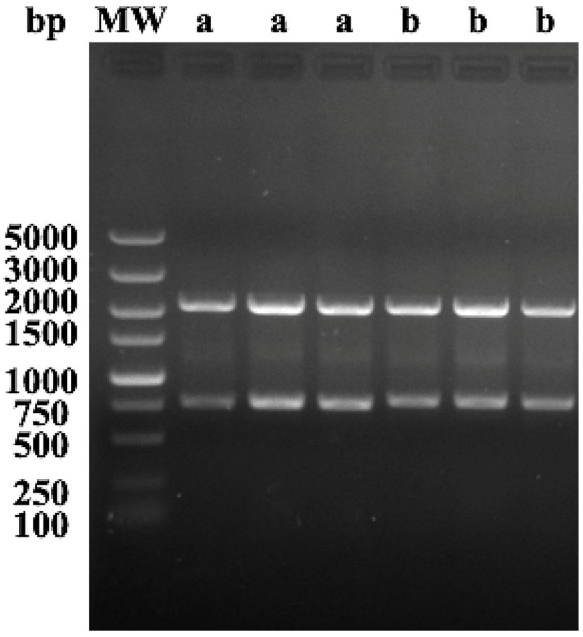

Fig. 3. Identification results of GS115/*Ac*-AMP2 and GS115/MiAMP1 recombinant strains by PCR. MW: 5000 bp DNA Marker; a: PCR result of G GS115/MiAMP1; b: PCR result of GS115/*Ac*-AMP2.

were the same as 2.7. 50 μL of sterile water (control), 1×10^{10} cells L^{-1} GS115, GS115/pPICZαA, GS115/*Ac*-AMP2 and GS115/MiAMP1 yeast suspensions were added to the wounds respectively. After drying for 2 h, each wound was inoculated with 30 μL of *P. expansum* (1×10^7 spores L^{-1}). Nine pears were selected as a treatment in a box, and stored at a thermostatic chamber of 25 °C. Recorded the mean lesion diameter (mm) and average incidence (%) of fruit every day. Nine pears for each replicate, three replicates for each treatment, and each experiment was repeated twice.

2.9. Statistical analysis

All the statistical operations of in this study were performed with software SPSS by one-way ANOVA of Duncan's multiple range test or independent sample *t*-test while the data were expressed as mean ± standard error ($n \geq 3$). Least significance between the mean values was determined at $p < 0.05$.

3. Results

3.1. Optimization and synthesis of Ac-AMP2 and MiAMP1

Ac-AMP2 and MiAMP1 were optimized and synthesized according to the preference of *Pichia pastoris* GS115. The synthetic product was ligated into pUC-SP vector. The sequencing results elucidated that the *Xho* I, *Not* I, Kex2 restriction sites and *Ac*-AMP2 and MiAMP1 target genes were correctly connected. The sequences after optimization and addition of the restriction sites are shown in Fig. 1.

3.2. Construction of pPICZαA/Ac-AMP2 and pPICZαA/MiAMP1

The antimicrobial peptides sequences were digested with *Xho* I and *Not* I. The pPICZαA vector were ligated with sequences by T4 ligase and transformed into *E. coli* DH5α competent cells which were then cultured on low-salt LB + *Zeocin* plates (25 mg L^{-1} *Zeocin*). The colonies growth is shown in Fig. 2. Some colonies were successfully verified by PCR.

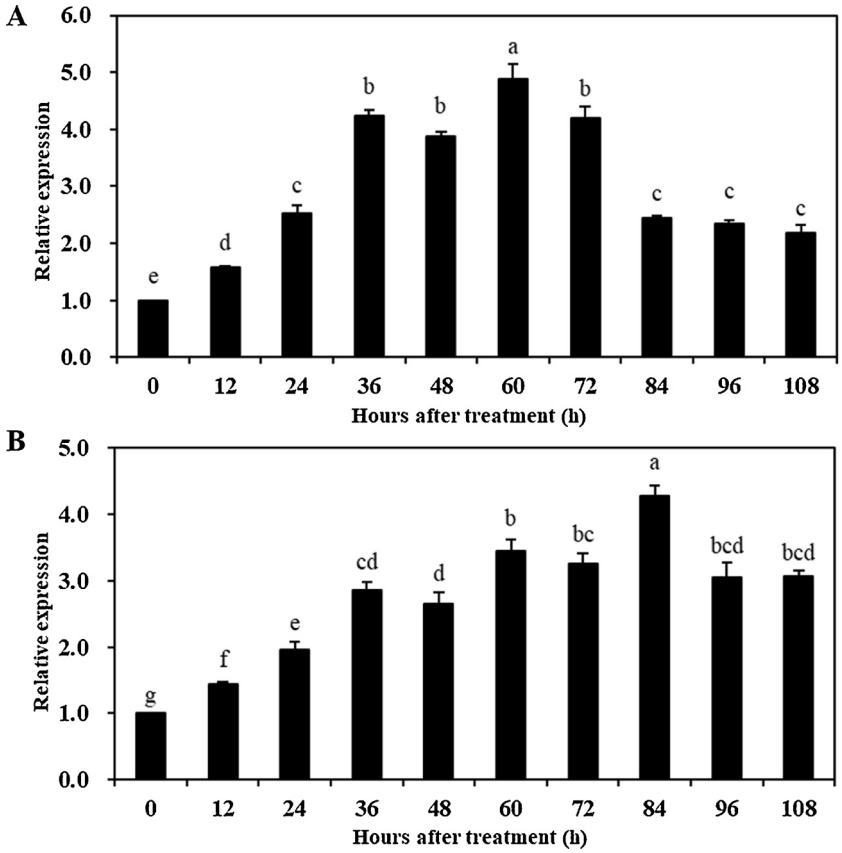

Fig. 4. Expression of *Ac*-AMP2 gene (A) and MiAMP1 gene (B) detected by qPCR. Different letters indicate a significant difference according to Duncan's multiple range tests ($p < 0.05$). Error bars represent the standard error of three replicates.

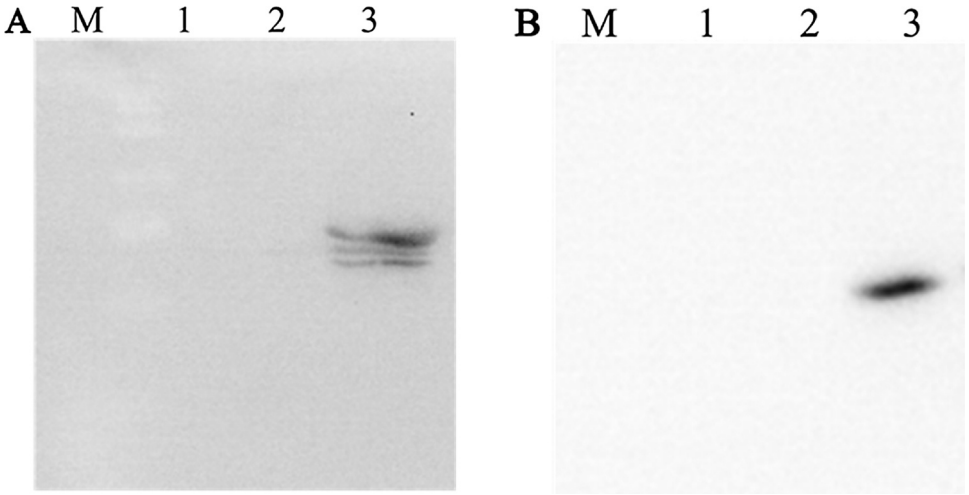

Fig. 5. Identification result of GS115/*Ac*-AMP2 recombinant strain (A) and GS115/MiAMP1 recombinant strain (B) by Western-blot. M: 6.5～270 kDa protein marker; 1: GS115; 2: GS115/pPICZαA; 3: GS115/*Ac*-AMP2 (A) or GS115/MiAMP1 (B).

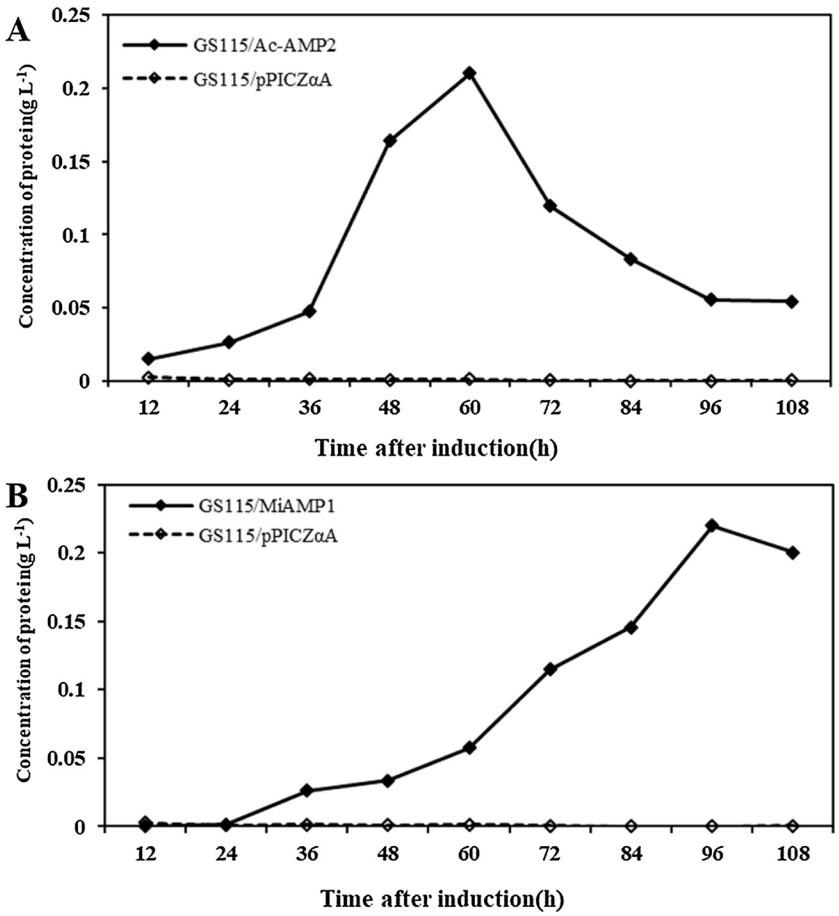

Fig. 6. Supernatant protein content of GS115/*Ac*-AMP2 (A) and GS115/MiAMP1 (B) recombinant strain.

3.3. Transformation and identification of GS115/Ac-AMP2 and GS115/MiAMP1

Single colonies were picked up for PCR amplification. The PCR results of GS115/*Ac*-AMP2 and GS115/MiAMP1 were plotted in Fig. 3. A common band appeared at around 2000 bp, which evince that GS115 has a recognition site of the universal primers 5′ AOX1 and 3′ AOX1. Additionally, the other different bands indicate that the GS115/*Ac*-AMP2 and GS115/MiAMP1 were recombined successfully.

3.4. Expression of Ac-AMP2 and MiAMP1 in P. pastoris

The results of qPCR are depicted in Fig. 4. After induction of recombinant yeast GS115/*Ac*-AMP2, *Ac*-AMP2 illustrated significant up-regulated expression from 24 h, and the gene expression level at 36－60 h was over 3 times compared with 0 h (Fig. 4A). As for the expression of GS115/MiAMP1, the antimicrobial peptide gene *MiAMP1* reached 4.3 times at 84 h, which was much higher than other induction time groups (Fig. 4B).

Table 2
Effects of *Ac*-AMP2 and MiAMP1 on spore germination rate of *P. expansum*.

Treatments (mg L^{-1})		Spore germination (%)
Ac-AMP2	0	72.7 ± 0.70 a
	25	55.0 ± 0.67 b
	50	25.5 ± 0.55 c
	75	25.3 ± 0.45 c
	100	22.5 ± 0.33 c
MiAMP1	0	72.7 ± 0.60 a
	25	54.7 ± 0.70 b
	50	49.3 ± 0.76 b
	75	24.7 ± 0.57 c
	100	21.8 ± 0.43 c

Germination rate was measured after 12 h of incubation at 25 °C (200 rpm) in PDB. Different letters indicate significant differences ($P < 0.05$) between different groups according to Ducan's tests. Each value is the mean ± standard error from three separate determinations.

Western-blot was able to detect the presence of antimicrobial peptides *Ac*-AMP2 and MiAMP1 (label 3) in the supernatants of GS115/*Ac*-AMP2 (11.9 kDa) and GS115/MiAMP1 (11.3 kDa). The strain GS115 (label 1) and the recombinant yeast GS115/pPICZαA (label 2) showed no band (Fig. 5), which elucidates that the antimicrobial peptide had been successfully transformed into *Pichia pastoris* GS115.

The antimicrobial peptides in the supernatant gradually increased with the induction time. The protein concentration of GS115/*Ac*-AMP2 reached a maximum value of 210 mg L^{-1} at 60 h (Fig. 6A). The maximum figure of GS115/MiAMP1 protein content of 220 mg L^{-1} was detected at 96 h (Fig. 6B). Thereafter, the content of the antimicrobial peptides gradually decreased. Meanwhile, the protein content of recombinant yeast GS115/pPICZαA kept a low value all the time.

3.5. Inhibition of *P. expansum* by antimicrobial peptides in vitro

Both antimicrobial peptides were reflected to be effective in suppressing the germination of *P. expansum* spores (Table 2). With the increase of the concentration of antimicrobial peptides, the germination rate of *P. expansum* spores decreased continuously. The spore germination rate of *Ac*-AMP2 group decreased to only 25.5 %, when the concentration of *Ac*-AMP2 increased to 50 mg L^{-1}. The MiAMP1 group had the most obvious decline trend at 75 mg L^{-1} (24.7 %), compared with the sterile water control group (72.7 %).

3.6. Inhibition of *P. expansum* by antimicrobial peptides in vivo

The direct inhibitory effect of two antimicrobial peptides on *P. expansum* is shown in Fig. 7. 50 mg L^{-1} *Ac*-AMP2 reflected a vastly inhibitory effect on the 5th day, while the MiAMP1 effectively suppressed the *P. expansum* at 25 mg L^{-1}. The general trend was consistent with the statistics in Table 2. As to the lesion diameter, the concentration of 25 mg L^{-1} seems strong enough to render the effect.

3.7. Effect of recombination strains against *P. expansum* on pears

As shown in Fig. 8, the recombinant yeasts could effectively suppress the pathogen. The incidence of the disease was respectively reduced by 42 % (GS115/*Ac*-AMP2) and 29.2 % (GS115/MiAMP1) on the 3rd day. There was little difference between the GS115 and GS115/pPICZαA compared with the sterile water treatment. The average lesion diameter reflects the detraction effect on the blue mold disease as well.

4. Discussion

In this study, *Ac*-AMP2 and MiAMP1 were transformed into *Pichia pastoris* GS115 for the first time. GS115/*Ac*-AMP2 and GS115/MiAMP1 with highly concentrated antimicrobial peptides and excellent biocontrol effect was obtained. It provides a basis for the development and utilization of postharvest diseases.

After induction of recombinant yeast GS115/*Ac*-AMP2 and GS115/MiAMP1, the max concentration of peptide was 210 mg L^{-1} and 220 mg L^{-1}, respectively. It was elaborated that the antibacterial peptide gene *snakin-1* was overexpression in *Pichia pastoris* and its extraction

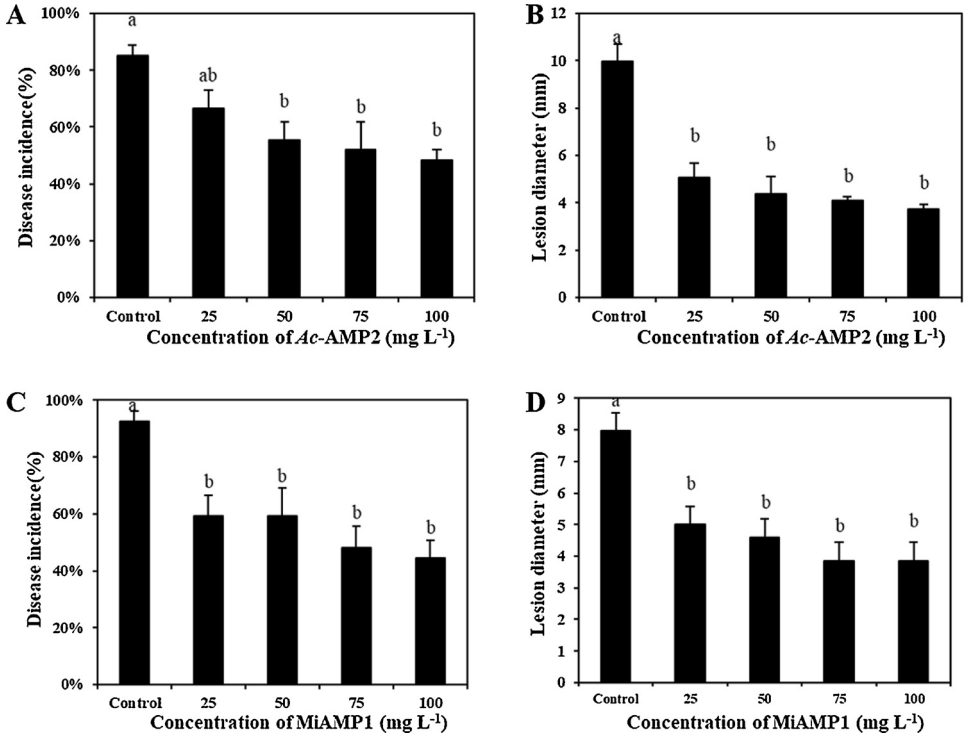

Fig. 7. Biocontrol efficacy of *Ac*-AMP2 (A, B) and MiAMP1 (C, D) against *P. expansum* in pears. The letters represent the significant differences ($p < 0.05$) according to Duncan's multiple range tests. Error bars represent the standard error of three replicates.

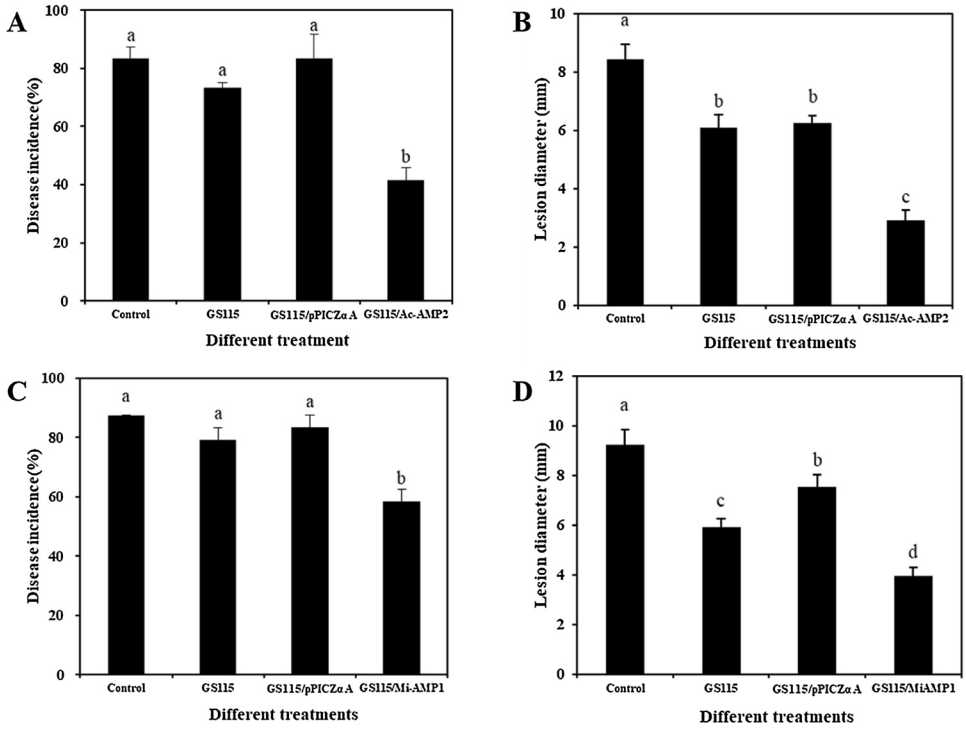

Fig. 8. Biocontrol efficacy of GS115/*Ac*-AMP2 (A, B) and GS115/MiAMP1 (C, D) against *P. expansum* in pears. The letters represent the significant differences ($p < 0.05$) according to Duncan's multiple range tests. Error bars represent the standard error of three replicates.

amount was 40 mg L^{-1} (Kuddus et al., 2016). Hispidalin is a novel antimicrobial peptide isolated from the seeds of *Benincasa hispida* and the amount of Hispidalin accumulation could reach as high as 98.6 mg L^{-1} in *Pichia pastoris* (Meng et al., 2019). The compared results of these experiments indicate that the GS115/*Ac*-AMP2 and GS115/MiAMP1 might have a potential to produce the antimicrobial peptides with high dose, which means a better efficacy.

Both in vivo and in vitro experiments have proved that *Ac*-AMP2 and MiAMP1 have the effect of inhibiting the expansion of *Penicillium expansum*. They can significantly reduce spore germination rate and disease incidence. Antimicrobial peptides mainly play a role in medicine, food and feed additives at present. In addition, antimicrobial peptides are under consideration as new substitutes for conventional pesticides and antibiotics in agriculture and food industry (Keymanesh et al., 2009; Leiter et al., 2017). In fact, a certain amount of studies had confirmed the inhibitory effect against *Penicillium expansum* of antimicrobial peptides. Bioactive peptides produced by *Lactobacillus plantarum* had the antifungal activity against *Aspergillus parasiticus* and *Penicillium expansum* in vitro (Luz et al., 2017). PAF26, characterized as a novel penetratin-type antimicrobial peptide, also had the activity against phytopathogenic fungi like *Penicillium digitatum*, *Penicillium italicum* and *Penicillium expansum* (Veyrat and Marcos, 2011). Relatively few studies have studied the antifungal effect of antimicrobial peptides directly on the fruit. And this is exactly a crucial step for the application.

It also can be found that the existence of GS115/*Ac*-AMP2 and GS115/MiAMP1 availably inhibits the occurrence of disease led by *P. expansum* in pears. The incidence of the disease was respectively reduced by 42 % (GS115/*Ac*-AMP2) and 29.2 % (GS115/MiAMP1) on the 3rd day, while there was no significant difference between the GS115 and GS115/pPICZαA compared with the sterile water treatment. According to this phenomenon, it could be justifiably ratiocinated that the introduced antimicrobial peptide genes played a vital role in the process of confrontation with etiological agent. Since *Pichia pastoris* is an established, FDA approved, safe (GRAS) yeast which fulfills recognized safety criteria pertinent to microbial production strains (Ciofalo et al., 2006). It will make more sense to enhance its resistance against diseases for primary industry.

A certain numbers of published works have highlighted the potential of yeasts for commercial biocontrol usage (Mukherjee et al., 2020). Many avenues have been clarified to improve the biocontrol effect of yeasts, including combining with other microorganisms (Janisiewicz et al., 2008) and chemicals (Cao et al., 2012; Farahani and Etebarian, 2012; Zhang et al., 2009, 2015), or using physical methods (Ou et al., 2016; Zhang et al., 2007). In general, molecular biology tools were rarely used in engineering biocontrol yeasts, even though these technologies have the potential to empower transforming yeast directionally. Due to the requirements for multi-targeted, efficacious biocontrol strategy in practical applications, it is predictable that exploiting these tools to transform yeast may take up a crucial spot for the agricultural field.

5. Conclusion

In summary, we have constructed yeasts that can efficiently express antimicrobial peptides *Ac*-AMP2 and MiAMP1. Both antimicrobial peptides can inhibit the growth of *Penicillium expansum* in vitro and in vivo. The transformed yeast also greatly enhanced the ability to directly compete with the pathogenic fungi, which can be proved by reducing the disease incidence of pears. The effectual means to improve the biocontrol effect by applying antimicrobial peptide-expression-yeast may become an attractive prospect of biocontrol technology.

CRediT authorship contribution statement

Yining Huang: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Liguang Gao:** Resources. **Ming Lin:** Methodology, Formal analysis. **Ting Yu:** Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled "**Recombinant expression of antimicrobial peptides in *Pichia pastoris*: a strategy to inhibit the *Penicillium expansum* in pears**".

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