Chloroplast Genetic Engineering of a Unicellular Green Alga
Haematococcus pluvialis with Expression of an
Antimicrobial Peptide

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Abstract
The purpose of this study was to express an antimicrobial peptide in the chloroplast to further develop the plastid engineering of H. pluvialis. Homologous targeting of the 16S-trnI/trnA-23S region and four endogenous regulatory elements, including the psbA promoter, rbcL promoter, rbcL terminator, and psbA terminator in H. pluvialis, were performed to construct a chloroplast transformation vector for H. pluvialis. The expression of codon-optimized antimicrobial peptide piscidin-4 gene (ant1) and selection marker gene (bar, biolaphos resistance gene) in the chloroplast of H. pluvialis was controlled by the rbcL promoter and psbA promoter, respectively. Upon biolistic transformation and selection with phosphinothricin, integration and expression of ant1 in the chloroplast genome were detected using polymerase chain reaction (PCR), southern blotting, and western blotting. Using this method, we successfully expressed antimicrobial peptide piscidin-4 in H. pluvialis. Hence, our results showed H. pluvialis promises as a platform for expressing recombinant proteins for biotechnological applications, which will further contribute to promoting genetic engineering improvement of this strain.

Keywords Haematococcus pluvialis · Chloroplast transformation · Antimicrobial peptide · Bar · Biolistic method

Introduction
Haematococcus pluvialis is a freshwater planktonic single-cell green alga, belonging to Chlorophyceae of order Volvocales (Hagen et al. 2002; Lee et al. 2016). It is capable of accumulating large amounts of astaxanthin (3, 3′-dihydroxy-diketo-β, β′-carotene-4, 4-dione), a liposoluble red ketocarotenoid with exceptional antioxidant properties (Galarza et al. 2018; Ma et al. 2018; Ota et al. 2018) and is considered to be the main source of natural astaxanthin (Ma et al. 2018; Ota et al. 2018). The application of astaxanthin in the field of human health has considerable potential (Liu 2018); in addition, it can also be used as a source of pigment and protein in the fish and poultry feed industry (Han et al. 2013).

Under stress conditions, H. pluvialis accumulates astaxanthin up to 1–4% of its dry weight (Shah et al. 2016); however, the production of astaxanthin by H. pluvialis is limited by low cell density, long growth cycle, and vulnerability to contamination, which is unfavorable in production and economy. Most studies on H. pluvialis have been aimed at obtaining high biomass and astaxanthin content by optimizing culture and induction conditions or the extraction method (Bauer and Minceva 2019; Fan et al. 1994; Li et al. 2019; Ma et al. 2018), albeit with limited progress. Genetic engineering
of *H. pluvialis* is a promising strategy of improving the properties of this important microalgal species. Hence, it has been proposed to express key enzymes for astaxanthin synthesis or high-value biological materials in cells through genetic engineering to improve the economics of *H. pluvialis* (Galarza et al. 2018; Specht et al. 2010).

Chloroplast genetic engineering is an emerging technology that can be used to produce recombinant proteins in industrial scale or for medicinal purposes and other new applications to improve wild algae strains. Overexpression of recombinant proteins via plastid engineering systems provides a cost-effective solution for using unicellular microalgae as bioreactors (Grevich and Daniell 2005). Owing to the safety of its application, this transformation system is becoming a new growth point in genetic engineering (Koo et al. 2013). *H. pluvialis* has the potential to be a vehicle for the production of large amounts of commercial proteins via transgenic expression from the chloroplast, as a part of the *H. pluvialis* chloroplast genome information is available and certain genes of unknown function have been annotated (Bauman et al. 2018; Zhang et al. 2019). In previous work, Gutierrez et al. (2012) firstly reported the location of a spectinomycin adenylyltransferase gene (*aadA*) regulated by the *rbcL* promoter and *rbcL* terminator in the chloroplast genome of *H. pluvialis*. Subsequently, another report showed that the endogenous phytoene desaturase nuclear gene (*pds*) and *aadA* was coexpressed in the chloroplast of *H. pluvialis* and increased the astaxanthin content by 67% (Galarza et al. 2018). These studies indicate that the chloroplasts of *H. pluvialis* are steerable, and plastid engineering is a potential strategy to improve wild *H. pluvialis*. However, although the chloroplasts of *H. pluvialis* are a potential platform for expressing recombinant proteins, there have been no reports of high-value recombinant proteins such as antimicrobial peptides expressed in *H. pluvialis*, which may be due to the lack of suitable genetic tools and the immature transformation system in the early stage of plastid engineering. Hence, it is necessary to accelerate the plastid engineering of *H. pluvialis* through more transformations.

In the present study, a chloroplast transformation vector was proposed using biolaphos resistance gene (*bar*) as selectable marker to further develop the plastid engineering of *H. pluvialis*. To the best of our knowledge, this is the first report of the successful expression of antimicrobial peptide piscidin-4 in the chloroplast of *H. pluvialis* via the optimized biolistic method. Additionally, the present study is one of the few reports on chloroplast transformation of *H. pluvialis*, which will be a prerequisite for strain improvement and the application of biotechnology to this important algal strain.

### Materials and Methods

#### Strain and Growth Conditions

*H. pluvialis* strain FACHB-712 was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences. The algae were cultured in modified MCM medium at 23 °C under indoor artificial light (10-μmol photons m⁻² s⁻¹ with a 12-h:12-h light: dark cycle) (Ota et al. 2018; Sun et al. 2008). *Escherichia coli* DH5α was cultivated in Luria-Bertani (LB) medium at 37 °C with shaking at 200 rpm (Yu et al. 2018).

#### Sequence Information and Primers

Four endogenous gene regulatory elements and the flanking sequences of the chloroplast genome insertion site in *H. pluvialis* were selected as the targets. These selected sequences were as follows: (i) *psbA* promoter (NCBI GeneBank Accession No. MT104459), (ii) *rbcL* promoter (NCBI GeneBank Accession No. MT104460), (iii) *rbcL* terminator (NCBI GeneBank Accession No. MT104461), (iv) *psbA* terminator (NCBI GeneBank Accession No. MT104462), (v) 165-*trnA*-23S (NCBI GeneBank Accession No. MG677935.1), and (vi) *trnA*-23S (NCBI GeneBank Accession No. MG677935.1). Antimicrobial peptide piscidin-4 gene (NCBI GeneBank Accession No. AKA60777.2) was determined to be *ant1* after codon optimized. Gene-specific primers were designed by Ruibo company (Qingdao, China) and all primer sequences are summarized in Table 1.

#### Cloning in the Chloroplast Expression Vector

Cells in the post-logarithmic phase were collected via centrifugation at 7000 rpm for 5 min. The total genomic DNA was extracted from the pelleted cells using the plant genomic DNA kit (Tiangen Biotech, China). The endogenous *psbA* promoter, *rbcL* promoter, *rbcL* terminator, and *psbA* terminator of *H. pluvialis* were amplified using sequence-specific primers (S3-For/S3-Rev, S4-For/S4-Rev, S5-For/S5-Rev and S6-For/S6-Rev). Similarly, primers (S1-For/S1-Rev and S2-For/S2-Rev) designed on the available sequence data of the chloroplast genome were used to amplify the chloroplast-specific regions 165-*trnA*-23S from the total genomic DNA of *H. pluvialis*. The *bar* marker gene was amplified from the pSVB vector (TaKaRa, China) using PCR and the codon optimized *ant1* was synthesized by Ruibo Company. All the PCR products were electrophoresed on 1% agarose gel, and the bands were detected using the ChemiDoc XRS+ system (Bio-Rad). All the target bands were recovered from agarose gel using the GeneJET gel extraction kit (Tiangen Biotech) and ligated with the pMD18T vector (TaKaRa, China). The recombinant vectors (pMD-*psbA*, pMD-*rbcL*, pMD-
TpsbA, pMD-TrbcL, pMD-trnl, and pMD-trnA) were transferred into *E. coli* using the heat shock method (Yu et al. 2018), and the accuracy of the sequences were analyzed via sequencing (Ruibo Company).

**Expression Cassette and Vector Construction**

The above recombinant plasmids were extracted from *E. coli* using the GeneJET plasmid miniprep kit (Thermo, USA). Primers (S1-For/S1-Rev, L1-For/L1-Rev, bar-For/bar-Rev, L2-For/L2-Rev and S4-For/S4-Rev) were designed to amplify 16S-trnI, psbA promoter, *bar*, *rbcL* terminator, and *rbcL* promoter, and the fragment-1 (16S-trnI-PpsbA:bar:TrbcL-trnA-TrbcL) was obtained as a part of the expression cassette using fusion PCR (Vazyme Biotech, China). Then, the above fragments were recovered using agarose gel electrophoresis and cloned in the pMD18T vector to obtain the recombinant pMD18T-F1 vector. Similarly, the *psbA* terminator and *trnA*-23S were amplified using primers (L3-For/L3-Rev and L4-For/L4-Rev) containing homologous sequences and suitable restriction sites. The fragment of *TpsbA-trnA*-23S with a SacI restriction site at the 5′ end and an EcoRI site at the 3′ end of the sequences was obtained using fusion PCR. *TpsbA-trnA*-23S and pMD18T-F1 were digested by SacI and EcoRI, and

| Table 1 | Primers used in this study |
|---------|-----------------------------|
| Name    | Primer sequence (5′-3′)     | Orientation | Special sequences | Target gene |
| S1-For  | CGCGCAAGCGTTGAGGAAGGTG     | For         | –                  | 16S-trnI    |
| S1-Rev  | CAACCCCTTTAACAATAGTATAGAC  | Rev         | –                  | 16S-trnI    |
| S2-For  | GGGGGCGCTAGCCAGAGGGC       | For         | trnA-23S           |
| S2-Rev  | CCTGGCTGATTCACAGGGGATTTC   | Rev         | trnA-23S           |
| S3-For  | AAAGCGCAACCTTTAAGTAGCTG    | For         | –                  | psbA promoter |
| S3-Rev  | ATGTTTGTITTTTTTTTTTTTTTAAATCT | Rev     | –                  | psbA promoter |
| S4-For  | AGCCCCAATGGCTGGGCTGCCATATGT | Rev      | –                  | rbcL promoter |
| S4-Rev  | TTATGTATGTATCAAAAGAATAGTTC | Rev      | –                  | rbcL promoter |
| S5-For  | TATATAGAATAAGTCGTACAGG     | For         | –                  | bar terminator |
| S5-Rev  | GGGGGTGCCCCAGAACACCTG      | Rev         | –                  | rbcL terminator |
| S6-For  | TTTTTTCTTAAAATATCAAACGTTA  | For         | –                  | psbA terminator |
| S6-Rev  | CGAGTCTGCGGGGCCCCAG        | Rev         | –                  | psbA terminator |
| bar-For | ATGAGCCAGAACACGCGCC        | For         | –                  | bar          |
| bar-Rev | TCATCAATCTCGGTGACGGG       | Rev         | –                  | bar          |
| L1-For  | GTCTATACCTAGTTTTAAGGTTGAAAG | For        | –                  | psbA promoter |
| L1-Rev  | CGCAACCTTTAAAATAGTACG      | Rev         | –                  | psbA promoter |
| L2-For  | CCCGTCACCGAGATTTGTGATAGT   | For         | rbcL terminator    |
| L2-Rev  | ATAGAATAGAAGCTAACC         | Rev         | –                  | psbA terminator |
| L3-For  | CGAGCTCTTTTTTTTTTTTTAAATCTA | For     | SacI               | psbA terminator |
| L3-Rev  | CGCTTCTGGCTAGGGGCCCCCGGA  | Rev         | –                  | psbA terminator |
| L4-For  | GGGGCCCTAGACGGAGGGCG (S2-For) | For     | trnA-23S           |
| L4-Rev  | GGAATCCCCCTCGCTGATTCACAGGG | Rev         | EcoRI              | trnA-23S    |
| F1-For  | CACGTAGGATACCATACATCACTACATCA | For     | XbaI, 6 × His      | ant1         |
| F1-Rev  | GGGATCCGGAATGTTGATGTTGATGTT | Rev         | BamHI              | ant1         |
| con-For | GTAGCTTGAATGCTGTCAGCAGCATC | For         | –                  | 16S-trnI-trnA-23S |
| con-Rev | TCCCACTGCTAGTAAAGCATAAG    | Rev         | –                  | 16S-trnI-trnA-23S |

Letters in underlined in the sequence of primers correspond to newly introduced restriction sites or histidine tag

Letters in italics in the sequence of primers correspond to the homologous sequences added on the side of the gene of interest
then ligated using T4 ligase to obtain the chloroplast homologous recombinant pHp/ch/bar vector of H. pluvialis without any foreign gene of interest. The primers (F1-For/F1-Rev) containing the 6× His tag and suitable restriction sites were designed to amplify ant1 with an XbaI restriction site at the 5’ end and a BamHI site at the 3’ end of the sequences. Then, ant1 was cloned in the pHp/ch/bar vector after double digestion to obtain the pHp/ch/bar/ant1 vector.

**Biological Transformation and Selection of Transformed Strains**

Cells in the logarithmic growth phase were adjusted to a density of 1 × 10^8 cells mL^-1 and 1-mL cell suspension was spread in the center of the solid plate. Based on the description of Gutierrez et al. (2012), the biolistic method was further optimized. The optimized bombardment parameters, i.e., 9-cm flight distance and 650-psi acceleration pressure, were used to obtain effective plastid transformation in H. pluvialis. As a negative control, the cultures were also transferred with empty plasmid.

The bombarded cells were regenerated on solid plates in dark conditions for 8 h and then cultured in liquid media under normal conditions for 40 h to restore the viability of cells. Upon regeneration, the cells transformed with pHp/ch/bar/ant1 were transferred to selective MCM medium with 15-μg mL^-1 phosphinothricin for 15 days. Subsequently, surviving cells were transferred onto fresh MCM agar plates containing 10-μg mL^-1 phosphinothricin and incubated for 2 weeks. Single clones were selected and streaked on MCM agar plates with 5-μg mL^-1 phosphinothricin. Finally, a total of 40 single algae clones were randomly selected and cultured in liquid MCM media with 5-μg mL^-1 phosphinothricin.

**Molecular Identification of Transformed Strains**

The transformed algal cells were collected at post-logarithmic phase after centrifugation at 7000 rpm for 5 min, and total genomic DNA was extracted as the template for PCR analysis. To identify homogenized algae strains, bar and the upstream and downstream homology arms of the expression cassette were amplified using specific primers (bar-For/bar-Rev and con-For/con-Rev) and sequenced. PCR on genomic DNA isolated from wild type H. pluvialis cells was performed as a control. A transgenic strain harboring both bar and ant1 were selected as the co-transformant and was used for subsequent analysis.

For Southern blot analysis, cells in the post-logarithmic phase were ground in liquid nitrogen and resuspended in 10-mL phosphate buffered saline (PBS). After centrifugation at 10,000 rpm for 5 min, the supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gradient gels and transferred to a PVDF (polyvinylidene difluoride) membrane (Immobilon®-PSQ Transfer Membrane, Merck, Germany) (Kang et al. 2015). The ant1 encoded protein containing 6× His tag was identified using mouse anti-His IgG and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma, USA). The membrane was washed with PBS, and signals were detected using Tanon-5200 (Tanon, China).

To further access the transgenic strain, the growth rate of T1 strain was compared with the wild type H. pluvialis. The growth rate of H. pluvialis in the liquid MCM medium was calculated by measuring OD680 once every 2 days.

**Statistical Analysis**

The experiments were performed in biological triplicates (n = 3) to ensure reproducibility. All values were presented as means ± SD. Statistical analyses were performed using the SPSS statistical package (http://www-01.ibm.com/software/analytics/spss/).

**Results**

**Chloroplast Transformation Vector of H. pluvialis Was Constructed**

To constructed the chloroplast transformation vector of H. pluvialis, the flanking regions of 16S-trnL (1040 bp) and trnA-23S (1060 bp) and endogenous regulatory elements containing the psbA promoter (507 bp), rbcL promoter (256 bp), rbcL terminator (195 bp), and psbA terminator (175 bp) were amplified. All fragments were sequenced and the results showed that all sequences were authentic. The final pHp/ch/bar/ant1 vector was constructed in the following way: 16S-trnL-PpsbA-bar:TrbcL:PrbcL:ant1:TpsbA-trnA-23S (Fig. 1).
After DNA sequencing, the results showed the correct assembly of the pHp/ch/bar/ant1 vector.

**Transformed H. pluvialis Cells Were Selected from Selective Agar Plates**

*H. pluvialis* cells were bombarded with plasmid pHp/ch/bar/ant1 using the optimized bombardment parameters. Bombarded *H. pluvialis* cells recovered on solid plates for 8 h after bombardments in liquid media before the DNA was extracted. The putative plastid transformants were initially identified using PCR. Amplification of a 550 bp PCR product using the bar-For/bar-Rev primer pair confirmed the presence of bar in transplastomic lines, while the wild type *H. pluvialis* did not show any amplification (Fig. 2a). The homoplasmic condition of the transplastomic cells was verified as a 4 kb size amplicon in transplastomic *H. pluvialis* and a 2.1 kb fragment in wild type *H. pluvialis* using the con-For/con-Rev primer pair (Fig. 2b). However, the 2.1 kb and 4 kb fragments coexisted in transgenic *H. pluvialis* (T1 strain), indicating that the transformants were not homogenized. Sequencing analysis also showed that the sequences of bar and the expression cassette were consistent with the vector sequence. The above results indicated that the foreign gene had been inserted into the chloroplast genome of *H. pluvialis*, although homogeneity was not achieved.

To further determine the insertion of ant1 in the chloroplast genome of *H. pluvialis*, genomic DNA was digested with *Xho*I and *Hind*III restriction enzymes and subjected to Southern blot analysis under stringent conditions. Strong hybridization signals were obtained using a 555-bp fragment of bar and a 220-bp fragment of ant1 as probes, respectively, while the wild type *H. pluvialis* did not show any hybridization signal (Fig. 2c, d).

The total soluble proteins were extracted and used for SDS-polyacrylamide gel electrophoresis. Expression of the protein product from the introduced ant1 gene was tested by western blot analysis using a monoclonal antibody against His tag. Results showed the expression of a protein with molecular weight of approximately 9.53 kDa in T1 strain, which was consistent with the expected size of 9.53 kDa based on the piscidin-4 amino acid sequence, while the protein was not detected in untransformed cells (Fig. 2e), indicating the stability of the transgene expression.

**Transformed H. pluvialis Showed the Similar Growth Rate with Wild Strain**

During 16 days of culture with the same initial concentration of the wild type and T1 strain, a typical sigmoidal behavior of microalgae growth was observed. Both wild type and T1 strain entered to the logarithmic phase after inoculation for 4 days, which lasted about 8 days and then entered the stationary growth phase (Fig. 3). Cell growth reached the highest cellular density at day 12; the cell concentration of the wild type *H. pluvialis* was 0.842 (OD680), while the T1 strains reached 0.853 (OD680). The statistical analysis showed no significant difference in growth rate between T1 strain and wild type *H. pluvialis*.

**Discussion**

One of the key requirements for the development of an efficient chloroplast expression system is the identification of appropriate promoters and regulatory sequences, which can provide the basis for vector construction (Gutierrez et al. 2012). However, only a few suitable genetic tools are currently available for *H. pluvialis*. Studies on microalgae and higher plants have shown that the *rbcL* promoter and *psbA* terminator have higher transcriptional activity (Klein et al. 1992; Klein et al. 1994; Lilly et al. 2002; Gutierrez et al. 2012; Gimpel et al. 2015). In addition, endogenous elements containing *psbA* promoter, *psbA* terminator, *rbcL* promoter, and *rbcL* terminator are available as previously reported by Galarza et al. (2018). Therefore, the endogenous promoters and terminators of *H. pluvialis* were cloned and used to regulate the expression of *ant1* and *bar* genes. Similarly, the inverted repeat 16S-trnI/trnA-23S of *H. pluvialis* was identified and used as a homologous target site in this study because it showed higher expression levels of foreign gene in chloroplast (Narra et al. 2018). Our results showed that *ant1* was expressed in *H. pluvialis*, indicating that an exogenous gene can be successfully expressed by endogenous regulatory sequences.

Since the first report of plastid transformation, a series of selectable marker genes have been developed. However, with the exception of the *aadA* cassette, most selectable markers are used only occasionally. Herbicides were shown to be specific to some processes that take place in the plastids; hence, the tolerance to various herbicides has been used to design chloroplast selection markers (Day and Goldschmidt-Clermont 2011). The sensitivity of *H. pluvialis* to phosphinothricin was investigated in our previous work.
Strong inhibition of cell growth by phosphinothricin appeared at a concentration of $5 \, \mu g \, mL^{-1}$ and the complete inhibition was at $15 \, \mu g \, mL^{-1}$ (data not shown). Therefore, it can be concluded that the \textit{bar} gene encodes phosphinothricin acetyl transferase is suitable selection markers for \textit{H. pluvialis}. To the best of our knowledge, this report showed the first use of

\textbf{Fig. 2} Identification of the expression cassette in transformed \textit{H. pluvialis}. \textbf{a} verification of \textit{bar} in transformed \textit{H. pluvialis} using PCR; \textbf{b} verification of the expression cassette in T1 strain using PCR; \textbf{c}, \textbf{d} southern blot analysis of genomic DNA from T1 strain. Genomic DNA was digested with restriction endonucleases \textit{XhoI} and \textit{HindIII} and then hybridized with \textit{bar} and \textit{ant1} as probes, respectively; \textbf{e} piscidin-4 protein accumulation in T1 strain determined by western blotting. M, DNA marker; Wild, wild type \textit{H. pluvialis}.

\textbf{Fig. 3} Cell growth curve of T1 strain and wild type \textit{H. pluvialis}. Significant difference is indicated at $P<0.05$ level. Each value represents mean $\pm$ SD ($n = 3$). WT, wild type \textit{H. pluvialis}.
the bar gene as a selectable marker in plastid transformation of *H. pluvialis*, which provides more options for the plastid engineering of *H. pluvialis*.

Currently, three main methods are used for microalgae chloroplast transformation as follows: biolistic method, glass bead method, and electroporation (Gan et al. 2018; Guo et al. 2013; Wannathong et al. 2016; Yamano and Fukuzawa 2020). Among these, the biolistic method is considered to be effective for *H. pluvialis* transformations (Galarza et al. 2018; Gutierrez et al. 2012; Teng et al. 2002). However, the transformation efficiency of *H. pluvialis* reported by Galarza et al. (2018) was rather low. Hence, we transformed *H. pluvialis* using the optimized biolistic method with a newly constructed chloroplast expression vector. The transformation efficiency was slightly higher than that observed by Gutierrez et al. (2012), which may be due to the higher cell density (10^8 cells mL^-1) during transformation, or the lower bombardment pressure (650 psi) reduced the mortality of transformed cells.

Algae chloroplasts show genomic polyploidy (Galarza et al. 2018). If the target gene is expressed from each set of genes, the expression of the target product will increase significantly. However, at the early stage of transformation, only a small part of the chloroplast genome contains the genes of interest, which does not guarantee stable inheritance of the obtained traits (Purton et al. 2013). Multi-passaging of the transformants on a screening medium for increasing the homogenization level of the chloroplast transformants of *H. pluvialis* can result in stable inheritance and increase the expression level of the target gene. Integration of the transgene into the chloroplast genome of transformed *H. pluvialis* was assessed 6 months post-bombardment selection and antibiotic plate re-streaking. Wild type *H. pluvialis* showed only a fragment of 2.1 kb (Fig. 2b), which represents the natural *trnI*/*trnA* site without the expression cassette. However, one of the transformants showed both the 2.1 kb and 4 kb fragments in PCR (Fig. 2b), indicating that the transformants were heteroplasmic. Analysis of the other transformants showed that all the strains were heterogeneous (showing two bands; data not shown), indicating that the transgene had been inserted into some copies of the chloroplast genome. Compared to that in other algal strains, obtaining homogeneity in the chloroplast genome of *H. pluvialis* is difficult, which has also been reported previously (Galarza et al. 2018; Gutierrez et al. 2012). However, although no homoplastids were obtained, our results showed stable expression of foreign protein in transformants and similar growth rate between transformants and wild type strain.

Antimicrobial peptides are considered as a potential substitute of traditional antibiotics due to their broad-spectrum antibacterial and environmentally friendly characteristics (Izadpanah and Gallo 2005). So far, some unicellular algae successfully expressed antimicrobial peptides were reported, such as *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, and *Nannochloropsis oculata* (Campos-Quevedo et al. 2013; Koo et al. 2013; Surendhiran et al. 2014). As a commonly used bait, *H. pluvialis* is expected to be used as a platform for expression of recombinant proteins in biotechnology applications, such as the development of oral vaccines for aquaculture. Research suggests that piscidin-4 exhibits specialized anti-bacterial and anti-protozoal activities in fishes (Salger et al. 2016). Thus, expressing antimicrobial peptide piscidin-4 in *H. pluvialis* has some benefits, enabling the production of stable, functional protein and improving animal resistance to pathogens via oral feed. In fact, microalgae expressing functional proteins can be fed directly without a subsequent protein purification process, which is also economically advantageous.

**Conclusions**

In this report, a chloroplast expression vector of *H. pluvialis* with bar as selectable marker was transformed using optimized biolistic method. We confirmed that recombinant antimicrobial peptide piscidin-4 could be expressed from the microalga *H. pluvialis*. Considering that the transgenes will not affect the growth rate, the recombinant proteins can be expressed in the industrial strain of *H. pluvialis* to further develop this important microalgal species.

**Author Contributions** Chunxiao Meng and Song Qin designed the experiments. Kang Wang and Yulin Cui analyzed the data and wrote the thesis with support from Tianzhong Liu. Zhengquan Gao and Yinchu Wang performed all experiments.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethics Approval** Not applicable.

**Statement of Informed Consent** Not applicable.

**Code Availability** Not applicable.
