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ORIGINAL ARTICLE

Preparation of transgenic *Dunaliella salina* for immunization against white spot syndrome virus in crayfish

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Abstract Although a white spot syndrome virus (WSSV) subunit vaccine could significantly enhance the immune response and benefit the shrimp host, its practical application is currently not feasible because of drawbacks in existing expression systems. We generated a transgenic *Dunaliella salina* (*D. salina*) strain by introducing the WSSV VP28 gene to produce a novel oral WSSV subunit vaccine. Following transformation of *D. salina*, VP28 gene expression was assessed by reverse transcription polymerase chain reaction (RT-PCR) assays, enzyme-linked immunosorbent assays (ELISAs), and western blot analysis. The RT-PCR results indicated that the VP28 gene was successfully expressed in *D. salina* cells. The presence of recombinant VP28 proteins with natural bioactivity was confirmed by western blot analysis and ELISA. Animal vaccination experiments indicated that transgenic *D. salina* can induce protection against WSSV by oral delivery in crayfish. Our findings indicate that the VP28 gene can be successfully expressed in transgenic *D. salina* and can be applied as an oral vaccine to protect crayfish against WSSV. We have demonstrated that it is feasible to produce an oral vaccine using *D. salina*, and thereby provide a new method for controlling other viral diseases in crustaceans.

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Introduction

White spot syndrome virus (WSSV) is the most serious pathogen that adversely impacts shrimp farms worldwide. An efficient vaccine would be a desirable and feasible method for controlling the spread of WSSV. There is growing evidence that various vaccines, such as inactivated WSSV vaccines [1, 2], recombinant viral subunit vaccines [3], DNA vaccines [4], and RNA vaccines [5, 6], can significantly enhance immune responses in the host and enhance resistance against WSSV-associated diseases.

More than 39 WSSV structural proteins have been identified [7, 8]. VP28, the main viral envelope protein, plays a pivotal role during viral infection, budding, entry, and virion assembly and has become the main target for exploitation of WSSV subunit vaccines [9, 10]. The expression of recombinant VP28 has been attempted in a variety of host systems, including *Pichia pastoris* [11], *Brevibacillus brevis* [12], *Escherichia coli* [13, 14], baculoviruses [15], silkworm pupae [16], and *Bacillus subtilis* [17, 18]. However, there are several disadvantages associated with these expression systems, including unsafe injection methods, high costs, long cultivation times, and difficulties in batch production. These disadvantages have resulted in the aforementioned expression systems not being considered feasible for practical applications in aquaculture. Thus, an optimal expression system for the production of a WSSV subunit vaccine is necessary and must be developed.

The eukaryotic green alga, *Dunaliella salina*, has been exploited as a novel bioreactor [19], as it has a very simple unicellular structure and can be cultured easily, rapidly, and cheaply. It has a high capacity for large-scale growth under natural conditions and can effectively accumulate protein products at high yields. *D. salina* lacks a rigid cell

wall, making it a naturally occurring protoplast, which in turn makes it relatively easy to introduce exogenous genes. As a eukaryotic organism, it can facilitate transcription and translation to produce crude proteins [20]. Because it is photosynthetic, *D. salina* can be cultured in open photobioreactors or natural seawater. *D. salina* cells have a single, large, cup-shaped chloroplast which can be used as an alternative and effective expression system to nuclear expression, as "position effect", "gene escaping" and "gene silencing" can be eliminated [21, 22]. Other valuable components, such as carotenoids, lipids, vitamins, and minerals, accumulate in *D. salina*, and therefore this alga has great potential for industrial and pharmaceutical applications [23]. As a bioreactor, *D. salina* is an optimal choice that provides the perfect opportunity for the production of a WSSV subunit vaccine.

Shrimp are the natural hosts for WSSV, and the WSSV structural gene can be introduced into *D. salina* cells by transformation; in turn, *D. salina* cells can be used as crude bait for shrimp. Furthermore, all these organisms live in the same seawater environment. Using these ecological interactions, we attempted to produce a live oral vaccine for controlling the spread of WSSV. This vaccine can be used more conveniently for shrimp than other vaccines, and it is expected to yield better immune protection.

Materials and methods

Algae strain and culture conditions

The UTEX-1644 strain of *D. salina* was purchased from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). Under a light intensity of 50 µmol photon m^{-2} s^{-1}, *D. salina* cells were cultured in modified PKS medium at 26 °C over 12-h night/day cycles [24]. At the logarithmic growth phase (10^5 cells ml^{-1}), cells were harvested by centrifugation for subsequent transformation. During this phase, *D. salina* cells had uniform size, shape, and movement (Fig. 1).

Cloning and sequencing the VP28 gene

Prior to VP28 gene cloning, WSSV was purified and titrated from an infected *Penaeus monodon* shrimp using sucrose gradient centrifugation [3, 25]. WSSV genomic DNA was extracted using the phenol-chloroform method [10]. Using the viral DNA as template, PCR amplification was conducted using specific primers 5′-CA *CCC GGG* ATG GAT CTT TCT TTC ACT CTT TC-3′ and 5′-TC *GAG CTC* TTA CTC GGT CTC AGT GCC AGA-3′; the italics sequences represent *Sma*I and *Sac*I endonuclease recognition sites, respectively), which were designed

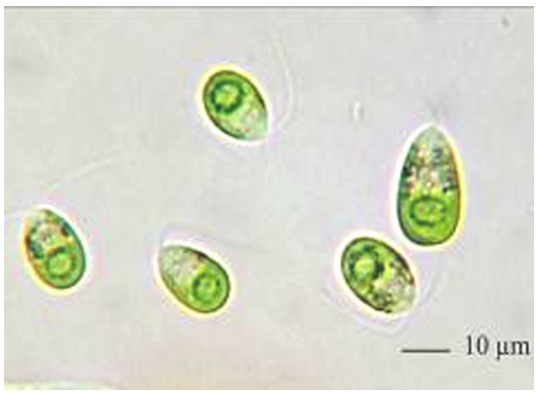

Fig. 1 Morphology of *D. salina* cells (400× magnification). The scale bar indicates 10 µm

according to the coding region of VP28 (GenBank accession no. AF502435.1). Amplification reactions comprised 5 µl of PCR buffer (10×), 1 µl of template DNA, 2 µl of each primer (25 µM), 2 µl of dNTPs (25 µM), 1 µl of Taq DNA polymerase (5 U/µl) and dH_2O to a volume of 50 µl. The thermal cycling conditions involved an initial denaturation step at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 10 min and termination at 4 °C. Amplifications were visualized by electrophoresis on 1 % (w/v) agarose gels. Amplified VP28 fragments were inserted into the pMD19-T vector (TaKaRa, Dalian, China) to yield pMD19-T-VP28 and sequenced (Sangon, Shanghai, China).

Generation of the eukaryotic expression vector

The pBI221-bar and pUΩ-GUS plasmids were obtained from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. The pUΩ-GUS plasmid was digested using endonucleases, and the linearized pUΩ- was then collected using a DNA gel extraction kit (Axygen Biosciences, USA). Using *Sma*I and *Sac*I, the VP28 fragment was excised from pMD19-T-VP28 and ligated into linearized pUΩ- to generate pUΩ-VP28. The bar box was removed from pBI221-bar by *Hin*dIII digestion and inserted into pUΩ-VP28 to produce pUΩ-VP28-bar (Fig. 2).

Transformation and screening of D. salina cells

Using glass beads [24], pUΩ-VP28-bar was introduced into *D. salina* cells using optimized transformation parameters. Transformed *D. salina* cells were cultured in fresh liquid medium under dim light for 24 h and then harvested by centrifugation and plated on solid selective PKS medium containing 10 g l^{-1} agarose and 3 µg ml^{-1} phosphinothricin (PPT) to screen for positive colonies. Negative controls were prepared by treating *D. salina* cells using the

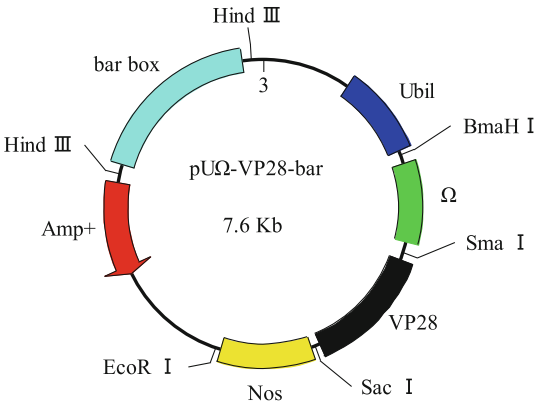

Fig. 2 Schematic diagram of the pUΩ-VP28-bar vector. Bar box, bialaphos resistance gene driven by the CaMV35S promoter; Ubil, maize ubiquitin promoter; Ω, 5′ leader sequence of tobacco mosaic virus RNA; VP28, WSSV VP28 gene; Nos, nopaline synthase gene terminator. Other labels are the restriction endonuclease sites

same transformation protocol without pUΩ-VP28-bar. Independent experiments were repeated at least three times.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of transformants

When the cell concentration reached 10^5 cells ml^{-1}, transformed cells were collected by centrifugation and washed three times with fresh liquid medium. Following the manufacturer's instructions, total RNA was extracted using Trizol Reagent (Invitrogen). Using the extracted total RNA as template, RT-PCR was conducted to detect VP28 gene expression. Amplification products were analyzed by electrophoresis on 10 g l^{-1} agarose gels. The transformed strain with the highest expression level of mRNA was selected from three transformants and used for further western blotting assays.

ELISA and western blot analysis of recombinant VP28 proteins

Proteins were extracted from the negative control and transformants using Trizol, and measured by enzyme-linked immunosorbent assay (ELISA) and western blot analysis. The concentration of proteins was determined using the Bradford assay [26]. Approximately 150 µl of total protein lysate from each transformant was used to coat ELISA plates at 37 °C overnight. After washing twice with Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris, pH 7.4), the plate was incubated with rabbit anti-VP28 IgG in TBS for 2 h at 37 °C. Subsequently, the plate was incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) at 37 °C for 1 h. The substrate solution was added to the plate for 20 min at 37 °C, and the absorbance at 492 nm was determined using a microplate reader.

The method of Yoganandhan et al. [27] was used for western blot detection of recombinant VP28. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking with 3 % (w/v) skim milk, membranes were incubated with rabbit anti-VP28 IgG for 2 h, and then with goat anti-rabbit IgG conjugated with alkaline phosphatase for 1.5 h at 37 °C. NBT and BCIP were used as substrates, and protein molecular weights were estimated with Band Scan 5 software.

Vaccination experiment and WSSV challenge

The oral vaccination experiment for transgenic *D. salina* in animals was carried out according to the method of Fu et al. [17]. Briefly, cultures (50 ml) of transgenic *D. salina* cells, including Ds-VP28 and Ds-empty (1×10^8 cells ml^{-1}; Table 1) were centrifuged, and cell pellets were resuspended in 15 ml of supernatant at 4 °C. Resuspended cells were subjected to two cycles of freeze-thawing for initial lysis of cells. Cells were then sonicated (250 W, 25 kHz, 7 s on/10 s off, 50 cycles) on ice, and lysates were observed with a microscope to ensure that *D. salina* cells were fully inactivated. Lysed cells were spread over 50 g of commercial crayfish feed, incubated on ice for 30 min to allow the feed to absorb lysed cellular material, and the feed was coated with fish oil. The negative and positive control pellets were coated with phosphate-buffered saline (PBS). Four groups of 20 WSSV-free crayfish were established. Each group was subjected to the vaccination experiment three times. The negative and positive control crayfish were orally administered PBS-coated feed, whereas the crayfish in groups 3 and 4 were orally administered Ds-VP28 and Ds-empty coated feed, respectively, for 10 consecutive days. Experimental crayfish were fed with coated food pellets to 5 % of their body weight. After the final

Table 1 Setup for the vaccination experiment

Group no.	Type	Mixture applied	Challenge	No. of crayfish
1	Negative control	Feed + PBS	PBS	20 × 3
2	Positive control	Feed + PBS	WSSV	20 × 3
3	Ds-VP28	Feed + *D. salina* harboring pUΩ-VP28-bar	WSSV	20 × 3
4	Ds-empty	Feed + *D. salina* harboring empty pUΩ-bar	WSSV	20 × 3

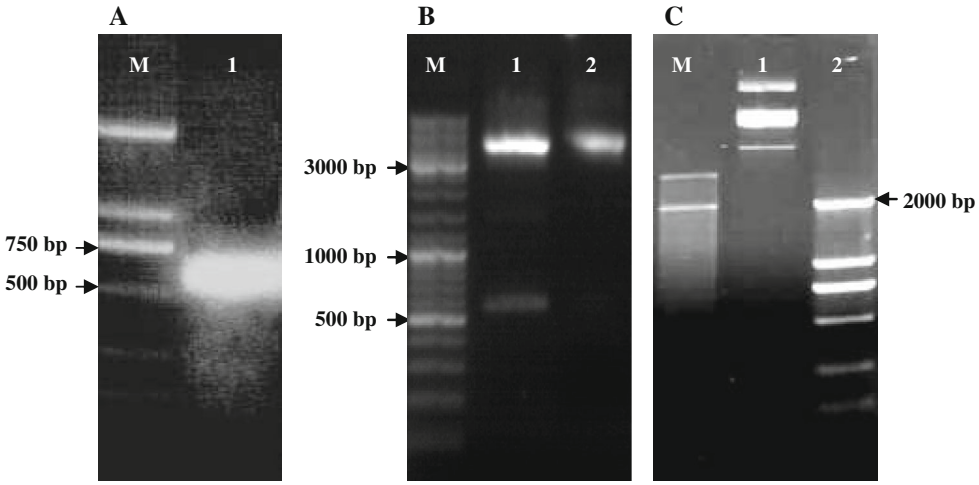

Fig. 3 Cloning the WSSV VP28 gene. (A) PCR amplification products of the WSSV VP28 gene. M, DNA marker; lane 1, amplification products. (B) Restriction enzyme analysis of pUΩ-VP28. Lane 1, pUΩ-VP28 digested with *Sma*I and *Sac*I; 2, pUΩ-VP28; M, DNA marker. (C) Restriction enzyme analysis of pUΩ-VP28-bar. Lane 1, pUΩ-VP28-bar digested with *Hin*dIII; 2, pUΩ-VP28-bar; M, DNA marker

vaccination, the crayfish in groups 2–4 were immediately challenged by immersing them in seawater containing WSSV stock solution (1×10^{-4} dilution) for 3 h. Negative control crayfish were challenged with PBS. All crayfish were then transferred to fresh WSSV-free seawater, and mortality at 20 days post-challenge was recorded. Dead crayfish were tested by one-step PCR to determine the presence of WSSV.

Statistical analysis

Data from all experiments were analyzed using a paired-samples *t*-test with SPSS (version16.0, Chicago, IL, USA). A *P*-value less than 0.05 was considered statistically significant.

Results

Gene cloning and vector construction

The PCR results revealed a specific fragment of 615 bp in lane 1 (Fig. 3A), consistent with expectations. Gene sequencing results showed that the sequence of the amplified DNA fragment was consistent with those in GenBank. Our findings demonstrate that the WSSV VP28 gene was successfully cloned and had the correct sequence, and that it could be liberated from pUΩ-VP28 (Fig. 3B). The bar box within pUΩ-VP28-bar was successfully excised (Fig. 3C).

Transformation of *D. salina*

Positive *D. salina* transformants appeared as uniform greenish algal colonies with diameters of about 2–3 mm (Fig. 4A and B). Three colonies were selected and inoculated in selective liquid selective containing 3 μg ml^{-1} PPT. After incubation for 7 days, all colonies exhibited cell growth with uniform size and movement. The recombinant eukaryotic expression vector was successfully introduced into the *D. salina* cells.

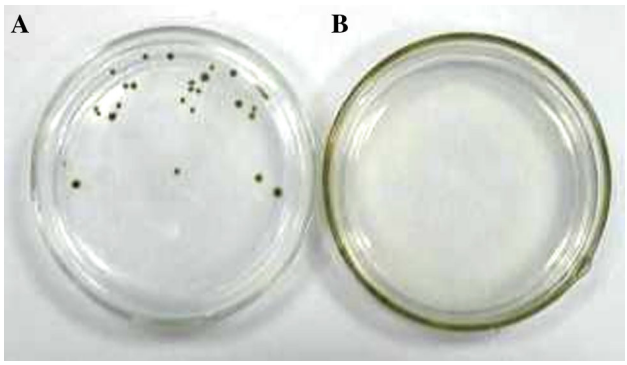

Fig. 4 Selective culture results for transformed *D. salina* cells. (A) Transformed *D. salina* cells containing pUΩ-VP28-bar. (B) Negative controls, *D. salina* cells lacking pUΩ-VP28-bar

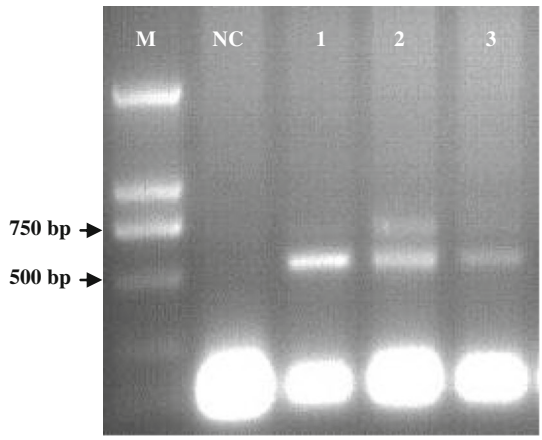

Fig. 5 RT-PCR analysis of the VP28 gene in different transformants of *D. salina*. M, DNA marker; NC, negative control; lanes 1–3, various *D. salina* transformants

RT-PCR analysis of transformants

RT-PCR results revealed that the predicted size of the VP28 gene, a 615-bp PCR fragment, was detected in all transformants (Fig. 5) but not in the negative controls. Sequence results indicated that the amplified DNA fragment was consistent with the coding region of the VP28 gene. PPT resistance tests demonstrated that all transformants survived repeated PPT selection and were stable for 3 months, indicating that the recombinant vector was successfully integrated into the genome of *D. salina* cells and stably expressed in progeny cells.

ELISA and western blot analysis of transformants

Total protein concentrations were 16.7, 16.9, and 17.1 μg μl^{-1} for each *D. salina* transformant, and 16.6 μg μl^{-1} for the negative control. ELISA results show that the levels of VP28 protein in transformants 1–3 were 3.04 ± 0.26, 0.99 ± 0.08, and 2.13 ± 0.17 ng mg^{-1}, respectively (Fig. 6). No protein was detected in the negative control (Fig. 6). Approximately 78 μg of recombinant VP28 protein was obtained from 100 ml cultures of *D. salina*.

Recombinant VP28 proteins appeared to be approximately 28 kDa in all three transformants and were lacking in the negative control (Fig. 7A). Our western blot results show that protein extracts from the transformant with maximal expression levels contained a band corresponding to 28 kDa (Fig. 7B). These results confirm that the recombinant VP28 protein was successfully expressed in *D. salina* cells and that it had specific immunologic activity.

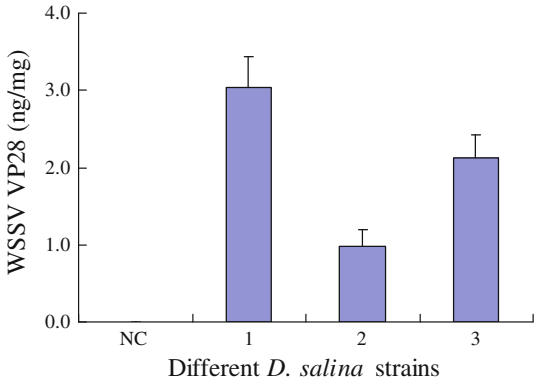

Fig. 6 Analysis of VP28 expression in different transgenic *D. salina* strains by ELISA. NC, negative control; 1–3, various transgenic *D. salina* strains

Vaccination experiment and WSSV challenge

After vaccination, experimental crayfish were observed twice a day. Ds-VP28-vaccinated crayfish had significantly higher survival rates (59 % mortality) than the Ds-empty control and the positive control groups (100 % mortality; Fig. 8). No deaths occurred among the crayfish in the negative control group throughout the experimental period. Using PCR, dead crayfish were found to be positive for the presence of WSSV, and all randomly selected survivors were found to be negative for virus.

Discussion

No adequate method or approach is currently available for controlling white spot disease. Initial treatments, such as

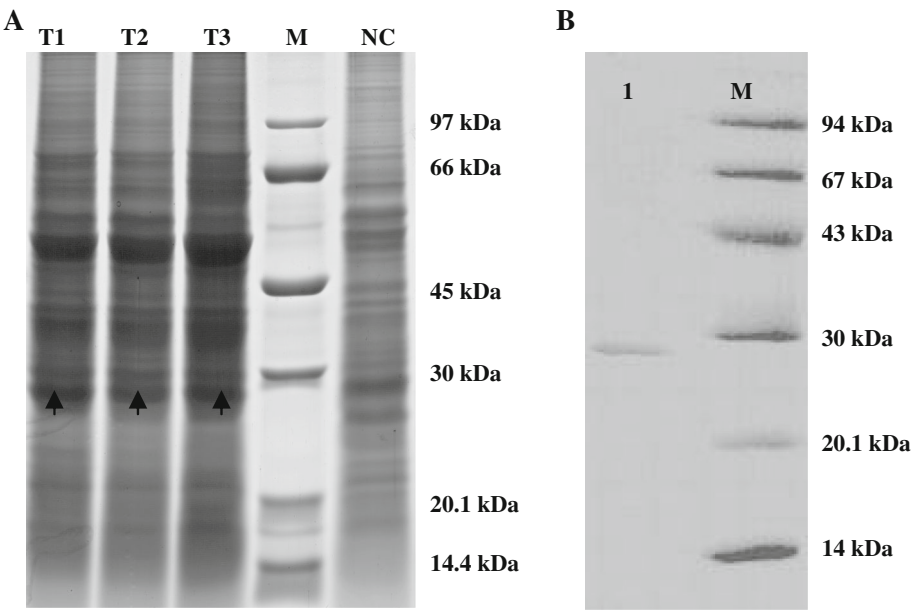

Fig. 7 SDS-PAGE and western blot analysis of *D. salina* transformants. (A) SDS-PAGE analysis of different *D. salina* transformants. M, protein marker; NC, negative control; T1–3, *D. salina* transformants. (B) Western blot analysis of the transformant with highest protein expression levels. Lane 1, recombinant VP28 protein; M, protein marker

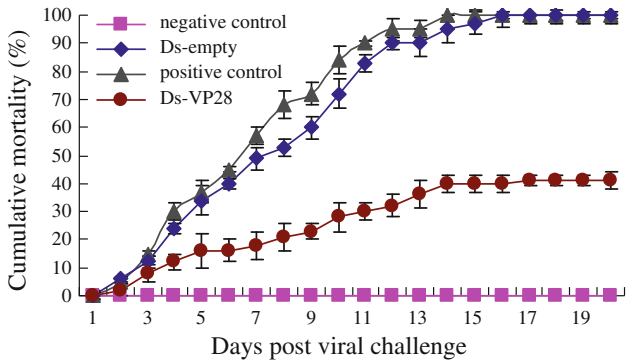

Fig. 8 The temporal-mortality relationship in orally vaccinated crayfish. Cumulative mortality rates of crayfish from the experimental groups vaccinated with Ds-VP28 (●), Ds-empty (◆), positive control (▲), and negative control (■), as indicated in Table 1

reducing densities and decreasing water carrying capacities, are often infeasible because of their inefficiency and high cost [11]. Therefore, studies have focused on alternative methods of disease control, in particular vaccines against WSSV [14–17]. However, given the drawbacks of most expression systems, vaccines have not been able to be suitably applied. Thus, we attempted to produce a novel subunit vaccine against WSSV using *D. salina* cells.

In the present study, *D. salina* was used as a novel expression system for producing the WSSV subunit vaccine. After transformation, PPT-resistant *D. salina* transformants were obtained. The WSSV VP28 gene was successfully introduced into the genome of *D. salina* cells and expressed, resulting in a protein with natural immunological activity. The Ds-VP28 vaccinated crayfish showed an approximate 59 % survival rate, whereas Ds-empty and positive control crayfish exhibited 100 % mortality. This result shows that protection against WSSV can be conferred in crayfish using transgenic *D. salina* as an oral vaccine.

The immune effects of the recombinant WSSV subunit vaccine have a direct relationship to the expression level of the target protein and its bioactivity [12]. In our previous study, the expression levels of the VP28 protein varied among different transformants. This may be attributed to the random integration of the VP28 gene into the *D. salina* genome at different sites. Therefore, more-detailed work involving optimization of codon dependence [28], transformation-associated genotypic modifications [29], reduction of sensitivity to proteases [30], and fusion of recombinant products with native proteins [31] should be conducted to obtain higher VP28 expression levels. Our next study will focus on the selection and effects of adjuvants, the optimal immunization procedure, the best formulation for the specific immunogen, immunization time, and dose of transgenic *D. salina* for shrimps.

In summary, transgenic *D. salina* strains successfully expressed the WSSV VP28 gene, and induced protection against WSSV in crayfish through an oral delivery approach. Our results further imply that producing an oral vaccine by *D. salina* is feasible for the control of WSSV infections and will also provide a new method for controlling other viral diseases in crustacean species.

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References

1. Namikoshi A, Wu JL, Yamashita T, Nishizawa T, Nishioka T, Arimoto M (2004) Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. Aquaculture 229:25–35
2. Zhu F, Du HH, Miao ZG, Quan HZ, Xu ZR (2009) Protection of *Procambarus clarkii* against white spot syndrome virus using inactivated WSSV. Fish Shellfish Immunol 26:685–690
3. Witteveldt J, Cifuents CC, Vlak JM, Van Hulten MCW (2004) Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. J Virol 78:2057–2061
4. Rajeshkumar S, Venkatesan C, Sarathi M, Sarathbabu V, Thomas J, Anver Basha K, Sahul Hameed AS (2009) Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV). Fish Shellfish Immunol 26:429–437
5. Mejía-Ruíz CH, Vega-Peña S, Alvarez-Ruiz P, Escobedo-Bonilla CM (2011) Double-stranded RNA against white spot syndrome virus (WSSV) vp28 or vp26 reduced susceptibility of *Litopenaeus vannamei* to WSSV, and survivors exhibited decreased susceptibility in subsequent re-infections. J Invertebr Pathol 107:65–68
6. Zhu F, Zhang X (2012) Protection of shrimp against white spot syndrome virus (WSSV) with β-1, 3-D-glucan-encapsulated vp28-siRNA particles. Mar Biotechnol (NY) 14:63–68
7. Tsai JM, Wang HC, Leu JH, Hsiao HH, Wang AH, Kou GH, Lo CF (2004) Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J Virol 78:11360–11370
8. Tsai JM, Wang HC, Leu JH, Wang AH, Zhung Y, Walker PJ, Kou GH, Lo CF (2006) Identification of the nucleocapsid, tegument, and envelope proteins of the shrimp white spot syndrome virus virion. J Virol 80:3021–3029
9. Van Hulten MCW, Witteveldt J, Snippe M, Vlak JM (2001) White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology 285:228–233
10. Yi GH, Wang ZM, Qi YP, Yao LG, Qian J, Hu LB (2004) Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. J Biochem Mol Biol 37:726–734
11. Jha RK, Xu ZR, Bai SJ, Sun JY, Li WF, Shen J (2007) Protection of *Procambarus clarkii* against white spot syndrome virus using recombinant oral vaccine expressed in *Pichia pastoris*. Fish Shellfish Immunol 22:295–307
12. Caipang CM, Verjan N, Ooi EL, Kondo H, Hirono I, Aoki T, Kiyono H, Yuki Y (2008) Enhanced survival of shrimp, *Penaeus*

(*Marsupenaeus*) *japonicus* from white spot syndrome disease after oral administration of recombinant VP28 expressed in *Brevibacillus brevis*. Fish Shellfish Immunol 25:315–320
13. Tang X, Hew CL (2007) Expression, purification and crystallization of two major envelope proteins from white spot syndrome virus. Acta Crystallogr Sect F Struct Biol Cryst Commun 63:624–626
14. Hou CL, Cao Y, Xie RH, Wang YZ, Du HH (2011) Characterization and diagnostic use of a monoclonal antibody for VP28 envelope protein of white spot syndrome virus. Virol Sin 26:260–266
15. Syed SM, Kwang J (2011) Oral vaccination of baculovirus-expressed VP28 displays enhanced protection against white spot syndrome virus in *Penaeus monodon*. PLoS One 6(11):e26428
16. Wei KQ, Yang JX (2011) Histological alterations and immune response in the crayfish *Procambarus clarkii* given rVP28-incorporated diets. Fish Shellfish Immunol 31:1122–1128
17. Fu LL, Shuai JB, Xu ZR, Li JR, Li WF (2010) Immune responses of *Fenneropenaeus chinensis* against white spot syndrome virus after oral delivery of VP28 using *Bacillus subtilis* as vehicles. Fish Shellfish Immunol 28:49–55
18. Ning D, Leng X, Li Q, Xu W (2011) Surface-displayed VP28 on *Bacillus subtilis* spores induce protection against white spot syndrome virus in crayfish by oral administration. J Appl Microbiol 111:1327–1336
19. Xue LX, Pan WD, Jiang GZ, Wang JR (2006) Patent No: US 7081567 B2: transgenic *Dunuliella salina* as a bioreactor
20. Walker TL, Purton S, Becker DK, Collet C (2005) Mocroalgae as bioreactors. Plant Cell Rep 24:629–641
21. Wang HH, Yin WB, Hu ZM (2009) Advances in chloroplast engineering. J Genet Genomics 36:387–398
22. Barzegari A, Hejazi MA, Hosseinzadeh N, Eslami S, Mehdizadeh Aghdam E, Hejazi MS (2010) *Dunaliella* as an attractive candidate for molecular farming. Mol Biol Rep 37:3427–3430
23. Hosseini Tafreshi A, Shariati M (2009) *Dunaliella* biotechnology: methods and applications. J Appl Microbiol 107:14–35
24. Feng SY, Xue LX, Liu HT, Lu PJ (2009) Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. Mol Biol Rep 36:1433–1439
25. Du HH, Fu LL, Xu YX, Kil ZS, Xu ZR (2007) Improvement in a simple method for isolating white spot syndrome virus (WSSV) from the crayfish *Procambarus clarkii*. Aquaculture 262:532–534
26. Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254
27. Yoganandhan K, Syed SM, Narayanan RB, Sahul Hameed AS (2004) Production of polyclonal antiserum against recombinant VP28 protein and its application for the detection of white spot syndrome virus in crustaceans. J Fish Dis 27:517–522
28. Rosales-Mendoza S, Paz-Maldonado LM, Soria-Guerra RE (2012) *Chlamydomonas reinhardtii* as a viable platform for the production of recombinant proteins: current status and perspectives. Plant Cell Rep 31:479–494
29. Surzycki R, Greenham K, Kitayama K, Dibal F, Wagner R, Rochaix JD, Ajam T, Surzycki S (2009) Factors effecting expression of vaccines in microalgae. Biologicals 37:133–138
30. Doran PM (2006) Foreign protein degradation and instability in plants and plant tissue cultures. Trends Biotechnol 24:426–432
31. Muto M, Henry RE, Mayfield SP (2009) Accumulation and processing of a recombinant protein designed as a cleavable fusion to the endogenous Rubisco LSU protein in *Chlamydomonas chloroplast*. BMC Biotechnol 9:1–11