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Chlorella sp. transgenic with Scy-hepc enhancing the survival of *Sparus macrocephalus* and hybrid grouper challenged with *Aeromonas hydrophila*

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ABSTRACT

Two marine antimicrobial peptides (AMPs), PC-hepc from large yellow croaker (*Pseudosciaena crocea*) and scygonadin from mud crab (*Scylla serrata*), are potently active against specific bacteria and thus they could be used as substitutes for antibiotics in aquaculture. However, how to utilize the AMPs feasibly for marine cultured animals has been so far confused. In our study, a 510 bp of the Scy-hepc sequence was cloned into pMDC85 expression vector, which was then electroporated into *Chlorella* sp., and thus a transgenic *Chlorella*, in which the Scy-hepc gene was effectively expressed, was developed. The Scy-hepc fusion protein was successfully expressed in *Chlorella* sp. and it showed obvious bactericidal activity. In addition, the *in vivo* efficacy of the transgenic *Chlorella* was evaluated using *Sparus macrocephalus* and the hybrid *Epinephelus fuscoguttatus* (♀) × *Epinephelus lanceolatus* (♂). Results showed that the survival rate of *S. macrocephalus* fed with transgenic *Chlorella* (80 ± 10% after 72 h) was significantly higher than that of fish fed with the same dosage of wild-type *Chlorella* (33.33 ± 11.55% after 72 h). Similarly, results showed that the survival rate of the hybrid grouper fed with transgenic *Chlorella* (55 ± 5% after 36 h) was much higher than that of fish fed with the same dosage of wild-type *Chlorella* (25 ± 5% after 36 h). Therefore, *in vitro* and *in vivo* results indicated that the constructed transgenic *Chlorella* with the marine AMPs Scy-hepc could exert effective protection for fish against the *Aeromonas hydrophila* infection, providing an encouraging prospect for the expected use of transgenic *Chlorella* in aquaculture in future.

1. Introduction

As is well known, antibiotics are widely and indiscriminately used in various fields including aquaculture to protect cultured marine animals from pathogenic diseases. Misuse or overuse of antibiotics increases the bacterial resistance of the pathogens and has an adverse effect on the environment [1]. The environment-friendly strategies in disease prevention of stopping the free use, or decreasing the dosage of antibiotics in aquaculture are proposed in many countries, including China in recent years [2,3]. Hence, a considerable amount of research has been focused in recent years on the development of substitutes for antibiotics in aquaculture.

Several potential substitutes for antibiotics in aquaculture, such as herbs, probiotics, vaccines and antimicrobial peptides (AMPs) have been studied in China [3–5], among which at least ten herbs are used as anti-viral, bacterial and fungal agents. And now, the probiotics are widely accepted in China [6]. The first fish vaccine was approved for production in 2011, and four fish vaccines are certificated as new veterinary drugs so far [3]. AMPs are one of the most potential alternatives which could be substituted of the conventional antibiotics [7,8]. In our previous studies, we identified dozens of new AMPs in marine animals, such as PC-hepc from *Pseudosciaena crocea*, and scygonadin, sphistin and sphyastatin from *Scylla paramamosain* [9–12]. PC-hepc has a rather wide spectrum of antimicrobial activity *in vitro* against the bacteria and fungi tested, and particularly shows strong activity against the principal fish pathogens, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *V. alginolyticus* and *V. harveyi* [9]. Previous studies reveal that both the native and recombinant products of scygonadin are active against Gram-positive bacteria such as *Micrococcus luteus*, *Staphylococcus aureus* and *Corynebacterium glutamicum*, and Gram-negative bacteria such as *A. hydrophila*, *Pseudomonas fluorescens* and *Shigella flexneri in vitro* [10,13]. Because of the low level of expression of AMPs *in vivo*, it is difficult and costly to extract and purify AMPs. The effective method to produce a large quantity of AMPs for commercial application

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at present is using genetic engineering techniques. Since the mature peptide of hepcidin is too small to be successfully expressed by engineering as reported [14], we used a fusion expression strategy to express the PC-hepc. We constructed a fusion sequence of PC-hepc combined with scygonadin and obtained the recombinant product of this fusion peptide, which is now produced as an additive of feedstuffs to be used for fish. In addition, some species of marine animals select growing plants, such as *Chlorella* for fish fries and *Gracilaria* for abalone, as edible food supplies other than eating feedstuffs. Therefore, we tried to develop an alternative approach by constructing transgenic microalgae with the AMPs gene as a different feed pattern for the special species living on plants.

In our study, we used the green microalgae *Chlorella*, which is widespread in freshwater and in the marine ecosystem, as the transgenic plant. Because it is unicellular and of small genome size, easy to culture, fast growing, low cost and able to serve as a food supplement, the microalgae *Chlorella* has attracted more and more attention as an ideal platform for larger production of pharmaceutical and industrial compounds [15]. Several functional proteins are successfully expressed in *Chlorella* cells, such as rabbit neutrophil peptide-1 gene [16], flounder growth hormone gene [17], and bovine lactoferrin *N*-lobe gene [18]. In addition, as a non-food, generally recognized as safe (GRAS), plant, the green alga *Chlorella* is edible for fish. Using the green microalgae *Chlorella,* the Scy-hepc fusion gene was transformed into the cells by electroporation, and thus a transgenic *Chlorella*, which could express Scy-hepc transcripts *in vivo*, was constructed in our study. The efficacy of the transgenic *Chlorella* sp. with the targeted Scy-hepc gene was evaluated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Strains and culture conditions

Chlorella sp. was obtained from the Center for Collections of Marine Algae, State Key Laboratory of Marine Environmental Science, Xiamen University, and cultured in f/2 medium at 25 °C under 70 μ mol m^2 s^{-1} on a 12:12 h light/day cycle.

Both plasmid pPIC9K and *Pichia pastoris* GS115 were purchased from Invitrogen (USA). The bacterial strains were purchased from the China General Microbiological Culture Collection Center.

The plant expression vector pMDC85 with 2 × 35S CaMV promoter was kindly provided by Professor Chen Liang (College of Life Science, Xiamen University).

2.2. Bactericidal activity of the Scy-hepc fusion protein produced by P. pastoris

The cDNA sequences separately encoding scygonadin (GenBank Accession No. AY864802) [19]and hepcidin (GenBank Accession No. EF156401) [9] were obtained from our previous work. Using overlap PCR amplification, the Scy-hepc fusion gene frame was obtained. Primers F1 with EcoR I site and primers R1 with overlap sequences (ACCGGAACCTGGGCCACC) were designed to amplify the scygonadin DNA fragment. The primer F2 with overlap sequences (GGTGGCCCA GGTTCCGGT) and Primers R2 with Not I site were designed to amplify the hepcidin DNA fragment. These primers sequences are shown in Table 1, the sequences of restriction enzyme sites are underlined and the overlap sequences of R1 and F2 are in bold. First, the scygonadin fragment was amplified using the primers F1 and R1, and then the hepcidin fragment was amplified using the primers F2 and R2 respectively. Secondly, the full length of the Scy-hepc frame was obtained using overlap PCR with F1/R2 as the primer, and the first PCR products of scygonadin and hepcidin frame as the template. Finally, the secondary PCR product was digested with *Eco*R I and Not I, and ligated into the *Eco*R I/Not I digested pPIC9K. Then the constructed plasmid pPIC9K-Scy/hepc was transformed into competent *P. pastoris* GS115 cells by electroporation using the Bio-Rad gene pulser Xcell™. A clone of GS115/pPIC9K-Scy/hepc was first cultured in BMGY medium, then resuspended in BMMY medium and grown at 28 °C with shaking at 230 rpm. After cultivation for 48 h, the culture medium was harvested using centrifugation at 10,000 g for 40 min. After 24–36 h dialysis, the supernatant containing the secreted component of Scy-hepc fusion protein was collected using centrifugation at 15,000 g for 40 min at 4 °C. Then the recombinant Scy-hepc fusion protein was purified using a HisTrap FF crude column (GE Healthcare Life Sciences). The protein concentration of purified Scy-hepc fusion protein was determined by Bradford method [20]. The purified recombinant Scy-hepc fusion protein was kept in ice before use for bactericidal activity assay.

Table 1
The Primers for PCR amplification.

Primer name	Sequence (5′to 3′)
Primers F1	GGGGAATTCGGCCAGGCACTCAACAA
Primers R1	**ACCGGAACCTGGGCCACC**GTAAGAAGCAATCCAGTCCTCGAC
Primers F2	**GGTGGCCCAGGTTCCGGT**GTCCCAGCCAATGAAGAGCAAG
Primers R2	CATGCGGCCGCTTAATGGTGATGGTGATGATGGAACCTGCAG CAGATACCA
Primers F3	CAA AAA AGC AGG CTT CAT GGG CCA GGC ACT CAA C
Primers R3	CAA GAA AGC TGG GTT GAA CCT GCA GCA GAT ACC
Primers F4	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT C
Primers R4	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT
Primers S1	ATG GGC CA G GC A CTC AA C
Primers H1	GAA CCT GCA GCA GAT ACC AC
Primers GF	GGA GAA GAA CTT TTC ACT GG
Primers GR	GTT CAT CCA TGC CAT GTG TA

Primers F1 and R2: the sequences of restriction enzyme sites are underlined.
Primers R1 and F2: the overlap sequences are in bold.
Primers F3, R3, F4 and R4: the sequences of attB sites are underlined.

The antimicrobial activity of the recombinant Scy-hepc fusion protein was determined against a panel of microorganisms, including Gram-positive *C. glutamicum*, *M. luteus*, *S. aureus* and *Staphylococcus epidermidis*, and Gram-negative *A. hydrophila*, *P. fluorescens*, *Pseudomonas stutzeri* and *Escherichia coli*. The minimal inhibitory concentration (MIC) for liquid growth inhibition assay was performed as previously reported [13]. Briefly, bacteria were diluted in 10 mM NaPB to OD600 = 0.003 (3–6 × 10^5 CFU mL^{-1}). The assay mixture consisted of 50 μL diluted purified peptide with different concentrations, 30 μL diluted bacteria suspension and 20 μL culture media. After 24 or 48 h of incubation at 28 °C, the MIC was determined after 24 h of incubation with bacteria. The lowest protein concentration induced no visible growth when compared with the negative control, and was defined as the MIC value [12]. This assay was performed in triplicate.

2.3. Expression vector construction using Gateway technology

Using two-step PCR amplification, the Scy-hepc fusion gene with attB DNA fragments was obtained. The constructed plasmid pPIC9K-Scy/hepc was used as a template. Primers F3, F4, R3 and R4 were designed following the User Guide: Gateway® technology (Invitrogen, USA) and the sequence of the Scy-hepc fusion gene, the sequences of attB sites in the primers are underlined, and the primers sequences are shown in Table 1. Primers F3 and R3 were used in the first PCR reaction under the following conditions: denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. Then, a final extension at 72 °C for 10 min. The resultant PCR product was electrophoresed in a 1.2% agarose gel containing DuRed DNA fluorescent dye (Fanbo Biochemicals, China) and observed on the FluorChem FC3 (Protein Simple, USA). Then the expected band about 510 bp was extracted from the agarose gel using a Gel Extraction Kit (Dongshengbio, China). This extracted attB-PCR product was used as the template DNA for the secondary PCR reaction. Using the primers F4 and R4, the Scy-hepc fusion

gene with attB DNA fragments was obtained.

The Scy-hepc fusion gene with attB DNA fragment was cloned into the Gateway donor vector pDONR207 *via* the BP recombination reaction (Invitrogen, USA). Consequently, the fusion fragment was cloned into the destination vector pMDC85 *via* the LR reaction (Invitrogen, USA). The resultant recombined plasmid pMDC85-scy/hepc was confirmed with DNA sequencing, and used for transformation.

2.4. Antibiotic sensitivity assay

The sensitivity of *Chlorella* sp. to antibiotic hygromycin B (Sigma, H7772) was investigated. 200 μL of culture at a cell density of approximately 1×10^7 cells mL^{-1} was spread on solid f/2 medium with different concentrations of hygromycin B (0, 100, 200, 300, 400 and 500 μg/mL). After an incubation period of 30 days, the sensitivity of *Chlorella* sp. to the antibiotic hygromycin B was evaluated as the lowest concentration yielding no visible growth.

2.5. Protoplast preparation of Chlorella sp.

Cells from 50 mL culture at a cell density of approximately 1×10^7 cells mL^{-1} were harvested using centrifugation at 6500 rpm for 5 min. The pellets were gently washed twice with sterilized seawater, then suspended in 5 mL of an enzyme mixture composed of 4.0% (w/v) cellulase R-10 (Yakult, Japan), 0.5% (w/v) pectinase (Yakult, Japan), and 0.6 M sorbitol, at pH 6.5 [21]. The cell suspension was incubated at 28 °C for 16 h in the dark with orbital shaking at 70 rpm for protoplast formation. Then the cell suspension was diluted twice with electroporation buffer (containing 500 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 20 mM HEPES, 300 mM mannitol and 300 mM sorbitol at pH 7.2) [22] and centrifuged at 1000 rpm for 5 min. The final protoplast pellets were resuspended in 1 mL electroporation buffer, and the final protoplast density adjusted to 10^8 cells mL^{-1}. The viability of freshly isolated protoplasts was determined using 0.01% w/v fluorescein diacetate (FDA) (Sigma, USA) and observed under a UV fluorescence microscope (DMLB, Leica).

2.6. Gene transfer by electroporation

Two hundred μL of the protoplasts resuspended in electroporation buffer were mixed with 10 μg plasmid pMDC85-Scy/hepc DNA and 30 μg calf thymus DNA, then incubated on ice for 10 min. The protoplast mixture was transferred to a 0.2 cm sterile electroporation cuvette and electroporated *via* a Genepulser XCell™ (Bio-Rad). Electrotransformation was conducted at 125 μF, 50 Ω and 2000 V/cm field strength. Electroporated protoplasts were incubated on ice for 5 min. Then a 5 mL aliquot of regeneration medium (f/2 medium plus 0.6 M sorbitol, 0.05 M glucose and 1% (w/v) yeast extract) [23] was added, and the cells were cultured in the dark at room temperature for 24 h.

Two hundred μL of the regenerated cells at a cell density of approximately 1×10^7 cells mL^{-1} were spread on a solid f/2 medium supplemented with 500 μg/mL hygromycin B at 25 °C for 30 days. Colonies grown on the agar plates (containing 500 μg/mL hygromycin B) were picked up and cultured in 5 mL liquid f/2 medium (containing 500 μg/mL hygromycin B) for secondary screening at 25 °C for 14 days. Next, the resistance colonies which grew well in the 5 mL liquid F/2 medium were extended cultured in 400 mL liquid f/2 medium (containing 500 μg/mL hygromycin B) at 25 °C for 60 days.

2.7. Screening the putative transgenic Chlorella sp. using PCR analysis

After 60 days extended culture, 100 mg of the transformed *Chlorella* sp. was collected by centrifuging at 6500 rpm for 5 min. The genomic DNA were extracted and purified using DNeasy® Plant Mini Kit (69104, Qiagen).

Primers S1 and H1 were synthesized for detecting the existence of the Scy-hepc fusion gene DNA fragments in genomic DNA, and primers GF and GR were synthesized for detecting the existence of GFP gene DNA fragments in genomic DNA, using PCR analysis. Primer sequences are shown in Table 1. Each PCR sample consisted of 20 μL of solution containing 1 μL genomic DNA, 1 μL primer F (10 μM), 1 μL primer R (10 μM), 10 μL HS taq mix (Dongsheng bio, China), 7 μL ddH_2O. PCR consisted of 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, followed by 10 min extension at 72 °C. PCR products (8 μL) were subjected to electrophoresis on a 1.2% agarose gel.

2.8. Fluorescence microscopy observation of GFP expression

The regenerated transformants which were confirmed using PCR analysis were selected for fluorescence microscopy observation. One hundred microliters of culture were added to a 96 well black glass bottom plate (*in vitro* Scientific), and placed at room temperature for 30 min. For confocal observation of the cells expressing GFP, images were taken on a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems) using 488 nm (GFP) and 633 nm (CphA) laser light for excitation. Detection windows ranged from 495 to 598 nm for GFP and from 647 to 721 nm for the chloroplast autofluorescence. Post-acquisition image analysis and processing was performed using the software ZEN 2011.

2.9. Protein extraction and western blotting analysis

Three hundred milligrams of the transformed *Chlorella* sp. were collected by centrifuging at 6500 rpm for 5 min. The pellets were gently washed twice with sterilized seawater and resuspended in 300 μL RIPA buffer (Beyotime, China) containing Pierce™ Protease Inhibitor Mini Tablets. The cell suspension was ground in liquid nitrogen with a mortar and transferred to a 1.5 mL centrifugal tube, then incubated on ice for 30 min. The supernatant was centrifuged for 10 min at 12,000 rpm at 4 °C, and 100 μL of 4 × sample loading buffer (1 mM EDTA, 250 mM Tris–HCl (pH 6.8), 4% SDS, 2% β-mercaptoethanol, 0.2% bromophenol blue, 50% glycerol) was added to the protein extract. Prior to SDS-PAGE analysis, samples were boiled for 10 min and then centrifuged for 5 min at 12,000 rpm. The supernatant was electrophoresed on a 12% SDS polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad) using *Trans*-Blot Turbo (Bio-Rad). The membrane was blocked with TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 8.0) containing 5% nonfat milk at room temperature for 60 min and incubated with either GFP antibody (2555S, Cell Signaling Technology), His antibody (TA-02, ZSGB Bio, China) or RbcL antibody (AS03037, Agrisera) in TBST containing 1% nonfat milk (overnight at 4 °C). Membranes were washed with TBST (three times for 8 min each) and then incubated with the HRP-conjugated goat anti-rabbit IgG or the HRP-conjugated goat anti-mouse IgG (ZSGB Bio, China) secondary antibodies in TBST at room temperature for 60 min. After washing three times, bound antibodies were visualized with an ECL substrate (Millipore). The result of immunoblotting was visualized on a Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology, China).

2.10. Bactericidal activity of the recombinant Scy-hepc fusion protein produced by transgenic Chlorella sp.

To detect the bactericidal activity of recombinant Scy-hepc fusion protein produced by the transgenic microalgae, we performed an inhibition zone assay. One hundred millilitres (about 1×10^7 cells mL^{-1}) of transformed *Chlorella* sp. were collected by centrifuging at 6500 rpm for 10 min. The pellets were gently washed twice with sterilized seawater and resuspended in 400 μL of Dulbecco's Phosphate Buffered Saline buffer. The cell suspension was ground in liquid nitrogen with a

mortar and transferred to a 1.5 mL centrifugal tube, then ultrasonicated for 10 min at 4 °C. The cell suspension was kept on ice for 1 h, then centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was used as the crude extract of the recombinant Scy-hepc fusion protein. The crude extract from the wild-type microalgae was used as a control.

The antimicrobial activity of crude extract from the transgenic microalgae was tested on agar plates. *A. hydrophila* was cultured on solid agar plates overnight. The next day, the cell density of *A. hydrophila* was diluted to 0.003 (OD_{600}). Then the cells were spread on the agar plates. Three wells were made in each plate and filled with 200 μL of crude extract from transgenic microalgae, crude extract from wild-type microalgae and Dulbecco's Phosphate-Buffered Saline buffer. Then the plates were grown overnight at 37 °C.

2.11. The efficacy of the transgenic Chlorella sp. with a high expression of Scy-hepc fusion protein

To determine the lethal condition of infection of *Sparus macrocephalus* by *A. hydrophila*, 10 *S. macrocephalus* (weight 67.43 ± 5.87 g) per group were infected with different concentrations of 1.5×10^7 cells, 1.5×10^8 cells and 1.5×10^9 cells of *A. hydrophila*, *via* peritoneal injection. Then the survival rate of *S. macrocephalus* was calculated at 48 h after infection. This assay was performed with two independent trials.

To estimate the *in vivo* efficacy of the transgenic *Chlorella, S. macrocephalus* and the hybrid grouper *Epinephelus fuscoguttatus* (♀) × *E. lanceolatus* (♂), two widely farmed species in China, were used. Ten *S. macrocephalus* (weight 69.52 ± 4.61 g) and 10 hybrid groupers (weight 62.89 ± 2.75 g) per group were used in this assay. We delivered 5×10^7 cells either wild-type or transgenic microalgae per fish into the stomach of *S. macrocephalus* (wild-type, n = 10; transgenic microalgae, n = 10) or hybrid grouper (wild-type, n = 10; transgenic microalgae, n = 10) by disposable catheter through the mouth once a day for three days. After three days, both wild-type and transgenic microalgae fed fish were infected with 1.5×10^8 cells of *A. hydrophila* per fish *via* peritoneal injection. Then the survival rate of microalgae-treated *S. microcephalus* or the hybrid grouper was calculated at different time points after infection. This *in vivo* assay was performed using two independent trials.

3. Results

3.1. Bactericidal activity of Scy-hepc fusion protein produced in P. pastoris

Using overlap PCR amplification, the fusion Scy-hepc sequence (the full length of 528 bp) was obtained, which was composed of scygonadin (309 bp), linker (18 bp), hepcidin (183 bp) and 6 × His-tag (18 bp) (Supplement Fig. 1). With the 6 × His-tag at the C-termimus of Scy-hepc, the recombinant Scy-hepc fusion protein was easily purified through a HisTrap FF crude column. Unspecific binding proteins were washed with 10 mM imidazole of the binding buffer (Supplement Fig. 1, lane3). Then the purified Scy-hepc was eluted with 300 mM imidazole, which appeared as a single band of 22 kDa of Scy-hepc fusion protein (Supplement Fig. 1, lane 4).

The recombinant Scy-hepc fusion protein produced in *P. pastoris* showed high activity against Gram-positive bacteria, such as *M. luteus* (MIC 1.6–3.2 μM), *C. glutamicum* (MIC 3.2–6.25 μM) and *S. aureus* (MIC 6.25–12.5 μM). In addition, it had high activity against Gram-negative bacteria, such as *A. hydrophila* (MIC 1.6–3.2 μM), *P. fluorescens* (MIC 1.6–3.2 μM) and *P. stutzeri* (MIC 6.25–12.5 μM) (Table 2). Results showed that the purified Scy-hepc fusion protein produced in *P. pastoris* had a rather wide spectrum of antimicrobial activities.

Table 2
The antimicrobial activities of Scy-hepc fusion protein produced by *P. pastoris*.

Microorganisms	CGMCC[b] No.	Scy-hepc from *P. pastoris* MIC[a] (μM)
Gram-negative bacteria		
Aeromonas hydrophila	1.2017	1.6–3.2
Pseudomonas fluorescens	1.0032	1.6–3.2
Pseudomonas stutzeri	1.1803	6.25–12.5
Escherichia coli	1.2389	> 50
Gram-positive bacteria		
Micrococcus luteus	1.029	1.6–3.2
Corynebacterium glutamicum	1.1886	3.2–6.25
Staphylococcus aureus	1.363	6.25–12.5
Staphylococcus epidermidis	1.2429	> 50

[a] MIC: minimal inhibitory concentration.
[b] CGMCC No.: China General Microbiological Culture Collection Center.

3.2. Plasmid construction

Using the recombinant plasmid pMDC85-Scy/hepc DNA as a template, the Scy-hepc fusion gene of 510 bp and GFP gene of 750 bp were found in the recombinant plasmid pMDC85-scy/hepc DNA using PCR assay. The recombinant plasmid pMDC85-scy/hepc was further confirmed using DNA sequencing. These results showed that the Scy-hepc fusion gene was successfully cloned into the pMDC85 expression vector using Gateway technology. The fusion gene with a GFP reporter gene and 6 × His tag was driven by the promoter of 2 × 35S CaMV, as described in Fig. 1.

3.3. Scy-hepc fusion gene effectively expressed in transgenic Chlorella sp.

As expected, the Scy-hepc fusion gene of 510 bp and GFP gene of 750 bp were found in the genomic DNA of transgenic *Chlorella* line Q5. The molecular mass of this positive band corresponded to that of the PCR product amplified from pMDC85- Scy/hepc plasmid DNA (Fig. 2A).

The GFP gene was linked with the Scy-hepc fusion gene downstreamed in the expression vector pMDC85-Scy/hepc. Visualization of GFP green fluorescence using a fluorescence microscope showed the expression of the Scy-hepc fusion gene. To detect the GFP green fluorescence, the regenerated transformants, which were confirmed using PCR analysis, were selected for fluorescence microscopy observation. Using 488 nm (GFP) laser light for excitation, the positive transgenic cells were visualized as bright green, but the wild-type cells were obscure in green fluorescence. Using 633 nm (CphA) laser light for excitation, both the positive transgenic cells and the wild-type cells showed red fluorescence. The green fluorescence of the positive transgenic cells was stronger than the red fluorescence. When the green fluorescence and the red fluorescence were merged, the positive transgenic cells were visualized as green with some yellow, whereas the wild-type cells were clearly visualized as red (Fig. 3).

To detect the Scy-hepc fusion protein in total soluble proteins extracted from transgenic *Chlorella*, the stable transgenic *Chlorella* line Q5 was used for immunological detection. Using rabbit monoclonal antibody against GFP and mouse monoclonal antibody against His tag, a specific peptide band with molecular mass of approximately 52 kDa was detected in the transgenic *Chlorella* line Q5, but no band was present in the wild type (Fig. 2B). Using mouse monoclonal antibody against scygonadin, a specific peptide band was detected in the transgenic *Chlorella* line Q5, and the molecular mass of this specific peptide was equivalent to that detected using rabbit monoclonal antibody against GFP. To further estimate the protein expression level of Scy-hepc in total soluble proteins, the purified Scy-hepc produced by *P. pastoris* with different concentrations was used as protein quantity control. When the loading quantity of total soluble proteins extracted from the transgenic microalgae line Q5 was 76.4 μg, the specific Scy-hepc band detected in the total soluble proteins lane was much clearer than the specific Scy-hepc band detected in the 0.04 μg purified Scy-hepc protein lane, but weaker than the specific Scy-hepc band detected

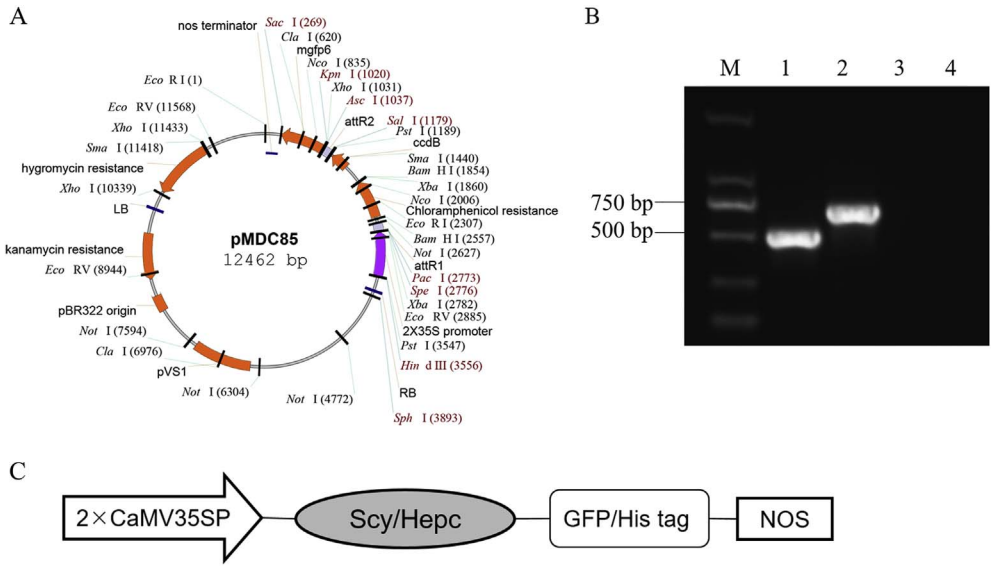

Fig. 1. Schematic diagram of the recombinant plasmid pMDC85-Scy/hepc. (A) Plant binary expression plasmid pMDC85. (B) PCR identification: result of the pMDC85-Scy/hepc expression vector. Lane 1: PCR product of Scy-hepc fusion gene; lane 2: PCR product of GFP gene; lane 3–4: negative control. (C) The expression frame of Scy-hepc fusion gene.

in the 0.06 μg purified Scy-hepc protein lane. Thus, the protein quantity of Scy-hepc fusion protein in total soluble proteins extracted from the transgenic *Chlorella* line Q5 was about 0.04–0.06 μg, and the expression level of Scy-hepc fusion protein in total soluble proteins extracted from the transgenic *Chlorella* line Q5 was about 0.052–0.078%, as shown in Fig. 2C.

In summary, both the results of fluorescence microscopy observation and western blotting showed that the Scy-hepc fusion protein was expressed successfully in the transgenic *Chlorella* line Q5.

3.4. The recombinant Scy-hepc fusion protein produced in transgenic Chlorella showing bactericidal activity

An inhibition zone assay was performed to detect the bactericidal activity of recombinant Scy-hepc fusion protein produced by the transgenic *Chlorella*. Results showed that the crude extracts from the transgenic strains exhibited significant inhibitory effects on the growth of *A. hydrophila*. An inhibition zone with a diameter of 3 ± 1 mm was visible around the well containing extracts from the transgenic *Chlorella* line Q5, but was absent around the well containing extracts from wild-type *Chlorella*, as shown in Fig. 4. Results indicated that the Scy-hepc fusion gene can be correctly transcribed and translated into active protein in the transgenic *Chlorella* line Q5.

3.5. The in vivo efficacy of the transgenic Chlorella sp. examined in two species of marine fish

The experimental *S. macrocephalus* were individually challenged with different concentrations of 1.5×10^7 cells, 1.5×10^8 cells and 1.5×10^9 cells of *A. hydrophila via* peritoneal injection. The survival

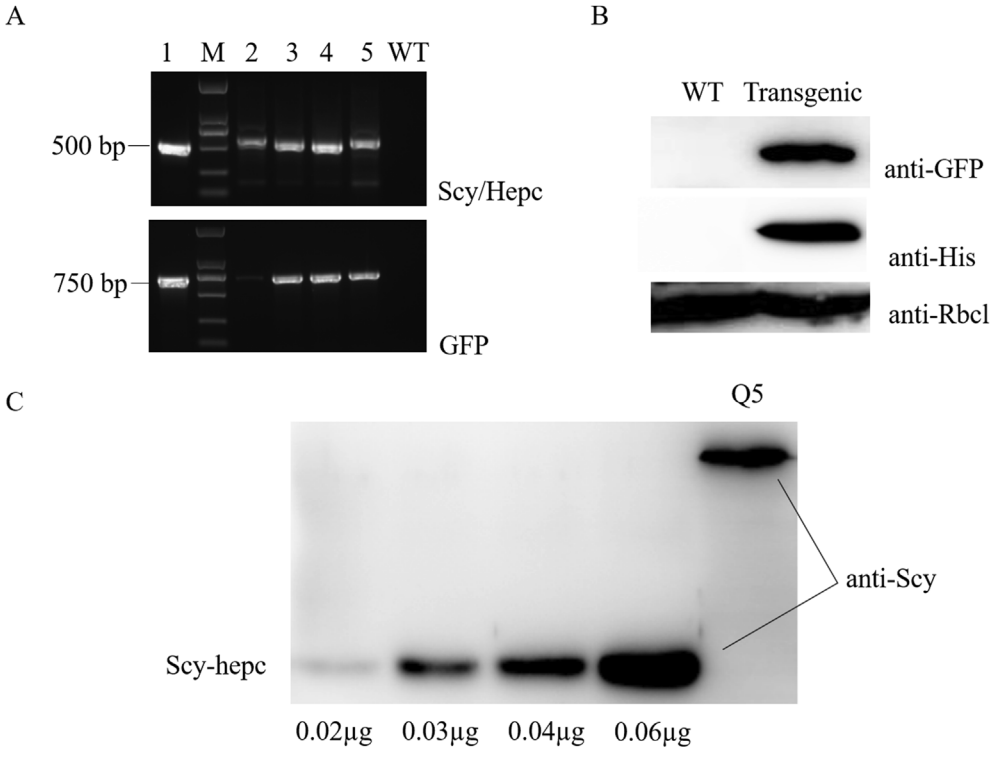

Fig. 2. Expression of the marine AMPs Scy-hepc fusion gene in transgenic *Chlorella* sp. (A) Scy-hepc fusion gene and GFP gene were detected in the genomic DNA of transgenic *Chlorella* line Q5. Lane 1: positive control (pMDC85-scy/hepc plasmid DNA used as a template); M: DNA marker (DL2000, Takara); lanes 2–5: the genomic DNA extracted from different generations of transgenic *Chlorella* line Q5 used as a template, 60 days as a generation, 2: T4 generation, 3: T3 generation, 4: T2 generation, 5: T1 generation; WT: the genomic DNA of the wild-type used as a template. (B) Using the *anti*-GFP antibody and anti-His antibody, a specific band was present in the transgenic *Chlorella* line Q5, but was absent in the wild type *Chlorella* sp. (C) Using the *anti*-Scy antibody and purified Scy-hepc fusion protein produced by *P. pastoris*, the expression levels of Scy-hepc fusion in total soluble proteins was estimated. Q5: total soluble proteins extracted from the transgenic *Chlorella* line Q5. Scy-hepc: purified Scy-hepc fusion protein produced by *P. pastoris,* the loading quantity was 0.02, 0.03, 0.04 and 0.06 μg.

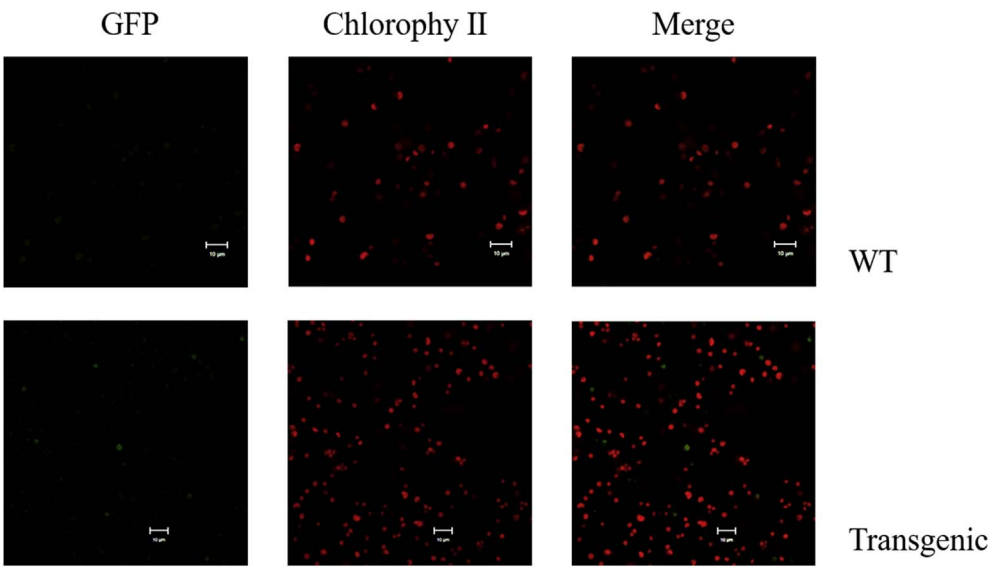

Fig. 3. Fluorescence microscopy observation of GFP expression. The positive transgenic cells were visualized as bright green and the wild-type cells were obscure in green fluorescence. The green fluorescence of the positive transgenic cells was stronger than the red fluorescence, when the green fluorescence and the red fluorescence were merged, the positive transgenic cells were visualized as green with some yellow, whereas the wild-type cells were clearly visualized as red. WT: wild-type cells, Transgenic: the transgenic *Chlorella* line Q5 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

rate of *S. macrocephalus* infected with 1.5×10^8 cells of *A. hydrophila* per fish was 43.33 ± 5.77% after 48 h infection; and that of *S. macrocephalus* infected with 1.5×10^7 cells of *A. hydrophila* per fish was 100% after 48 h infection; but 100% were dead within 24 h infection with 1.5×10^9 cells. Thus, a dosage of 1.5×10^8 cells of *A. hydrophila* per fish was selected to infect the fish in the subsequent study.

To estimate the *in vivo* efficacy of the transgenic *Chlorella, S. macrocephalus* and hybrid grouper *E. fuscoguttatus* (♀) × *E. lanceolatus* (♂), two widely farmed species in China, were used. *S. macrocephalus* were fed with 5×10^7 cells either of wild or transgenic *Chlorella* types once a day for three days, and then infected with 1.5×10^8 cells of *A. hydrophila via* peritoneal injection. Results showed that the survival rate of *S. macrocephalus* fed with transgenic *Chlorella* (80 ± 10% after 72 h) was significantly higher than that of fish fed with the same dosage of wild-type *Chlorella* (33.33 ± 11.55% after 72 h) (Fig. 5A). *S. macrocephalus* fed with transgenic *Chlorella* enhanced the resistance to *A. hydrophila.* Similarly, results showed that the survival rate of the hybrid grouper fed with transgenic *Chlorella* (55 ± 5% after 36 h) was much higher than that of fish fed with the same dosage of wild-type *Chlorella* (25 ± 5% after 36 h) (Fig. 5B).

4. Discussion

In our study, the Scy-hepc fusion gene was successfully cloned into the pMDC85 expression vector using Gateway technology. The recombinant plasmid pMDC85- Scy/hepc was transformed into the *Chlorella* sp. *via* electroporation. The Scy-hepc fusion gene was highly expressed in the transgenic *Chlorella* sp., and the recombinant Scy-hepc fusion protein produced by the transgenic *Chlorella* sp. showed strong bactericidal activity. In addition, the *in vivo* production of Scy-hepc fusion protein could enhance the survival rate of *S. macrocephalus* fed with transgenic *Chlorella* sp. compared to fish fed with wild-type *Chlorella* sp. This is the first report of a transgenic *Chlorella* sp. in which a fusion gene Scy-hepc from two marine AMPs was effectively developed.

Since it was realized that it was time to consider decreasing the dosage or stopping the misuse or overuse of antibiotics, the constant exploration for antibiotics substitute has been underway in many laboratories in the world [24,25]. AMPs are an integral part of the innate immune system in all organisms, and can protect a host from various invading pathogenic bacteria. In particular, they possess the unique features described in an AMPs: small size, heat-stable, no drug residues and drug resistant. Thus, AMPs are considered as one of the most potential alternatives for antibiotics [26]. Many people have become more and more interested in the therapeutic application of various AMPs from different species, since the first AMPs (cecropin) was discovered in 1980; and, in recent years, AMPs are commonly used in aquaculture to protect animals from bacterial infection [27]. At present, oral administration and intraperitoneal injection are two reported methods commonly employed for the administration of AMPs in aquaculture. Oral administration of the recombinant Epi-1 protects the grouper (*Epinephelus coioides*) and zebrafish (*Danio rerio*) against the Gram-negative *V. vulnificus* infection [28]; and, oral administration of the synthesized FSB-AMP also shows the potential protection of white shrimp against the infection caused by *V. parahaemolyticus* [27]. However, oral administration of AMPs is found to be susceptible to proteolytic degradation *in vivo* [29,30], and the cost of synthesized AMPs is too high to be used in aquaculture. Intraperitoneal injection has the most rapid response and be the most-effective method for fish [31]: zebrafish intraperitoneally injected with the synthesized epinecidin-1 peptide have increased resistance against *V. vulnificus* [32]; and intraperitoneal injection of AMPs CEME and pleurocidin amide also increases the resistance against the pathogen *V. anguillarum* in coho salmon [33]. However, it is not practical to quickly inject fish in large numbers, and intraperitoneal injection is difficult to carry out on fish fries and juveniles [34].

Confronted with the overall real problems, we developed an efficient AMPs delivery system using microalgae as an alternative

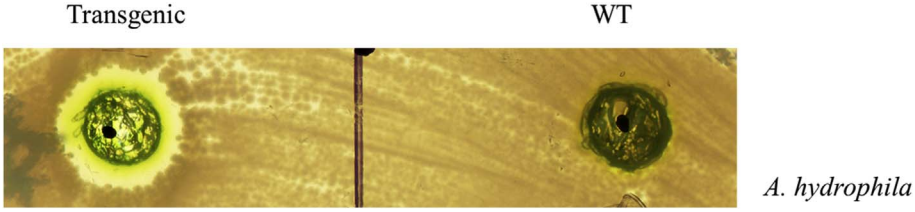

Fig. 4. Bactericidal activity of recombinant Scy-hepc fusion protein produced by transgenic *Chlorella* sp. Transgenic: crude extracts from the transgenic *Chlorella* line Q5. WT: crude extracts from wild-type *Chlorella* sp.

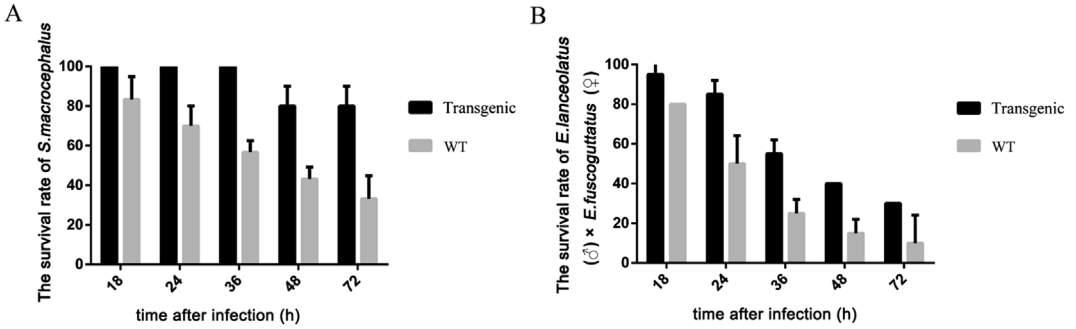

Fig. 5. The survival rate of *Sparus macrocephalus* and the hybrid *E. fuscoguttatus* (♀) × *E. lanceolatus* (♂) infected with *A. hydrophila*. (A) The survival rate of *Sparus macrocephalus* fed with transgenic *Chlorella* sp. (Transgenic) and wild-type *Chlorella* sp. (WT). (B) The survival rate of *E. lanceolatus* ♂ × *E. fuscoguttatus* ♀ fed with transgenic *Chlorella* sp. (Transgenic) and wild-type Chlorella sp. (WT).

approach. The major obstacle to be overcome for oral administration of AMPs is to prevent their digestion of the targeted peptides in the stomach. AMPs delivered by microalgae overcome this kind of problem by relying on the plant cell wall [35]. In addition, microalgae have rapid growth and easy cultivation, in particular the capability of performing post-transcriptional and post-translational modifications [36], by which they can be used as cell factories for large production of certain exogenous proteins such as AMPs [1]. The second concern in our study was whether the fish fed with transgenic microalgae had higher survival than the controls. Medaka fed with transgenic *Nannochloropsis oculata* with bovine lactoferrin gene have enhanced resistance against *V. parahaemolyticus*; and the survival rate of medaka fed with 1×10^8 transgenic microalgae and then infected with 1×10^5 *V. parahaemolyticus* cells per fish is significantly higher than that of medaka fed with the same dosage of wild-type algae and infected with the same dosage of *V. parahaemolyticus* [1]. In our study, *S. macrocephalus* fed with the transgenic *Chlorella* expressing the Scy-hepc fusion gene achieved similar results. *S. macrocephalus* fed with transgenic *Chlorella* enhanced the resistance to *A. hydrophila.* The survival rate of *S. macrocephalus* fed with transgenic *Chlorella* (80 ± 10% after 72 h) was significantly higher than that of fish fed with the same dosage of wild-type *Chlorella* (33.33 ± 11.55% after 72 h). Our artificial infective experiment on fish indicated that microalgae transgenic with AMPs genes could produce efficient resistance against invading pathogens with the production of AMPs in fish. For AMPs with a broad antimicrobial spectrum, microalgae transgenic with the AMPs gene would respond to more specific pathogenic bacteria.

One of the major requirements for developing a highly efficient *Chlorella* transformation expression system is selection of a suitable promoter [16]. The 35S CaMV promoter that is most often used in transgenic plants is used in the *Chlorella* transformation expression system [18,22,35]. In our study, we used the dual 35S CaMV promoter in the destination vector pMDC85 (Fig. 1A) because of its highly activity in most transgenic plant cells [37,38]. In comparison with the expression levels driven by other promoters, the expression level of our constructed transgenic microalgae was relatively higher than the GUS gene by rbcL promoter in *Chlamydomonas reinhardtii* (around 0.01% of total soluble proteins) [39]. However, there is still much left to be done to increase the expression level of the *Chlorella* transformation expression system, which is relatively weakly expressed under the 35S CaMV promoter than LFB by the hsp 70A promoter in *Nannochloropsis oculata* (around 4.27% of total soluble protein) [1]. The numbers of positive cells in cultures were lower, approximately 10% (Fig. 3), and, thus, collection of the positive cells in cultures by means of a flow cytometer may be useful to enhance the Scy-hepc fusion protein content in total soluble protein.

The green fluorescent protein (GFP) and its variants are now widely used as reporters for monitoring gene expression in many microbes [40]. The GFP is used as a reporter in transgenic *Chlorella* since the GFP signal can be easily and conveniently detected without manipulation of the samples [23,41]. In our study, the GFP gene followed the Scy-hepc fusion gene in expression vector pMDC85-scy/hepc. Visualization of GFP using a fluorescence microscope showed the expression of the Scy-hepc fusion gene. Since the fluorescence from GFP is green and the auto-fluorescence from chloroplasts red, the transformants could be easily distinguished from wild-type cells. In our study, the green fluorescence was unnoticeable until the plateau period of cell growth was completed (about 60 days), and the intensity of green fluorescence was enhanced as culture time progressed. This phenomenon was similar to that of another report [41], which indicates that the GFP proteins are synthesized mainly in late growth stages, and require long-term accumulation for microexamination.

In summary, the transgenic *Chlorella* sp. with a high level of expressing the Scy-hepc fusion protein has been developed as an oral delivery system for fish to effectively utilize its AMPs. The AMPs Scy-hepc generated in transgenic *Chlorella* cells enhanced the efficacy of the transgenic *Chlorella* against infection of *A. hydrophila* and increased the survival rate of the transgenic *Chlorella* over that of the wild-type cells. This study provides us with a convenient and feasible method of feeding AMPs to marine cultured animals.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.11.051.

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