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Development of an oral vaccine using recombinant viral haemorrhagic septicaemia virus glycoproteins produced in tobacco

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Abstract: The viral haemorrhagic septicaemia virus (VHSV) causes high mortality in many marine and freshwater fish species, resulting in heavy economic losses in fish farming. Previously, cholera toxin B subunit (CTB)-fused recombinant viral haemorrhagic septicaemia virus glycoproteins (rec-VHSV-GPs) have been successfully expressed in tobacco, *Nicotiana benthamiana*. Here, we evaluated the potential of rec-VHSV-GPs as an oral vaccine against a live viral challenge. After immunisation of mice and fish (olive flounder, *Paralichthys olivaceous*) with those antigenic proteins in a feed additive form, the antibody titres were increased statistically, especially in the primed groups ($P < 0.0001$) in both the mouse and fish. After the viral challenge under low water temperature culture conditions (below 18 °C), the immunised fish were protected successfully against the challenge, showing a significantly lower mortality rate ($P < 0.05$). This result suggests that this plant-based immunisation system could induce an effective immune response. It could be used as a candidate to develop an oral vaccine for fish.

Keywords: flounder; oral vaccine; plant expression; viral haemorrhagic septicaemia virus

The viral haemorrhagic septicaemia virus (VHSV) is an envelope ribonucleic acid (RNA) virus that belongs to the genus *Novirhabdovirus* of the family Rhabdoviridae (Tien et al. 2017). VHSV occurs in freshwater and marine fish worldwide (Skall et al. 2005). It is a fish pathogen that causes high mortality of many marine and freshwater fish species. The olive flounder, *Paralichthys olivaceous* (Temminck and Schlegel), is known as one of the main species susceptible to the VHSV infection in Korea (Kim et al. 2009). Interestingly, VHSV is most likely to occur at low water temperatures (below 20 °C). It is not infectious or pathogenic at temperature above 20 °C (Goodwin and Merry 2011; Avunje et al. 2012). VHSV is known to have 6 genes in the order 3'-N-P-M-G-NV-L-5', encoding a non-structural protein (Nv) with an unclear function and 5 structural proteins: nucleocapsid-, phospho-, matrix-, glyco, and RNA polymerase proteins (Schutze et al. 1999). Of these proteins,

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glycoprotein (GP) is known as a neutralising antigen (Bernard et al. 1983; Lorenzen and Olesen 1997; Lorenzen et al. 1999; Rocha et al. 2002). Thus, GP might be a promising antigen for vaccination against VHSV.

Previous experiments on fish immunisation have employed a parenteral immunisation route which is very stressful to the fish. It is also labour-intensive, time/cost-consuming (Cho et al. 2018). Recently, plant-based expression systems have been found to have the potential to be an alternative platform to produce recombinant proteins owing to their relatively low cost, easy scale-up, efficient/convenient storage, and no animal pathogen contamination (Twyman et al. 2012). In view of these plant-based system advantages, we evaluated the efficacy of using this system for oral immunisation.

Previously, fragments of the VHSV GP gene have been fused with a cholera toxin B subunit (CTB) to stimulate immune responses in mucosal immune systems. They have been successfully expressed in tobacco (*Nicotiana benthamiana*) using an *Agrobacterium*-mediated transient expression system (Tien et al. 2017). The aim of this study was to induce an immune response (antibody production) using this recombinant protein from tobacco as a feed additive via an oral route and to investigate its protection efficacy against the VHSV challenge.

MATERIAL AND METHODS

Recombinant proteins from tobacco and *E. coli*. The CTB-fused rec-VHSV-GPs from tobacco leaves was prepared as described previously (Tien et al. 2017). Briefly, two fragments of VHSV-GP (GP-1: amino acid 99-235th and GP-2: 258-417th, GenBank accession no. AY167587) were analysed and selected based on the antigenic determinant analysis on a Hopp-Woods plot (Hopp and Woods 1981). The GP-1 and GP-2 fragments were fused with CTB known to function as an effective carrier molecule for the fused mucosal vaccine antigens (Sanchez and Holmgren 2008). The infiltration of the CTB-fused GPs was mediated by the transfer of the transgenes from the T-DNA region of the bacterial Ti-plasmid (Tien et al. 2017).

The rec-GP from the *E. coli* was prepared as described previously (Kang et al. 2015). Briefly, whole GP gene was cloned into a pRSET plasmid (Invitrogen, Carlsbad, CA, USA), transformed into BL21(DE3)pLysS (Invitrogen) for the expression, and then purified using a Probond™ purification system (Invitrogen) according to the manufacturer's instructions.

Animals. All the animal experiments complied with the guidelines for the care and use of experimental animals of the Canadian Council on Animal Care. They were approved by the Animal Care Committee of Chonnam National University, the Republic of Korea.

Mouse immunisation and antibody titration. A total of 25 6-week-old ICR female mice (Orient Bio Inc, Seongnam, Republic of Korea) were divided into five groups as shown in Table 1. The mice were primed with PBS (Group 1) or 50 μg of rec-GP from the *E. coli* (Group 2) (Kang et al. 2015) with Freund's complete adjuvant. For oral boosting, the mice of Groups 1-2 and 2-1 received a tobacco-derived CTB-fused rec-VHSV-GP-1, while Groups 1-3 and 2-2 received a CTB-fused rec-VHSV-GP2 (Tien et al. 2017) every Monday for a total of four times. The desired concentration of the rec-VHSV-GP in the feed formulae was adjusted by mixing the transformants with wild-type (W/T) leaf powders. The Group 1-1 sample was used as a negative control.

The blood samples (sera) were collected from the tail veins of all the mice before the experiments (1st sampling) as controls. One day before each immunisation, the sequential blood samples (2nd to 5th) were collected. The presence of an antibody against VHSV GP was determined using ELISA (an enzyme-linked immunosorbent assay) as described (Kang et al. 2015). Briefly, the rec-GP from the *E. coli* was used as antigen. It was absorbed onto the microtiter plates (Maxisorp™, Nunc, Roskilde, Denmark). The experimental sera were used as the primary antibody followed by incubation with the goat anti-mouse IgG HRP conjugate (Pierce™, IL, USA). Using an ELISA reader, the absorbance at 405 nm was measured and interpreted as the induction of the antibody. The results are expressed as a mean ± standard deviation of the optical density value interpreted as the antibody induction.

Flounder immunisation and antibody titration. A total of 85 apparently healthy (without clinical signs) olive flounders, *Paralichthys olivaceous* (weight: 40–50 g, length: 15–17 cm, approximately two months after moving 7 cm-long fingerlings into culture farms from a nursery) were obtained

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Table 1. The immunisation design, methods, and trial numbers in the mice

Groups	Priming with	Oral immunisation with	Number
1-1	PBS	W/T tobacco leaf (without GP)	5
1-2	PBS	CTB-fused rec-VHSV-GP-1*	5
1-3	PBS	CTB-fused rec-VHSV-GP-2*	5
2-1	rec-GP from *E. coli*†	CTB-fused rec-VHSV-GP-1*	5
2-2	rec-GP from *E. coli*†	CTB-fused rec-VHSV-GP-2*	5

*Oral immunisation (fed) with lyophilised transformant tobacco leaf powder (30% w/w powder containing 30 μg of each CTB-fused rec-VHSV-GP in 2.5 g feed/per head)

†Administrated subcutaneously. For the oral immunisation, the total amount of feed additives per Group was fed randomly

from the culture farms and kept in separate tanks supplied with ample aeration and artificial sea water at 22 °C (± 1 °C). After acclimatisation for two weeks, five fish were selected randomly and the presence (infection) of VHSV in the fish's kidney was tested using the VHSV-specific RT-PCR method (Kang et al. 2015).

The fish in Groups 2 and 3 were primed intraperitoneally with 5 μg of purified rec-GP expressed in the *E. coli* (Kang et al. 2015). To prepare the priming injection, the Montanide™ adjuvant (Seppic, Fairfield, NJ, USA) was used. The fish in Groups 1 and 4 received PBS instead. After the injections, the fish in Groups 2 and 3 were fed with a tobacco-derived CTB-fused rec-VHSV-GP-1 or a CTB-fused rec-VHSV-GP-2 every Monday at 2-week intervals for a total of 4 times while the fish in Groups 1 and 4 received the W/T tobacco leaf or the normal flounder feed as described in Table 2. At 7 days after the final immunisation, the blood samples were collected from 5 randomly selected fish per Group (a total of 20 fish) and the presence of IgM against VHSV-GP was screened by ELISA as described above. The fish sera (diluted 1 : 100 in PBS) were used as the primary antibody while the mouse anti-flounder IgM (diluted 1 : 10, Aquatic Diagnostics, Stirling, Scotland) was used as a secondary antibody. The goat anti-mouse IgG HRP (1 : 500, Pierce™, IL, USA) was used as a tertiary antibody.

Viral challenge. The virus isolation was conducted using a previously published method (Kim et al. 2009) with some modifications. Briefly, the VHSV (genotype IVa from Jeju Island, confirmed by sequence analysis; Han et al. 2014) was prepared by inoculating the Epithelioma Papulosum Cyprini (EPC) (ATCC, Rockville, USA) cell line. The spleen samples positive in the RT-PCR assay were homogenised, centrifuged, filtered, and added into the cell culture plates containing the EPC cells at 60–70% confluency. The culturing water temperature was reduced gradually from 22 °C to 18 °C for a week. Fish were then maintained at a temperature below 18 °C ± 1 °C (the low water temperature culture condition). After the $TCID_{50}$ calibration (data not shown), 1 ml of the viral culture (corresponding to $10^{5.86}$ TCID/ml) was injected intraperitoneally and the fish were monitored daily for four weeks. The anterior kidney and liver samples from the dead or moribund fish were collected for RT-PCR to confirm the viral infection using VHSV-specific primers as described previously (Kang et al. 2015).

Statistical analysis. If not indicated separately, the significance of variation among the different groups was determined by the one-way analysis

Table 2. The immunization design, methods, and trial numbers in the fish (olive flounder)

Groups	Priming with	Oral immunisation with	Number
1	PBS	W/T tobacco leaf (without GP)	20
2	rec-GP from *E. coli*	CTB-fused rec-VHSV-GP-1*	20
3	rec-GP from *E. coli*	CTB-fused rec-VHSV-GP-2*	20
4	PBS	normal feed	20

*Oral immunisation (fed) with lyophilised transformant tobacco leaf powder (30% w/w powder containing 30 μg of each CTB-fused rec-VHSV-GP). For the oral immunisation, the total amount of feed additives per Group was fed randomly

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of variance (ANOVA) and the difference among the groups was determined by Dunnett's multiple comparisons test using GraphPad® Instat Software 3.05 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Antibody titration in the mouse

The statistical differences in the antibody titre were compared and calculated over Group 1-1 according to the sampling time. The significant increase in antibody titre in primed groups (Group 2-1 and 2-2, $P < 0.0001$) occurred at the 2nd, 3rd, 4th, and final 5th sampling time. At the 5th sampling time, significant increases in the antibody titre were also observed in the non-primed groups ($P < 0.01$ in Group 1-2 and $P < 0.05$ in Group 1-3; see Figure 1).

Antibody titration and the protective effects of the immunisation in the fish

A significant ($P < 0.0001$) increase in the IgM contents was found in the immunised groups (Group 2 and 3; see Figure 2). The protective efficacy of the immunisation was examined using a viral challenge study. After the viral challenge, dead fish were observed from 2 to 12 days after the infection

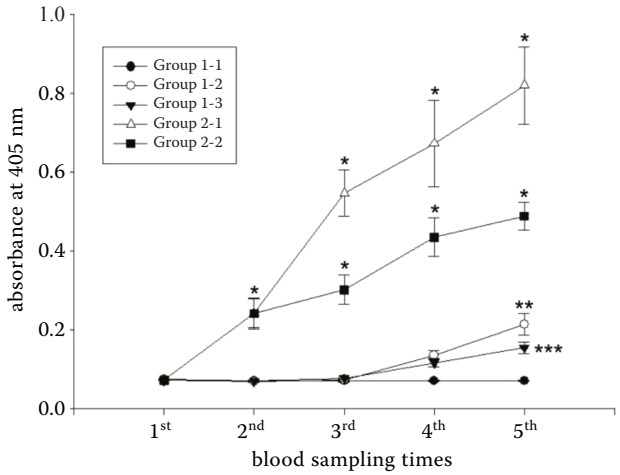

Figure 1. The changes in the mouse IgG titres based on ELISA. The significant (*$P < 0.0001$) increases in the primed groups (Group 2-1 and 2-2) occurred from the 2nd to the 5th sampling times. At the 5th sampling time, significant increases also occurred in the non-primed (oral immunisation only) groups (**$P < 0.01$ in Group 1-2 and ***$P < 0.05$ in Group 1-3)

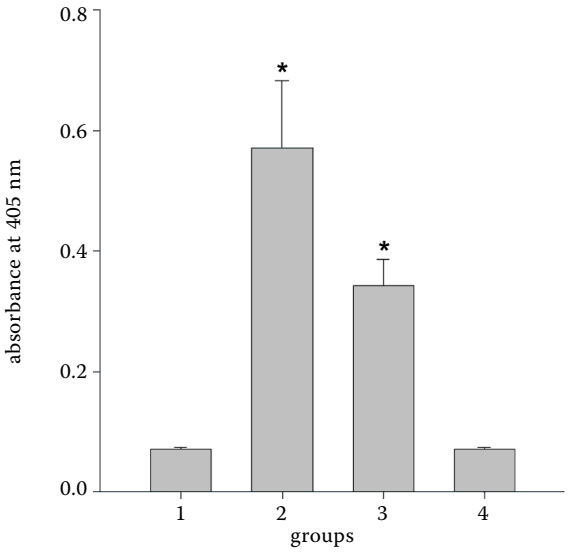

Figure 2. The changes in the olive flounder IgM titres based on ELISA. Significant (*$P < 0.0001$) increases were found in all the immunised groups (Groups 2 and 3). Group 1 (W/T tobacco leaf control) and Group 4 (normal feed) were used as negative controls as described in Table 2

Table 3. The number of dead fish in each Group after the viral challenge and cumulative mortality over four weeks of observation

DPI	2	3	4	7	8	9	10	11	12	Total	Mortality (%)†
Group 1	0	0	1	1	1	2	0	1	1	7	46.67
Group 2	0	1	1	0	0	0	0	0	0	2	13.33
Group 3	1	0	0	0	0	0	0	0	0	1	6.67
Group 4	0	1	1	1	2	2	1	1	0	9	60

DPI = day post infection

†The two-sided *P*-values over Group 1 were 0.1086 (Group 2), 0.0352 (Group 3, which was significant), and 0.7152 (Group 4) based on Fisher's exact test

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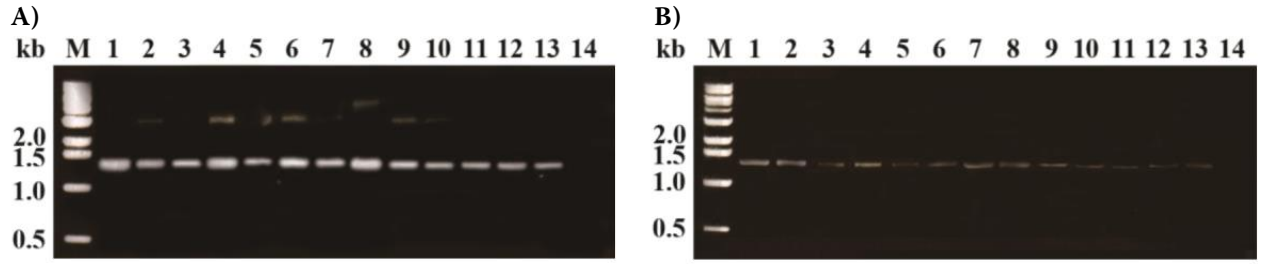

Figure 3. The RT-PCR verification of the VHSV infection in the fish's anterior kidney (**A**) and the liver (**B**) samples. Lane 1–7: samples from Group 1; lanes 8–9: Group 2; lane 10: Group 3; lane 11–13: three randomly selected samples from Group 4; lane 14: uninfected control

M = the DNA size marker

(DPI – day post infection). The dead fish showed one or more typical clinical signs/pathological lesions such as petechial haemorrhaging in the eyes and muscles, ascites, exophthalmia, and an abdominal hernia. From 13 DPI, no death was observed. Lower cumulative mortality rates were observed in the immunised groups compared to those in the control Group without immunisation (Table 3).

The RT-PCR assay for the viral infection confirmed that the dead or moribund fish were infected with VHSV (Figure 3A = anterior kidney, 3B = liver).

DISCUSSION

VHS (viral haemorrhagic septicaemia) is a highly pathogenic disease that causes about 50% mortality in fish. VHSV infects all sizes/ages of fish and occurs in the winter/spring at low temperatures (Skall et al. 2005). In the olive flounder farms of East Asia, high mortality due to VHS usually occurs during the cold-water season from water temperature 5 °C to 15 °C (Isshiki et al. 2001). However, no mortality by the VHSV-challenge has been observed in the olive flounder reared at temperature of 20 °C (Sano et al. 2009). Based on a previous report, the fish were reared at 22 °C (± 1 °C) to increase the antibody production (Makrinos and Bowden 2016) followed by a water temperature reduction to 18 °C (± 1 °C) for the viral challenge. Although not described here, we performed a separate viral challenge experiment at 22 °C with the same immunisation and challenge conditions which showed no mortality in any Group (the control or experimental group).

In the mouse experiment, the antibody induction was significantly ($P < 0.0001$) increased in the priming + oral immunisation groups (Group 2-1 and 2-2, Figure 1). This suggests the possibility of a systemic immune response induction by the oral immunisation.

In the fish experiments, the successful antibody induction was also observed in the priming + oral immunisation groups (Group 2 and 3, Table 2 and Figure 2). After the viral challenge, the fish in Group 3 showed a statistically lower mortality rate (6.67% vs. 46.67% over Group 1 and 6.67% vs. 60% over Group 4) than the controls. The fish in Group 2 also showed a remarkably lower mortality rate (13.33% vs. 46.67% over Group 1 and 13.33% vs. 60% over Group 4) than the controls, although the difference was not statistically significant. This is a very promising result to us since there is no commercial vaccine available.

The affected fish are known to be characterised by clear ascites fluid in the peritoneal and pericardial cavities, congested liver, splenomegaly, enlarged kidney, and haemorrhages in the lateral musculature (Isshiki et al. 2001). In our experiments, the dead/moribund fish also showed similar findings (Figure not shown).

Our results confirmed that the serum IgM titres against GP were significantly ($P < 0.001$) higher in the immunised fish when compared to those in the controls with lower mortality rates (Table 3). Considering these findings, we conclude that the antibodies against GP can act as neutralising ones, similar to previous reports (Bernard et al. 1983; Lorenzen and Olesen 1997; Lorenzen et al. 1999; Rocha et al. 2002).

In conclusion, GP expressed in tobacco leaves can induce the production of an antibody that is neutralising. It can protect the fish against the VHSV infection. The oral administration protocol developed here could be used as an effective (less labour and stress) means to accomplish practical vaccine trials.

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