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Expression of Tandem Repeat *Cecropin B* in *Chlamydomonas reinhardtii* and Its Antibacterial Effect*

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Abstract To overcome the negative effects of antibiotics commonly employed in most aquaculture, here we present a study to examine the feasibility of expressing an antimicrobial peptide by microalga as alternative. An antimicrobial peptide *Cecropin B* gene was modified according to the codon bias of the nuclear genome in *Chlamydomonas reinhardtii*. Four repeats of the *Cecropin B* gene were fused in tandem and each repeat was separated by inserting a cleavable linker peptide sequence (LWMRFA). The artificial DNA (522 bp in length) was inserted into a site between hsp70-RBCS2 promoter and RBCS2 terminator for constructing the expression vector pCB124. A cell-wall deficient strain of *C. reinhardtii* CC-849 was transformed by using glass bead method with pCB124. A large number of transformants were selected on Tris-acetate-phosphate media containing 10 mg/L Zeomycin. PCR and RT-PCR analyses on the transformants revealed that tandem repeated *Cecropin B* gene had been integrated into the genome of *C. reinhardtii* and could express at transcriptional level. The Western blot results confirmed the presence of recombinant antimicrobial peptide Cecropin B in the transgenic algal cells. The total protein was extracted from transgenic algae and its antimicrobial activity was tested. The results indicated that the extracted proteins from transgenic alga showed very strong antimicrobial activity against both Gram negative bacterium (*E. coli* JM109) and Gram positive bacteria (*Bacillus subtilis* and *Micrococcus lysodeikticus*). This finding has provided a new approach for production and utilization of antibacterial bait-algae.

Key words antimicrobial peptide, *Chlamydomonas reinhardtii*, bait-algae, tandem *Cecropin B*
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Antimicrobial peptides were originally characterized as a type of active polypeptide from insect haemocytes upon immunization. In 1975, a new type of peptide was found in *Hylophora cecropia* after infected with *Bacillus cereus* [1]. This peptide was recognized as a new member of antimicrobial peptide family, named "Cecropins". To date, over 1 700 species of antimicrobial peptides have been found in different species, including bacteria, fungi, amphibian, mammals, plants and humans[2].

Antimicrobial peptides are a class of active peptides of 20 to 60 amino acids, They contain usually 2 or 3 positively charged amino acids, such as arginine or lysine, which made them also known as positive antimicrobial peptides. They are soluble in water and highly thermal stable; moreover, they can not easily be enzymatic hydrolysis[3]. As for antimicrobial activity, they are effective against a broad spectrum of bacteria, fungi and protozoa[4-5]. Besides, it exists some special peptides which can even kill viruses and tumor

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cells[6-8]. What is more interesting, these antimicrobial peptides are highly efficient in killing bacteria even at concentration of 0.25～4.0 mg/L[9]. Since antimicrobial peptides have different mechanisms of action from common antibiotics, it is expected to become a new generation of antimicrobial drugs without the problem of bacterial resistance.

Cecropin B is an insect antimicrobial peptide isolated from *Bombyx mori*. It is known as the strongest natural antimicrobial peptides in its activity. It is effective against a broad spectrum of bacteria, fungi and protozoa. In contrast, Cecropin B has no harmful effect on eukaryotic organisms, and its activity is strictly limited to prokaryotic or eukaryotic cells which are involved in pathogenesis process.

In this study, the *Cecropin B* gene was transferred into the green algae *Chlamydomonas reinhardtii*. Recently, Li and Tsai[10] showed that medaka fish fed with transgenic marine microalgae containing an antimicrobial peptide LFB will have bactericidal defense against *V. parahaemolyticus* infection, which suggested the possibility of preventing the diseases in aquaculture using transgenic alga. In our study, we selected a freshwater microalgae *C. reinhardtii* as candidate, which is considered as a safe food because they are free from human pathogens and endotoxins [11]. The transgenic algal cells expressed the *Cecropin B* gene at both transcript and protein levels. In addition, the proteins extracted from the transgenic alga demonstrated significant antibacterial activity against both Gram-negative bacterium (*E. coli*) and Gram-positive bacteria(*Micrococcus lysodeikticus* and *Bacillus subtilis*). These encouraging results provide the feasibility for further feed-based delivery application in aquaculture.

1 Materials and methods

1.1 Algal species, plasmids and reagents

The *Chlamydomonas reinhardtii* CC-849 was provided from *Chlamydomonas* Genetics Center at Duke University. Plasmid pSP124 containing *ble* gene which confers resistance to phleomycin and zeomycin were obtained from Dr. Saul's laboratory (University College London, UK). Plasmid pH105 contains *Chlamydomonas* hsp70-RBCS2 promoter and RBCS2 terminator, *E. coli* and *B. subtilis* strains were maintained in our laboratory. *M. lysodeikticus* was a gift from Dr. Philippe Roch, Université de Montpellier 2. T4 DNA ligase, restriction enzymes *Nhe* Ⅰ, *Sal* Ⅰ and *Eco*RⅠ, DNA Fragment Purification Kit Ver. 2.0, and RNA PCR Kit (AMV) Ver. 3.0 were purchased from TaKaRa Biological Limited Company (Dalian, China). DNA Extraction kit was purchased from Jingmei Biological Engineering Limited Company, and E.Z.N.A® Plasmid Miniprep Kit Ⅰ was purchased from Promega.

1.2 Experimental methods

1.2.1 Modification and synthesis of the tandem *Cecropin B* gene. After modification according to the codon bias in *C. reinhardtii*, the *Cecropin B* gene was designed and synthesized (Gene bank accession AAA29184). Four identical *Cecropin B* gene sequences were fused in tandem, and a self splicing linker peptide LWMRFA was inserted between each repeat. The construct had a 6 × His tag at C-terminal of the construct, and *Nhe* Ⅰ and *Sal* Ⅰ cleavage sites were added at each end of the complete sequence. The total length of the fragment was 522 bp, which was synthesized by Shanghai Bioengineering Limited Company, China.

1.2.2 Construction of the recombinant expression vector. The tandem repeat *Cecropin B* was cloned into pH105 vector between hsp70-RBCS2 promoter and RBCS2 terminator, which was called pCB105. Next we digested the pCB105 with *Eco*RⅠ in order to connect with the vector pSP124 which contains the *ble* selection gene. This final expression vector was called pCB124. An enzymatic digestion and PCR analysis were used to verify the vector sequence after the construction.

1.2.3 Genetic transformation of *C. reinhardtii*. Recombinant plasmid pCB124 was linearized after digestion with *Not* Ⅰ enzyme. The glass-bead transformation method was used for the genetic transformation of *C. reinhardtii* cells with pCB124. The transformation procedure was as following: (1) The cell-wall deficient algal strain cc-849 was cultured until exponential stage under continuous light in TAP nutrient solution. When cell density reached 1×10^6～2×10^6 cells/ml, the alga were collected by centrifuging at 5 000 r/min at room temperature for 5 min. (2) The cell pellets were resuspended in sterile TAP nutrient solution to a final cell density of 2×10^8 cells/ml. (3) An aliquot of 300 μl suspension was transferred into a 1.5 ml Eppendorf tube which contained sterilized tin alloy beads. For genetic transformation, 1 μg of

linearized pCB124 plasmid was added to the tube, and the control was replaced by sterile water. (4) The algal cells/tin alloy beads/plasmid DNA mixture was shaken brutally for 25 s. (5) The mixture solution was transferred into 10 ml of fresh TAP medium, and cultured in an incubator under dim light overnight (22～25℃) to allow the cells to recover. (6) The overnight culture was centrifuged at 3 000 r/min at room temperature for 5 min. After the centrifugation, the cells pellet was resuspended in 4 ml 0.5% TAP medium, which was then spread on TAP plate containing 10 mg/L Zeomycin. The inverted plates were incubated at 22～25℃ in a lighted incubator until single green colonies were formed.

1.2.4 Identification of transgenic algal cells using PCR and RT-PCR.

The primer pair, which consists of P1 (5′ GCTA-GCATGAAGTGGAAGGTGTTC 3′) and P2 (5′ GTC-GACTTAGTGGTGGTGGTGGT 3′), was synthesized by Shanghai Bioengineering limited company, China for detection of the existence of the transferred DNA fragment. Both wild type and the putative positive transformants selected against antibiotics were cultured until late exponential growth stage, and then the cultures were centrifuged at 5 000 r/min at 4℃ for 5 min. After a step of centrifugation, genomic DNA and total RNA were extracted from the algal cells for further analysis by PCR and RT-PCR. PCR was performed with P1 and P2 primers using the following program: the first step of 94℃ for 3 min was followed by 30 cycles of 94℃ for 30 s, 60℃ for 1 min and 72℃ for 1 min, and a final extension at 72℃ for 10 min. PCR products were cloned for DNA sequence analysis after verified on 1% agarose gel.

For RT-PCR analysis, cDNA was reverse transcribed from 1 μg total RNA using RNA PCR Kit (AMV) Ver. 3.0.RT. The RT reaction was performed at 30℃ for 10 min followed by 50℃ for 40 min. A final step at 99℃ for 5 min was included to inactivate the transcriptase. The cDNA was used in PCR reaction which was performed with P1 and P2 primers following the same PCR program as described above. RT-PCR products were also analyzed on 1% agarose gel.

1.2.5 Western blot analysis of transgenic algal cells. The expression vector pH105 contains HSP70A-RBCS2 promoter-terminator, and the insert gene was placed under the heat inducible HSP70A promoter[12–13]. Both the wild type and transgenic cells were subcultured in fresh TAP medium under lighted condition. Upon reaching the exponential growth stage, protein expression was induced by twice heat shock treatments[12]. For heat induction, the cell cultures were placed at 40℃ under lighted condition for 30 min, followed by returning to 22℃ in a lighted incubator for 5 h. After final culture at 22℃ for 1 h, the cells were collected to extract total protein. For Western blot analysis, the first antibody used the monoclonal anti-His6 antibody and the second antibody was phosphatase labeled goat anti-mouse IgG.

1.2.6 Detection of antibacterial activity of Cecropin B protein using the zone of inhibition assay. To test the antimicrobial activity of transgenic alga, both Gram-negative bacterium(*E. coli* JM109) and Gram-positive bacteria (*M. lysodeikticus* and *B. subtilis*) were used. Aliquot of 100 μl of each bacterial culture at late exponential stage was spread onto each LB plate. After evaporation of the culture, holes were made in the plate, and then one drop of LB broth was added into each hole to cover the bottom of the plate. After algal protein extracts were added (80 mg/L), the plate was incubated for 24 h to observe the formation of the zone of inhibition.

1.2.7 Detection of antibacterial activity using the turbidimetry method. Three bacteria as mentioned previously were also used to test the antibacterial activity of the transgenic alga by turbidimetry method. For each bacterium tested, the following items were added, including each bacterial overnight culture 5 μl (A=1), LB broth 50 μl, and 75 μl protein extracts (80 mg/L) from either transgenic or wild type algal cells. A blank control was also included which replaced the protein extracts by equal amount of LB both. After mixing thoroughly, the reaction was carried out in a 37℃ water bath for 3 h with constant shaking. The culture was finally diluted into 3 ml LB broth, and incubated for 1 h. The absorbance (A) at 600 nm wavelength was read on a 721spectrophotometer.

2 Results

2.1 Modification of *Cecropin B* gene and construction of the tandem antimicrobial peptide gene

C. reinhardtii cell nuclear genome has a bias toward G and C nucleotides, therefore the original *Cecropin B* gene was modified according to the

degenerate codon and codon bias in the species. The modified *Cecropin B* gene is shown in Figure 1a. The resultant sequence had higher GC content. Four repeats of the modified *Cecropin B* gene sequence were cloned in tandem, and a self-splicing linker peptide LWMRFA was inserted between each repeat. The construct contained a 6×His tag at the C-terminal, and *Nhe* Ⅰ and *Sal* Ⅰ cleavage sites were added at N and C terminal, respectively (Figure 1b). The fragment was 522 bp in length with 64.2% of GC content. The construct met the *C. reinhardtii* genome preference for GC base pairs, which made it easier for *Cecropin B* gene to be integrated into the genome of *C. reinhardtii* and be stably expressed in transgenic cells.

(a) GCTAGCATGAAGTGGAAGGTGTTCAAGAAGATCGAGAAGATGGGCCGCAACATCCGCAACGGC
ATCGTGAAGGCCGGCCCCGCGATCGCGGTGCTGGGCGAGGCCAAGGCGCTGCAGCTGTGGATG
CGCTTCGCCAAGTGGAAGGTGTTCAAGAAGATCGAGAAGATGGGCCGCAACATCCGCAACGGC
ATCGTGAAGGCCGGCCCCGCGATCGCGGTGCTGGGCGAGGCCAAGGCGCTGCAGCTGTGGATG
CGCTTCGCCAAGTGGAAGGTGTTCAAGAAGATCGAGAAGATGGGCCGCAACATCCGCAACGGC
ATCGTGAAGGCCGGCCCCGCGATCGCGGTGCTGGGCGAGGCCAAGGCGCTGCAGCTGTGGATG
CGCTTCGCCAAGTGGAAGGTGTTCAAGAAGATCGAGAAGATGGGCCGCAACATCCGCAACGGC
ATCGTGAAGGCCGGCCCCGCGATCGCGGTGCTGGGCGAGGCCAAGGCGCTGCAGCACCACCAC
CACCACCACTAAGTCGAC

(b)

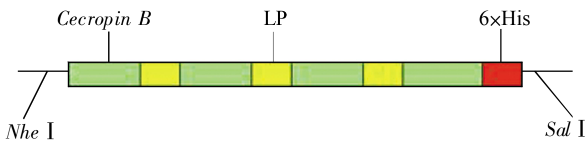

Fig. 1 The tandem repeat *Cecropin B* gene structure and sequence

(a) The modified DNA sequence of tandem repeat *Cecropin B* gene designed using codon bias of *C. reinhardtii* genome. (b) The DNA structure of tandem repeat *Cecropin B* gene. LP: Sequence of the self splicing linker peptide LWMRFA.

2.2 Construction of expression vector including the tandem antimicrobial peptide gene

The construction procedure of the expression vector is shown in Figure 2. After the digestion with *Nhe* Ⅰ and *Sal* Ⅰ, a 522 bp tandem *Cecropin B* gene fragment was isolated. Then it was ligated with pH105 vector which was previously digested with the same enzymes. This construct was called pCB105. After digestion of pCB105 plasmid with *Eco*R Ⅰ, the expression frame (1 300 bp) containing hsp70-RBCS2 promoter, tandem *Cecropin B* gene, and RBCS2 terminator were generated. The expression frame was connected into pSP124 vector which was digested with *Eco*R Ⅰ. This final recombinant expression vector was called pCB124.

2.3 PCR and RT-PCR analysis of transgenic *C. reinhardtii*

Using glass bead method, pCB124 containing the construct of *Cecropin B* gene was integrated into the nuclear genome of *C. reinhardtii*. The transgenic clones appeared in the TAP plates with 10 mg/L Zeomycin after 7 weeks culture.

Total DNA was extracted from alga collected from both transgenic and wild type cultures and PCR was performed using P1 and P2 primers. When the PCR products were analyzed on 1% agarose gel, transgenic cells produced a 522 bp band which was absent in wild type culture (Figure 3a). These results indicated that the tandem *Cecropin B* gene was inserted into the genome of *C. reinhardtii*.

Total RNA was extracted from transgenic and wild type cell cultures and cDNA was synthesized using TaKaRa PCR(AMV) kit. RT-PCR amplification with P1 and P2 primers produced a 522 bp band in the transgenic culture, but no signals in the wild type culture (Figure 3b). These results confirmed the transcriptional expression of *Cecropin B* gene in transgenic *C. reinhardtii*. The tandem *Cecropin B* gene was replicated and processed into mature mRNA in transgenic alga.

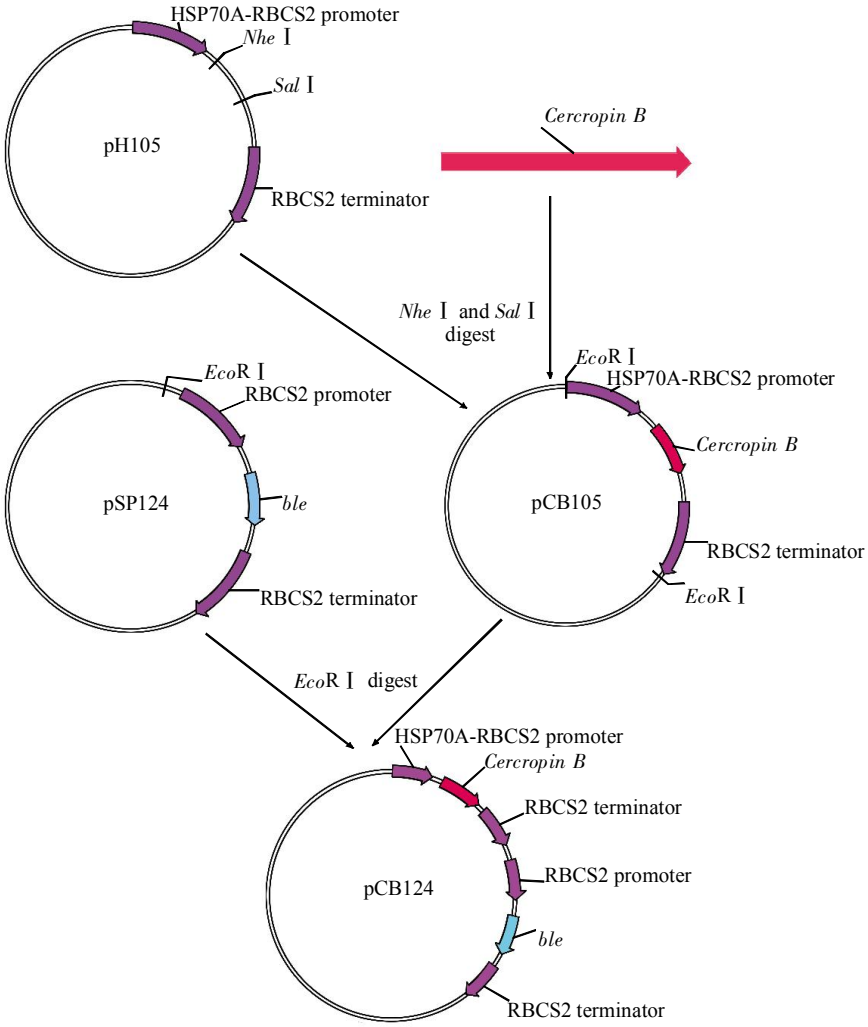

Fig. 2　Construction of the recombinant *Cecropin B* gene expression vector

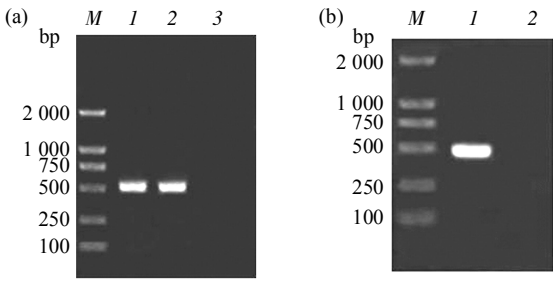

Fig. 3　Confirmation of transgenic *C. reinhardtii* using PCR(a) and RT-PCR(b)

(a) *M*: Marker; *1*: PCR analysis of transgenic type; *2*: The positive control; *3*: PCR analysis of wild type. (b) *M*: Marker; *1*: RT-PCR of transgenic type; *2*: The negative control.

2.4　Western blot analysis of transgenic *C. reinhardtii*

Total protein was extracted from both transgenic algae and wild type of *C. reinhardtii* CC-849. Two bands at 14 ku and 20 ku were detected with Western blot analysis against the monoclonal 6 × His antibody in transgenic culture, but no signals were observed in wild type culture (Figure 4). These results confirmed

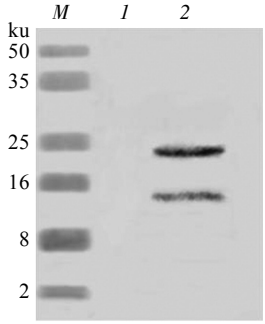

Fig. 4　Protein expression of *Cecropin B* gene in transgenic *C. reinhardtii* on Western blot

M: Protein marker; *1*: Total protein from wild type; *2*: Total protein from transgenic type.

the protein expression of *Cecropin B* gene in transgenic *C. reinhardtii*.

2.5 The antimicrobial activity assay of transgenic algae

Figure 5 showed the results from antimicrobial activity assay using the zone of inhibition assay. 100 μl of total protein at concentration of 80 mg/L extracted from transgenic *C. reinhardtii* was added, and we observed that clear zone was formed around the hole. The highest zone of inhibition was observed with *M. lysodeikticus*(51 mm) and lowest zone of inhibition was observed with *E. coli* JM109 (20 mm). Moreover, the protein extract from transgenic algal cells was also positive against *B. subtilis* with 32 mm zone of inhibition. In contrast, these results were absent from the test with protein extracted from wild type cells. Based on these tests, total protein from transgenic *C. reinhardtii* was active against *E. coli* JM109, *M. lysodeikticus* and *B. subtilis*. These results indicated that tandem *Cecropin B* gene had been integrated into *C. reinhardtii* and the protein was expressed as functional antimicrobial peptide.

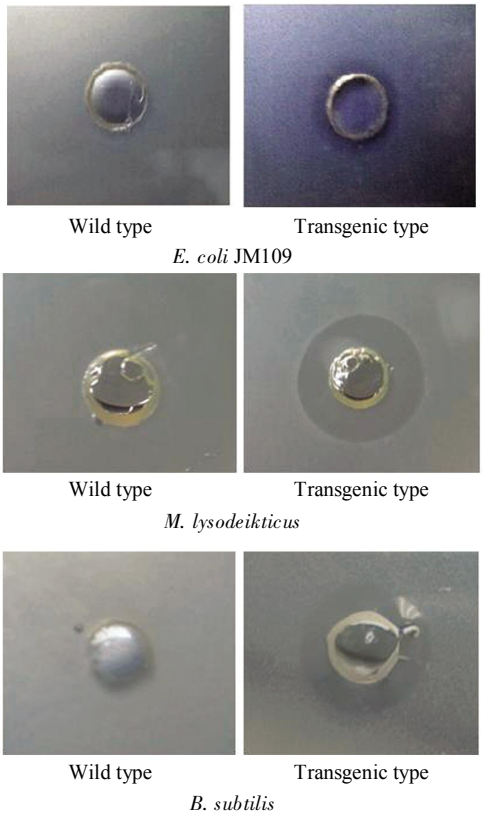

Fig. 5 The zone of inhibition assay with protein extracts from transgenic *C. reinhardtii*

In addition, antibacterial test was also carried out by turbidimetric method with the same strains of bacteria as previously mentioned. When the selected bacterial cells reached the exponential phase, they were treated with 80 mg/L total protein extracted from both transgenic and wild type cultures, respectively. By adding total proteins from transgenic *C. reinhardtii*, the biomass of *M. lysodeikticus*, *B. subtilis* and *E. coli* was 72.6%, 64.7% and 66.0% less than that of blank control. The most significant effect on *M. lysodeikticus* confirmed the result observed by the zone of inhibition assay. Moreover, compared with the same bacteria treated with the proteins extracted from wild type alga, the biomass was 37.1%, 51.0% and 24.0% less, respectively, which suggested the antibacterial activity of transgenic *C. reinhardtii* (Figure 6).

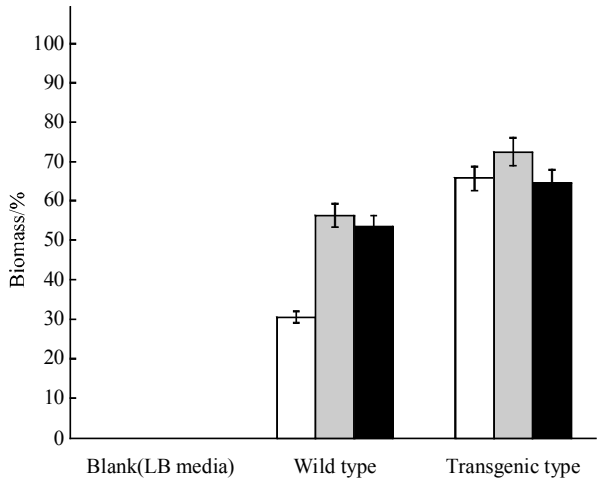

Fig. 6 Determination of antibacterial activity of total protein extracts from transgenic algae and wild type

□: *E. coli*; ▨: *M. lysodeikticus*; ■: *B. subtilis*.

3 Discussion

HSP70A-RBCS2 promoter is a heat-inducible promoter. After heat shock at 40℃, it could significantly enhance expression of the inserted genes[12–13]. In this paper, the heat shock at 40℃ significantly enhanced expression of the tandem *Cecropin B* gene in the transgenic algae (Figure 4). Compared to wild type, transgenic algal culture produced two unique bands at 14 ku and 20 ku on Western blot. It is probably that the transgenic *C. reinhardtii* synthesized three or four

repeats of the tandem Cecropin B peptides (Figure 4). The monomer of Cecropin B antimicrobial peptide has 36 amino acids, which equals to 4.3 ku. Two self-splicing linker peptides each made of 6 amino acids should be 1.4 ku. A sequence consisting of three repeats of Cecropin B peptides and two self splicing linker peptides will produce a peptide of 14.3 ku. Four repeats of Cecropin B peptide with three self splicing linker peptides and the 6 × His tag should have an apparent molecular size of 20.08 ku. These match the sizes of the two bands on Western blot (Figure 4). These results confirmed expression of the tandem *Cecropin B* gene at protein level in transgenic *C. reinhardtii*.

Pathogenic bacteria are one of the major causes of severe losses in aquaculture. Utilization of antibiotics can effectively control infection of pathogenic microbes, but the residue contamination has become a problem for food safety. Using antimicrobial peptides that are degradable by microbes provides a safe alternative to address the contamination issue of aquacultural products. However, it is quite difficult to produce large amount of antimicrobial peptide in prokaryotic organisms[14]. Cabral *et al.*[15] attempted to massively produce active antimicrobial peptides in *Pichia pastoris* expression system. However, the yeast expression system requires a lot of fermentation substrates, and its over-glycosyslation of the products makes its application limited. Oard *et al.*[16] succeeded to express β-purine sulfur in transgenic *Arabidopsis*, which has strong antimicrobial activity. It provided a new way to produce and utilize antibacterial bait-plant. Green algae provide food for many species of aquatic animals. Transgenic algae with antimicrobial activity in the feeding materials can help resolve the problem of bacterial contamination in aquatic animals. Moreover, it also may reduce contamination from chemical residues.

In this study, transgenic *C. reinhardtii* expressing successfully the tandem *Cecropin B* gene demonstrated very strong antibacterial activity against both Gram-negative bacterium(*E. coli*) and Gram-positive bacteria (*M. lysodeikticus* and *B. subtilis*). The bacterial biomasses treated with total proteins from transgenic *C. reinhardtii* were significantly lower than that treated with total protein from the wild type (Figure 6). These results suggested that it could be a good approach to produce transgenic algae that has antibacterial activity. In conclusion, the antimicrobial peptide Cecropin B was shown to be a suitable candidate for feed-based delivery using *C. reinhardtii*. A feed-based delivery system of antimicrobial peptides would be a valuable tool in preventing eventual aquatic diseases. Our data also suggested that a bait-algae system could be developed to effectively and economically treat bacterial infections in aquaculture.

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串联抗菌肽 *Cecropin B* 基因在莱茵衣藻中的表达及其抗菌活性分析 *

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摘要 为了应对各种抗生素在水产养殖业所带来的副作用，我们在本文中尝试利用微藻对一种抗菌肽进行表达的可行性研究．根据莱茵衣藻核基因组偏爱密码子对抗菌肽 *Cecropin B* 基因进行改造，并将 4 个经改造的 *Cecropin B* 基因依次串联起来，中间加上莱茵衣藻的自剪切连接肽序列 LWMRFA，人工合成总长度为 522 bp 的串联 *Cecropin B* 基因．将串联 *Cecropin B* 基因克隆到含 hsp70-RBCS2 启动子和 RBCS2 终止子的 pH105 载体上，再与携带 *ble* 筛选基因的表达框架连接，构建重组表达载体 pCB124．采用玻璃珠转化法将载体 pCB124 导入莱茵衣藻 cc-849 中，筛选得到能表达抗菌肽 Cecropin B 的转基因衣藻．经过 6 个月的保持培养后，进一步对转基因藻细胞提取液进行抗菌活性分析，发现转基因藻具有明显的抑制革兰氏阴性菌(大肠杆菌)和革兰氏阳性菌(枯草芽孢杆菌和溶壁微球菌)生长的特征．这一结果为具有抗菌活性的饵料藻的生产和应用提供了新的途径．

关键词 抗菌肽， 莱茵衣藻， 饵料藻， 串联 *Cecropin B*

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