Journal of Microbiology (2017) Vol. 55, No. 8, pp. 655–664
DOI 10.1007/s12275-017-7218-5
eISSN 1976-3794
pISSN 1225-8873

Development of recombinant *Yarrowia lipolytica* producing virus-like particles of a fish nervous necrosis virus§

Van-Trinh Luu[1†], Hye Yun Moon[1†], Jee Youn Hwang[2], Bo-Kyu Kang[3], and Hyun Ah Kang[1,4*]

[1]*Department of Life Science, College of Natural Science, Chung-Ang University, Seoul 06974, Republic of Korea*
[2]*Aquatic Disease Control Division, National Institute of Fisheries Science, Busan 46083, Republic of Korea*
[3]*Green Cross Veterinary Products Co. LTD., Yongin 17066, Republic of Korea*
[4]*Bio-Integration Research Center for Neutra-Pharmaceutical Epigenetics, Chung-Ang University, Seoul 06974, Republic of Korea*

(Received May 29, 2017 / Revised Jul 4, 2017 / Accepted Jul 4, 2017)

Nervous necrosis virus (NNV) causes viral encephalopathy and retinopathy, a devastating disease of many species of cultured marine fish worldwide. In this study, we used the dimorphic non-pathogenic yeast *Yarrowia lipolytica* as a host to express the capsid protein of red-spotted grouper nervous necrosis virus (RGNNV-CP) and evaluated its potential as a platform for vaccine production. An initial attempt was made to express the codon-optimized synthetic genes encoding intact and N-terminal truncated forms of RGNNV-CP under the strong constitutive *TEF1* promoter using autonomously replicating sequence (ARS)-based vectors. The full-length recombinant capsid proteins expressed in *Y. lipolytica* were detected not only as monomers and but also as trimers, which is a basic unit for formation of NNV virus-like particles (VLPs). Oral immunization of mice with whole recombinant *Y. lipolytica* harboring the ARS-based plasmids was shown to efficiently induce the formation of IgG against RGNNV-CP. To increase the number of integrated copies of the RGNNV-CP expression cassette, a set of 26S ribosomal DNA-based multiple integrative vectors was constructed in combination with a series of defective Y*lura3* with truncated promoters as selection markers, resulting in integrants harboring up to eight copies of the RGNNV-CP cassette. Sucrose gradient centrifugation and transmission electron microscopy of this high-copy integrant were carried out to confirm the expression of RGNNV-CPs as VLPs. This is the first report on efficient expression of viral capsid proteins as VLPs in *Y. lipolytica*, demonstrating high potential for the *Y. lipolytica* expression system as a platform for recombinant vaccine production based on VLPs.

*For correspondence. E-mail: hyunkang@cau.ac.kr; Tel.: +82-2-820-5863; Fax: +82-2-825-5206
§Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Keywords: *Yarrowia lipolytica*, capsid proteins, multicopy integration, nervous necrosis virus, ribosomal DNA, virus-like particles

Introduction

Nervous necrosis virus (NNV), a member of the genus *Betanodavirus*, is the causative agent of viral encephalopathy and retinopathy, also known as viral nervous necrosis, which is a devastating disease of many species of marine fish cultured worldwide (Thiéry *et al.*, 2006). NNV is a family of positive-sense single-stranded RNA viruses surrounded with a non-enveloped, icosahedral-shaped capsid. The NNV genome consists of two positive-sense RNAs: a 3.1-kb RNA that encodes the viral replicase and a 1.4-kb RNA that encodes the 37-kDa capsid protein (CP) (Lin *et al.*, 2001; Choi *et al.*, 2013). Various types of vaccines against NNV infection have been developed, including formalin-inactivated NNV, recombinant NNV-CPs, synthetic peptides with neutralizing NNV epitopes, and a DNA vaccine (Gomez-Casado *et al.*, 2011). Recombinant NNV-CPs can self-assemble into icosahedral virus-like particles (VLPs) that mimic the organization and conformation of native viruses but that do not encapsidate a viral genome (Tang *et al.*, 2002). The VLPs of recombinant NNV-CPs produced in baculovirus, *Escherichia coli*, and *Saccharomyces cerevisiae* expression systems have been shown to efficiently elicit protective antibody responses in fish, indicating that the recombinant VLPs are promising vaccine candidates (Lin *et al.*, 2001; Lu *et al.*, 2003; Liu *et al.*, 2006; Thiéry *et al.*, 2006; Choi *et al.*, 2013).

Among various expression systems, yeasts have several advantages in the production of heterologous proteins with potential applications as vaccines. As eukaryotic microorganisms, yeasts can be grown cheaply and rapidly and are amenable to high cell-density fermentation. They are feasible for genetic manipulation and have the capacity to carry out most of post-translational modifications. Several yeast species are microorganisms in the status of Generally Recognized As Safe (GRAS) (Schmidt, 2004; Gomes *et al.*, 2016). In particular, oral use of whole yeast cells eliminates additional manufacturing processes such as purification of a desired product and provides additional immune stimulation because of the presence of cell walls containing beta 1,3/1,6 glucans. We have previously shown that not only parenteral administration of the purified recombinant capsid protein of red-spotted grouper nervous necrosis virus (RGNNV-CP) produced by *S. cerevisiae* but also oral administration of whole recombinant *S. cerevisiae* cells expressing RGNNV-CP efficiently provoked immune responses in

Table 1. List of yeast strains used in this study

Strains	Description	References
Yarrowia lipolytica IL3	*MATA ade1 ura3 xpr2 leu2::tc*	Bal *et al.* (2013)
IL3-L/NNV	IL3 harboring pINATX2-NNVI	This study
IL3-L/NNV(F)	IL3 harboring pINATX2-NNVI(F)	This study
IL3-L /Δ49NNV	IL3 harboring pINATX2-NNVII	This study
IL3-L /Δ49NNV(F)	IL3 harboring pINATX2-NNVII(F)	This study
IL3-L /Δ81NNV	IL3 harboring pINATX2-NNVIII	This study
IL3-L /Δ81NNV(F)	IL3 harboring pINATX2-NNVIII(F)	This study
IL3-U/NNV	IL3 harboring pIMR53-NNVI	This study
IL3-U/NNV(F)	IL3 harboring pIMR53-NNVI(F)	This study
IL3-LU/NNV-X2	IL3 harboring pINATX2-NNVI and pIMR53-NNVI	This study
IL3-LU/NNV(F)-X2	IL3 harboring pINATX2-NNVI(F) and pIMR53-NNVI(F)	This study
Yarrowia lipolytica Po1f	*MATA leu2-270 ura3-302 xpr2-322*	Madzak *et al.* (2000)
Po1f/ud0-NNV(F)	Po1f integrants of p26SrDNA2-ura3d0-NNV(F)	This study
Po1f/ud4-NNV(F)	Po1f integrants of p26SrDNA2-ura3d4-NNV(F)	This study
Po1f/ud8-NNV(F)	Po1f integrants of p26SrDNA2-ura3d8-NNV(F)	This study
Po1f/ud15-NNV(F)	Po1f integrants of p26SrDNA2-ura3d15-NNV(F)	This study
Po1f/ud41-NNV(F)	Po1f integrants of p26SrDNA2-ura3d41-NNV(F)	This study
Po1f/URA3-NNV(F)	Po1f integrants of p26SrDNA2-URA3-NNV(F)	This study
Saccharomyces cerevisiae Y2805	*MATA pep4::HIS3 prb1-d can1 GAL2 his3d ura3-52*	Sohn *et al.* (1991)
Y2805/NNVcp	Y2805 harboring YEGa-MCS-NNVcp	Choi *et al.* (2013)

mice (Kim *et al.*, 2014; Moon *et al.*, 2016).

In recent decades, several non-conventional yeasts, including *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*, have been developed as practical alternative hosts to *S. cerevisiae* for the production of recombinant proteins (Müller *et al.*, 1998; Kim *et al.*, 2015). *Y. lipolytica* is a dimorphic alkane-assimilating yeast, classified as GRAS based on its association with several food-related processes (Zinjarde, 2014). This yeast can use diverse carbon sources, including glucose (but not sucrose), alcohols, acetate, and hydrophobic substrates such as alkanes, fatty acids, and oils (Barth and Gaillardin, 1997). The glycosylation pattern of *Y. lipolytica* is closer to the mammalian high-mannose type of glycosylation than that of *S. cerevisiae* (Song *et al.*, 2007; Moon *et al.*, 2013; Oh *et al.*, 2016).

Table 2. List of plasmids used in this study

Plasmids	Description	References
YEGa-MCS-NNVcp	YEGa-MCS:*pGAL10*-NNV	Choi *et al.* (2013)
pINAScTRP1	p*TEF1*-Sc*TRP1*-t*XPR2* Yl*LEU2 ARS68*	Cheon *et al.* (2003)
pINATX2-NNVI	ARS68-based vector carrying Yl*LEU2* and the p*TEF1-NNVI-tXPR2* expression cassette	This study
pINATX2-NNVI(F)	ARS68-based vector carrying Yl*LEU2* and the p*TEF1-NNVI(F)-tXPR2* expression cassette	This study
pINATX2- NNVII	ARS68-based vector carrying Yl*LEU2* and the expression cassette of RGNNV-CPΔ49	This study
pINATX2- NNVII(F)	ARS68-based vector carrying Yl*LEU2* and the expression cassette of RGNNV-CPΔ49 tagged with FLAG	This study
pINATX2-NNVIII	ARS68-based vector carrying Yl*LEU2* and the expression cassette of RGNNV-CPΔ81	This study
pINATX2-NNVIII(F)	ARS68-based vector carrying Yl*LEU2* and the expression cassette RGNNV-CPΔ81 tagged with FLAG	This study
pIMR53-AUX	p*XPR2*-t*XPR2* Yl*URA3 ARS18*	Yang *et al.* (2001)
pIMR53-NNVI	ARS18-based vector carrying Yl*URA3* and the p*TEF1-NNVI-tXPR2* expression cassette	This study
pIMR53-NNVI(F)	ARS18-based vector carrying Yl*URA3* and the p*TEF1-NNVI(F)-tXPR2* expression cassette	This study
p26SrDNA2-ura3d0-NNV(F)	26S rDNA2-based integration vector carrying a defective Yl*ura3* with a 0-bp promoter and the expression cassette of RGNNV-CP tagged with FLAG	This study
p26SrDNA2-ura3d4-NNV(F)	26S rDNA2-based integration vector carrying a defective Yl*ura3* with a 4-bp promoter and the expression cassette of RGNNV-CP tagged with FLAG	This study
p26SrDNA2-ura3d8-NNV(F)	26S rDNA2-based integration vector carrying a defective Yl*ura3* with an 8-bp promoter and the expression cassette of RGNNV-CP tagged with FLAG	This study
p26SrDNA2-ura3d15-NNV(F)	26S rDNA2-based integration vector carrying a defective Yl*ura3* with a 15-bp promoter and the expression cassette of RGNNV-CP tagged with FLAG	This study
p26SrDNA2-ura3d41-NNV(F)	26S rDNA2-based integration vector carrying a defective Yl*ura3* with a 41-bp promoter and the expression cassette of RGNNV-CP tagged with FLAG	This study
pT26SrDNA2-URA3-NNV(F)	26S rDNA2-based integration vector carrying Yl*URA3* with an intact promoter and the expression cassette of RGNNV-CP tagged with FLAG	This study

In this study, we evaluated *Y. lipolytica* as a host to produce VLPs by expressing codon-optimized RGNNV-CPs. We constructed a set of RGNNV-CP expression vectors based on autonomously replicating sequence (ARS) elements and the 26S rDNA fragment of *Y. lipolytica* to achieve a high level of RGNNV-CP expression in *Y. lipolytica*. Here, we show that oral immunization of mice with whole recombinant *Y. lipolytica* expressing RGNNV-CP provoked efficient immune responses and that recombinant RGNNV-CP was expressed in the form of VLPs in *Y. lipolytica*. Our data suggests the potential for *Y. lipolytica* to serve as a host for the production of VLP-based recombinant vaccines for fish and other animals.

Materials and Methods

Strains, media, and growth conditions

The *Y. lipolytica* strains and plasmids used in this study are summarized in Tables 1 and 2, respectively. *Y. lipolytica* strains were grown at 28°C in YPD (yeast extract-peptone-dextrose) or synthetic complete medium without uracil (SC-URA) or leucine (SC-LEU) with 2% glucose. *Y. lipolytica* was transformed by a one-step transformation as previously described (Chen *et al.*, 1997). *E. coli* DH5α was used for transformation and amplification of the constructed plasmid vectors. *E. coli* transformants were grown on Luria-Bertani medium supplemented with 100 μg/ml ampicillin for selection (Sambrook *et al.*, 1989).

Construction of RGNNV-CP expression vectors

The synthetic cDNA of RGNNV-CP for expression in *Y. lipolytica* was optimized by Blue Heron Biotech for the preferred codons of *Y. lipolytica* and to reduce secondary structure of its mRNA (Supplementary data Fig. S1). The *Y. lipolytica* codon-optimized cDNAs of full-length RGNNV-CP (YlNNVI) and two truncated forms of RG-NNVCP (YlNNVII and YlNNVIII), which encoded modified forms of the RGNNV-CP protein with a deletion of the N-terminal 49 (ΔN49) or 81 (ΔN81) amino acid residues, respectively, were amplified by polymerase chain reaction (PCR) using the primer sets listed in Supplementary data Table S1. The PCR fragments encoding RGNNV-CP fused with or without a FLAG tag at the C-terminus were digested and inserted

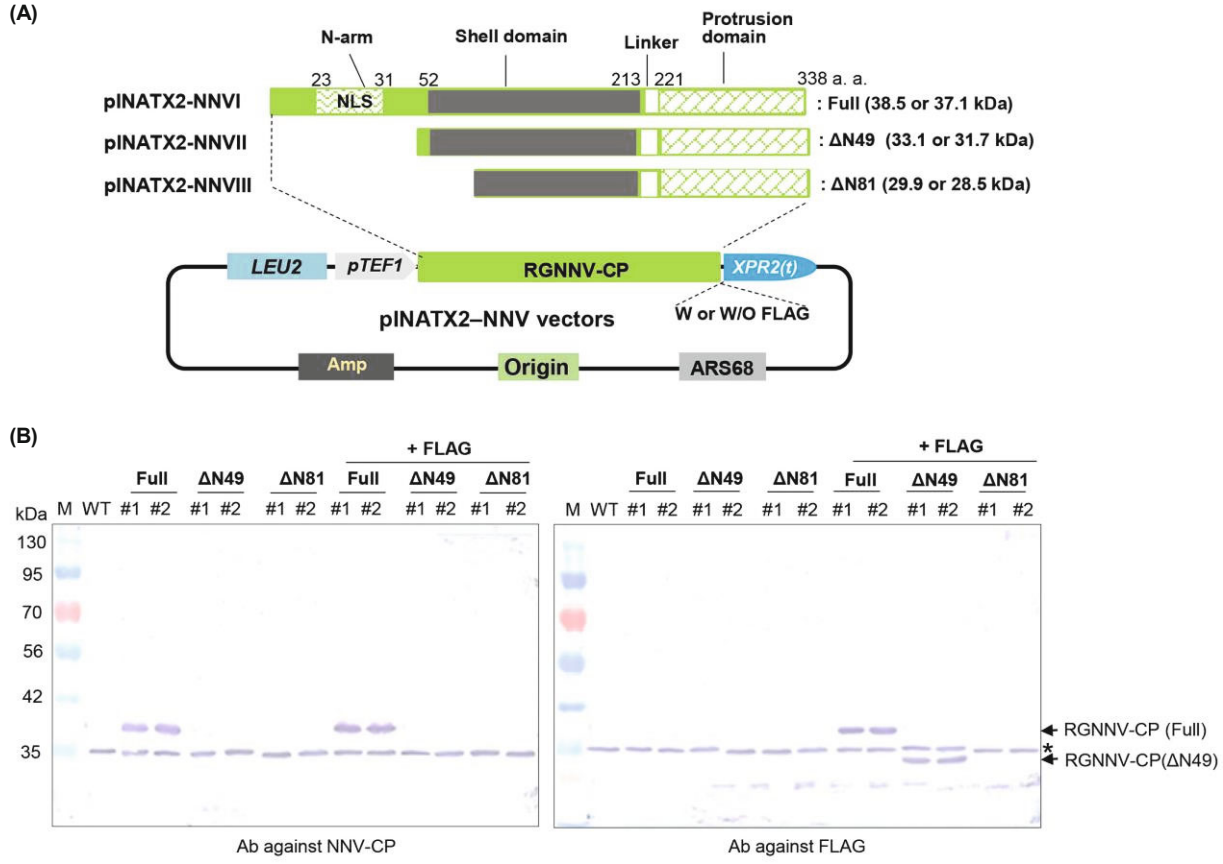

Fig. 1. Expression of full-length and N-terminally truncated capsid proteins of red-spotted nervous necrosis virus (RGNNV) in *Yarrowia lipolytica* using the autonomously replicating sequence (ARS)-based pINATX2–NNV vector. (A) Schematic representation of the ARS68-based vectors pINATX2–NNVI, pINATX2–NNVII, and pINATX2–NNVIII expressing the full-length and truncated (Full, ΔN49, ΔN81) forms of RGNNV capsid proteins (RGNNV-CP). NLS: nucleus localization sequence. The codon-optimized RGNNV-CP synthetic genes, with or without FLAG epitope sequence tags, were placed under the control of the Yl*TEF1* promoter and *XPR2* terminator. (B) Expression of the recombinant full-length and truncated RGNNV-CP (Full, ΔN49, ΔN81) in *Y. lipolytica*. Western blot analysis was performed using anti-RGNNV-CP (left panel) and anti-FLAG (right panel) antibodies, respectively. Two randomly selected transformants (#1 and #2) of each vector construct were used in the expression analysis.

into vector pINAScTRP1 at *Bam*HI and *Xba*I restriction sites to generate the autonomously replicating sequence (ARS)-based vectors expressing YlNNV proteins, pINATX2-NNVI or pINATX2-NNVI(F), pINATX2-NNVII, or pINATX2-NNVII(F), and pINATX2-NNVIII or pINATX2-NNVIII(F) (Fig. 1A). The *Eco*RI/*Not*I-digested fragment containing the YlNNVI expression cassette under the control of the *TEF1* promoter and *XPR2* terminator, obtained from pINATX2-NNVI or pINATX2-NNVI(F), was inserted into the *Eco*RI/*Not*I site of pIMR53_AUX, resulting in pIMR53-NNVI or pIMR53-NNVI(F) (Fig. 2A).

For construction of the pT-26SrDNA-ura3ds-NNV series, a 1-kb 26S ribosomal DNA (rDNA) fragment that contained an *Eco*RI site in the middle was PCR amplified from the genomic DNA of *Y. lipolytica* strain Po1f. The amplified 26S rDNA fragment was cloned into pGEM-T Easy Vector (Promega), generating pT-26SrDNA. Then, the amplified fragment containing the Yl*TEF1* promoter, YlNNVI(F), and the *XPR2* terminator was introduced into pT-26SrDNA at the *Sph*I site, resulting in the vector p26SrDNA-NNV(F). Finally, the Yl*URA3* gene with intact promoter (385 bp) and the defective Yl*ura3* genes with truncated promoters (0, 4, 8, 15, or 41 bp), amplified by PCR from pIMR53 using the primers in Table 3, were inserted at the *Bgl*II sites of p26SrDNA-NNV(F), generating p26SrDNA-URA3-NNV(F), p26SrDNA-ura3d0-NNV(F), p26SrDNA-ura3d4-NNV(F), p26SrDNA-ura3d8-NNV(F), p26SrDNA-ura3d15-NNV(F), and p26-SrDNA-ura3d41-NNV(F) (Fig. 4A). All of the constructed p26SrDNA-ura3-NNV(F) vectors were confirmed by sequencing.

Oral immunization of mice by recombinant yeasts expressing RGNNV-CP

Oral immunizations using freeze-dried *S. cerevisiae* and *Y. lipolytica* were prepared based on the methods used in our previous study (Kim *et al.*, 2014). Briefly, mice (5-week-old female BALB/c) received four oral doses of 10 mg of the recombinant *S. cerevisiae* and *Y. lipolytica* expressing RGNNV-CP with 5 mg of saponin (Sigma) at 2-week intervals. The control groups received oral doses of 100 ml phosphate-buffered saline (PBS) per day or 10 mg of a non-transformed yeast strain for 3 days. Ten days after the 3rd and 4th immunizations, mouse sera were obtained from tail veins. Animal experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and the Guidelines for Animal Experiments of Chung-Ang University.

Copy-number determination by quantitative real-time PCR (qPCR)

Chromosomal DNA was prepared from each of the *Y. lipolytica* transformants cultivated on the selective SC-URA medium and used as a template for qPCR, which was performed using a qPCR cocktail that contained 12.5 µl MaximaSYBR Green qPCR Master Mix (Fermentas) and 10 pmol forward

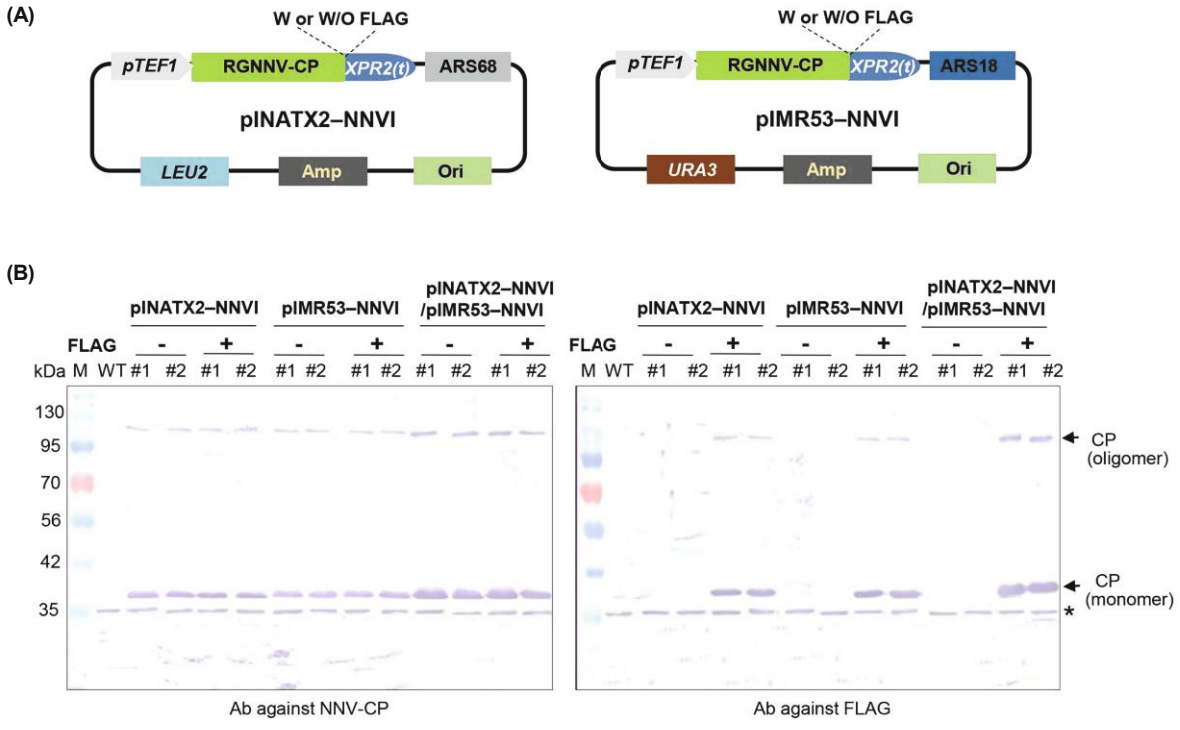

Fig. 2. Co-expression of recombinant RGNNV-CP using two autonomously replicating sequence (ARS)-based vectors in *Yarrowia lipolytica*. (A) Schematic representation of the two ARS-based RGNNV-CP expression vectors pINATX2-NNVI and pIMR53- NNVI that carry different selection markers and ARS sequences. (B) Western blot analysis of *Y. lipolytica* IL3 transformants harboring one (pINATX2-NNVI or pIMR53- NNVI) or two (pINATX2-NNVI and pIMR53- NNVI) expression vectors. Soluble cell lysates were subjected to western blot analysis with anti-RGNNV-CP (left panel) or anti-FLAG (right panel) antibodies (Ab). Arrows indicate the position of the RGNNV capsid protein in monomers and oligomers, and a star indicates a non-specific protein band.

and reverse oligonucleotide primers targeting *URA3*. The PCR reaction was monitored on a CFX96 Real-Time PCR Detection System (Bio-Rad). The copy numbers of *URA3* in the expression vectors were estimated by comparison with the copy number of *ACT1* in the host genome as an internal reference gene (one copy per genome).

Sucrose density gradient and transmission electron microscopy of recombinant RGNNV-CP

Yeast cell pellets were resuspended in ice-cold TNE buffer (50 mM Tris-HCl; pH 7.4, 100 mM NaCl, 0.1 mM EDTA) containing 1 mM PMSF, 1× PIC (phenylmethylsulfonyl fluoride, protease inhibitor cocktail, protease inhibitors), mixed with an equal volume of glass beads and lysed with a Precellys Bead Homogenizer (Life Science Product). Yeast cell lysates were obtained by centrifugation at 15,000 rpm at 4°C for 10 min and subjected to sucrose density gradient ultracentrifugation. Sucrose density gradients in the range of 20–70% (w/w) were prepared in 1× TNE buffer containing 1 mM PMSF and 1× PIC. Yeast cell lysates were loaded onto a 20–70% sucrose gradient in 3.5-ml centrifuge tubes (Beckman Coulter) and centrifuged at 100,000×*g* (Beckman Optima LE-80K Ultracentrifuge) at 4°C for 2 h. Thereafter, 0.5 ml of each fraction was collected and analyzed by western blotting using rabbit anti-RGNNV-CP (Bethyl) or anti-FLAG (Sigma-Aldrich) antibodies. Fractions enriched with RGNNV-CP were collected and concentrated using an Amicon Ultra-15 Centrifugal Filter in 1× modified PBS buffer (137 mM NaCl and 2.7 mM KCl, without Na_2HPO_4 and KH_2PO_4) containing 50 mM $CaCl_2$ at 4°C. The partially purified recombinant RGNNV-CPs were absorbed onto carbon-coated grids and negatively stained with 2% phosphotungstic acid. Electron microscopy was performed on an energy-filtering transmission electron microscope (Libra 120, Carl Zeiss).

Results

Expression of the codon-optimized RGNNV-CP gene using ARS-based plasmids

In an effort to express RGNNV-CP in *Y. lipolytica*, the composition of the synthetic gene encoding RGNNV-CP was optimized according to the codon preferences of *Y. lipolytica*. As an initial trial, we expressed a set of the codon-optimized RGNNV-CP cDNAs that encoded a full-length RGNNV-CP (338 amino acids of approximately 37 kDa) and one of two N-terminally truncated RGNNV-CPs, ΔN49 (~33.1 kDa) or ΔN81 (~29.9 kDa) (Fig. 1A). The cDNAs encoding the truncated ΔN49 and ΔN81 CPs were constructed by deleting the majority of the N-terminal arm, which contains a nuclear localization signal (NLS, amino acid residues 23–31), and a part of the shell domain (S domain, residues 52–213) (Costa and Thompson, 2016). The synthetic gene fragments encoding RGNNV-CP with or without a FLAG tag at the C-terminus were placed under the control of a constitutive Yl*TEF1* promoter and a *XPR2* terminator, and the RGNNV-CP expression cassettes were introduced into an ARS-vector containing a selection maker Yl*LEU2* and ARS68 (Fig. 1A). Soluble cell lysates from the *Y. lipolytica* IL3 transformants harboring pINATX2-NNV expression vectors were analyzed by western blotting using antibodies against RGNNV-CP (Fig. 1B, left) and FLAG (Fig. 1B, right), respectively. Interestingly, the full-length RGNNV-CPs were efficiently detected with the RGNNV-CP antibody, but the ΔN49 and ΔN81 truncated proteins were barely detected using the same antibody. However, the ΔN49 RGNNV-CP tagged with FLAG was efficiently detected using anti-FLAG antibody, indicating that deletion of N-terminal arm might affect the antigenicity of RGNNV-CP, decreasing detection by RGNNV-CP antibody. The ΔN81 truncated RGNNV-CP tagged with FLAG was not detected even with anti-FLAG

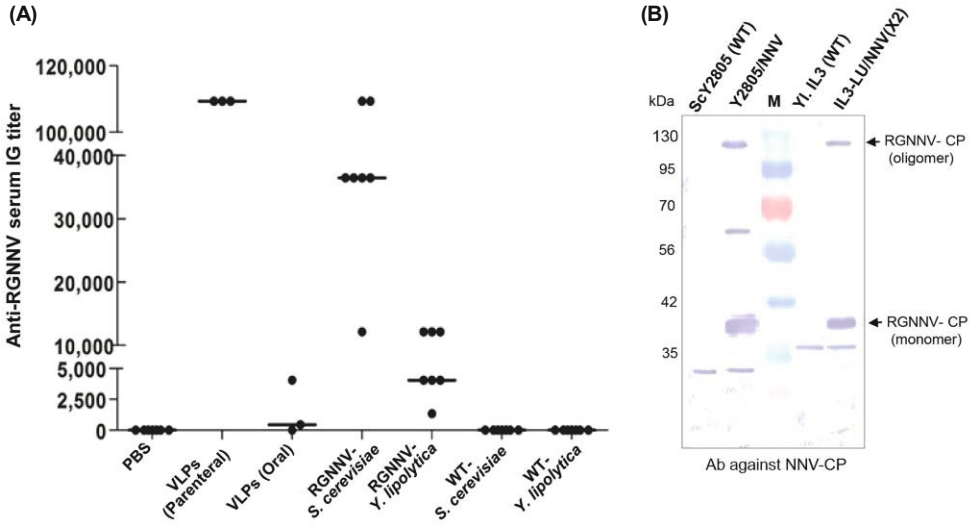

Fig. 3. Immunogenicity of *Yarrowia lipolytica* whole recombinant cells expressing nervous necrosis virus virus-like particles (NNV-VLPs) administered orally to mice. (A) Serum IgG responses following oral immunization with the whole recombinant *S. cerevisiae* Y2805/NNVcp (RGNNV-*S. cerevisiae*) and *Y. lipolytica* IL3-LU/NNV-X2 (RGNNV-*Y. lipolytica*) strains expressing intracellular RGNNV capsid proteins, respectively. Mouse sera were obtained from tail veins 10 days after the 4th immunization. The line represents the median anti-RGNNV serum IgG titers. (B) Western blot analysis of soluble cell lysates prepared from the recombinant *S. cerevisiae* Y2805/NNVcp and *Y. lipolytica* IL3-LU/NNV-X2 strains, which were orally administered to mice.

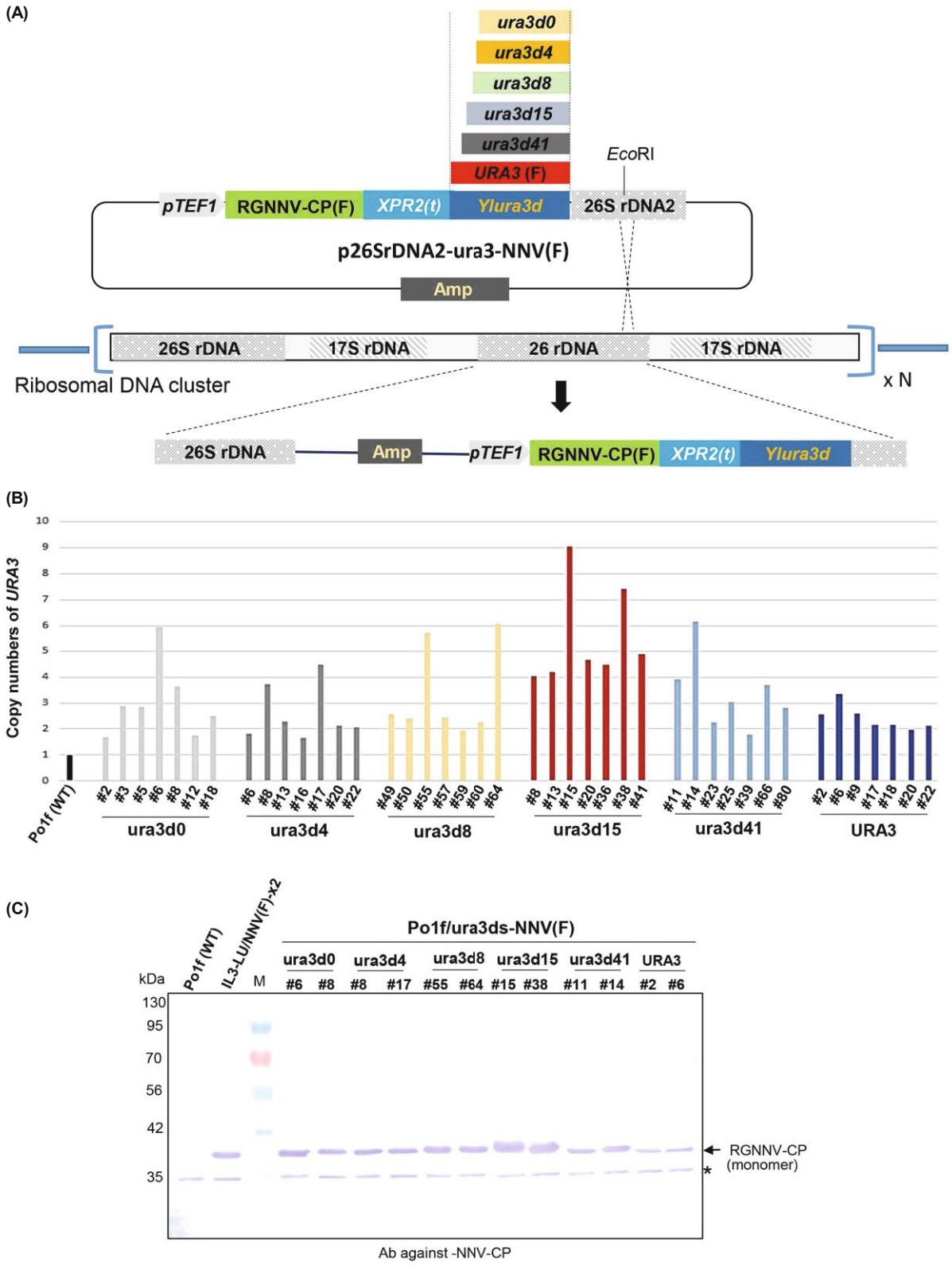

Fig. 4. Expression analysis of integrated capsid proteins using rDNA-targeting in *Yarrowia lipolytica*. (A) Schematic representation of 26S rDNA-based integration vectors for the expression of full-length RGNNV capsid protein with FLAG tagging in combination with defective *ura3* markers with a truncated promoter (0-, 4-, 8-, 15-, or 41-bp) or *URA3* (full promoter) as a selection marker. The *Eco*RI-cleaved vectors integrated into the chromosomal loci of *Y. lipolytica* rDNA units, which are present in tandem repeats. (B) Copy number analysis of *Y. lipolytica* Polf/ura3-NNV(F) transformants by qPCR. (C) Western blot analysis of *Y. lipolytica* transformants that showed the highest copy numbers of each integrated p26SrDNA2-ura3-NNV(F) construct.

antibody, implying that the N-terminal and partial S domain deletion might significantly decrease the stability of RGNNV-CP.

To increase the levels of recombinant RGNNV-CP expression in *Y. lipolytica*, we constructed another ARS-based vector, pIMR53-NNVI, which contained the YlNNVI expression cassette under the control of a constitutive *TEF1* promoter and a *XPR2* terminator, the Yl*URA3* gene as a selection marker, and ARS18 (Fig. 2A). The two expression vectors pINATX2-NNVI and pIMR53-NNVI, with or without FLAG tags, were co-transformed into *Y. lipolytica* IL3. As expected, the *Y. lipolytica* transformant IL3-LU/NNV-X2 harboring two ARS-based NNV expression vectors showed enhanced levels of RGNNV-CP expression compared to the transformants IL3-L/NNV and IL3-U/NNV harboring only a single expression vector (either of pINATX2-NNVI or pIMR53-NNVI) (Fig. 2B). These results suggested that RGNNV-CP expression in *Y. lipolytica* could be further improved by increasing the copy number of the YlNNVI expression cassette. Notably, the recombinant CPs expressed in *Y. lipolytica* were detected not only as monomers (~37 kDa) but also as trimers (~120 kDa), which is the basic unit for formation of the $T = 3$ icosahedral structure of NNV virus-like particles (Chen *et al.*, 2015).

Evaluation of whole recombinant *Y. lipolytica* expressing RGNNV-CP as an oral vaccine candidate

To evaluate the potential of whole recombinant *Y. lipolytica* as an oral vaccine candidate to prevent NNV infection, mice were orally immunized with the recombinant *Y. lipolytica* strain harboring two RGNNV-CP expression vectors, IL3-LU/NNV(X2), and compared to those immunized with the RGNNV-CP-expressing *S. cerevisiae* Y2805/NNVcp strain (Fig. 3). As reported in a previous study (Kim *et al.*, 2014), oral immunizations of mice with the freeze-dried whole recombinant *S. cerevisiae* Y2805/NNVcp efficiently provoked the generation of anti-RGNNV-CP antibodies in the sera of immunized mice after four immunizations. Immunization with the recombinant *Y. lipolytica* IL3-LU/NNV-X2 strain also provoked anti-RGNNV-CP IgG at a level comparable to that of *S. cerevisiae* Y2805/NNVcp when considering the much lower level of RGNNV-CP expression in *Y. lipolytica*, compared to that in *S. cerevisiae* (Fig. 3B). Expression of RGNNV-CP in the recombinant *S. cerevisiae* strain Y2805/NNVcp was directed by a 2μ-based vector with more than 25 copies, whereas expression of RG-NNVCP in the recombinant *Y. lipolytica* strain IL3-LU/NNV-X2 was directed by two ARS-based vectors with only one or two copies per cell. Moreover, we also observed that sera from mice immunized with the recombinant *Y. lipolytica* IL3-LU/NNV-X2 showed neutralizing activity against RGNNV (data not shown). These results indicate that orally administration of RGNNV-CP expressed in *Y. lipolytica* can induce systemic neutralizing antibodies and confer protective immunity against virus challenge.

Expression of RGNNV-CP using integration vectors based on *Y. lipolytica* 26S rDNA

Once we confirmed the immunogenicity of RGNNV-CP expressed in *Y. lipolytica*, we adopted another strategy to further increase the levels of RGNNV-CP expression in *Y. lipolytica*. We constructed a set of multiple integration vectors based on the rDNA cluster and a defective auxotrophic marker. *Y. lipolytica* contains rDNA units of two different size classes, viz. 7.7 kb (P-type) and 8.7 kb (G-type), with each unit containing 26S and 17S rRNAs (van Heerikhuizen *et al.*, 1985). The 26S rDNA-based vectors were designed to carry an approximately 1 kb G unit rDNA fragment with a unique *Eco*RI site that is used for linearization of vectors to be specifically integrated into rDNA loci of *Y. lipolytica* chromosomes by single homologous recombination. As a selection marker to direct multiple integrations, a set of defective Yl*ura3d* markers with 0-, 4-, 8-, 15-, and 41-bp truncated promoters or an intact Yl*URA3* with a 385-bp promoter were employed. The constructed p26SrDNA2-ura3-NNV(F) vectors were linearized by *Eco*RI digestion and transformed into *Y. lipolytica* Po1f, which exhibited much higher homologous recombination efficiency than *Y. lipolytica* IL3 in our experiments (Fig. 4A).

A copy number analysis by qPCR of the obtained transformants was performed to compare the amount of *URA3* amplified from the chromosomal *URA3* and those from the integrated p26SrDNA2-ura3-NNV(F) vectors with selection markers. The results showed that the transformants obtained from vectors carrying the defective *ura3d* with truncated promoters (0, 4, 8, 15, or 41 bp) exhibited 2 to 8 integrations per cell, whereas the transformants from the vector carrying intact *URA3* exhibited mostly single copy integrations. The highest average copy numbers were observed in the 26SrDNA-ura3d15-NNV(F) transformants harboring the defective *ura3* with 15-bp promoter (Fig. 4B). The constructs with the shortest promoters (0, 4, and 8 bp) appeared to generate transformants by integration into a mutated Yl*ura3* gene on the host chromosome to recover full expression of Yl*URA*3. The highest level of RGNNV-CP expression was observed in the 26SrDNA-ura3d15-NNV(F) transformant, Pol1f/ura3d15-15, carrying eight copies of integrated vector, which was estimated by western blot analysis to be more than two-fold greater than that of transformant IL3-LU/NNV(F)-X2 harboring two ARS-vectors (Fig. 4C). These results indicated that the 26S rDNA-based vectors carrying defective *URA3* genes were suitable for multicopy integration of the expression cassettes into rDNA repeats in the *Y. lipolytica* chromosome to achieve a high level of RGNNV-CP production.

Formation of VLPs from the recombinant RGNNV-CPs expressed in *Y. lipolytica*

To investigate the formation of VLPs from the recombinant RGNNV-CPs expressed in *Y. lipolytica*, soluble cell lysate of the *Y. lipolytica* transformant Pol1f/ura3d15-15, which expressed the full-length codon-optimized RGNNV-CP tagged with FLAG, was analyzed by 20–70% sucrose gradient centrifugation. The sucrose gradient fractions after ultracentrifugation were analyzed from bottom to top by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting, revealing RGNNV-CP enrichment with high purity in the bottom fractions. This strongly indicated that a substantial amount of RGNNV-CPs was

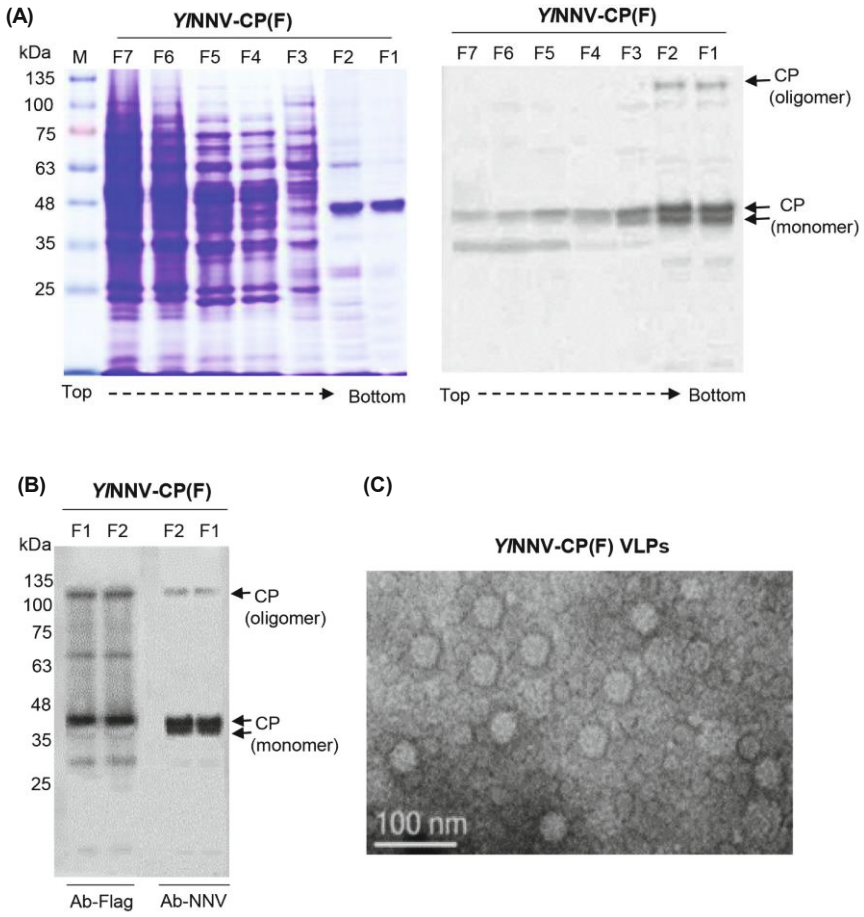

Fig. 5. Virus-like particle (VLP) formation of RGNNV capsid proteins expressed in *Yarrowia lipolytica*. (A) Sucrose gradient centrifugation analysis of RGNNV capsid proteins expressed in *Y. lipolytica*. Yeast cell lysates were loaded onto a 20–70% sucrose gradient, and the samples of each fraction were analyzed by SDS-PAGE and western blotting using anti-RGNNV-CP antibody. The fractions were numbered from bottom to top of the sucrose gradient. (B) Analysis of FLAG tag cleavage in the recombinant RGNNV-CP(F) expressed in *Y. lipolytica* by western blotting using an anti-FLAG antibody (left) or anti-RGNNV-CP antibody (right). (C) Transmission electron microscopy (TEM) analysis of the recombinant RGNNV capsid proteins expressed in *Y. lipolytica*. Fractions (F1 and F2) from the sucrose density gradient centrifugation that were enriched with the RGNNV capsid protein were combined and concentrated using Amicon Ultra-15 Centrifugal Filters (left). The samples with enriched RGNNV capsid proteins were absorbed onto carbon-coated grids and negatively stained with 2% phosphotungstic acid (right). TEM was performed with an energy-filtering microscope (Libra 120, Carl Zeiss) at a final magnification of 200,000× (bar, 100 nm).

expressed in VLPs in *Y. lipolytica* (Fig. 5A). Interestingly, we observed two monomer bands of RGNNV-CP tagged with FLAG in *Y. lipolytica* in the western blotting analysis using rabbit RGNNV-CP serum. We speculated that this might be caused by C-terminal cleavage of RGNNV-CP(F), which generated a lower-weight protein band of the same size as RGNNV-CP without a FLAG tag. As expected, only the higher-weight protein band of RGNNV-CP monomers, but not the lower-weight band, was detected in western blotting with mouse anti-FLAG antibody (Fig. 5B). These results suggested that the recombinant RGNNV-CP with a FLAG-tag underwent C-terminal cleavage, probably during sample preparation, generating the lower-weight protein band.

For the transmission electron microscopy analysis of VLPs, the two ultracentrifugation fractions (numbered F1 and F2) that were highly enriched with RGNNV-CP were combined and concentrated in 1× modified PBS buffer supplemented with 50 mM $CaCl_2$, which is reported to play a crucial role in particle stability (Chen *et al.*, 2015). Heterogeneous spherical particles, approximately 10–30 nm in diameter, were detected in the sucrose gradient fractions enriched with RGNNV-CP expressed by *Y. lipolytica* transformants (Fig. 5C). These particles resembled the structure and morphology of previously described recombinant RGNNV-like particles. These results strongly suggest that *Y. lipolytica* can be used as a host organism to produce RGNNV-CP as VLPs that can be used in a vaccine to prevent RGNNV infection.

Discussion

There is an increasing demand for recombinant vaccine antigens or immunotherapeutic molecules, which require efficient heterologous expression systems. In particular, recombinant VLP-based vaccines have emerged as efficient, safe, and economical in protecting animals against viral infection. Several recent studies have indicated that yeasts are favorable expression systems for the production of VLPs to be used as vaccine candidates (Kim and Kim, 2016). In the present study, we expressed the codon-optimized RGNNV-CP gene in *Y. lipolytica* to evaluate the potential of this yeast to serve as a host for production of VLP-based recombinant vaccines for fish and animals. Natural episomal plasmids have not yet reported in *Y. lipolytica*; thus, high-copy episomal vectors such as the 2μ-based vectors of *S. cerevisiae* are not available for use with *Y. lipolytica* (Ludwig and Bruschi, 1991; Madzak *et al.*, 2004). Therefore, as an initial trial to express RGNNV-CP in *Y. lipolytica*, we used ARS-based vectors that are often present in one or two copies per yeast cell (Madzak *et al.*, 2004). The initial level of recombinant RGNNV-CP expression using *Y. lipolytica* ARS-based vectors were not high, because of the copy numbers of the expression cassette in the host cell were limited compared to that of the *S. cerevisiae* transformant harboring the 2μ-based RGNNV-CP expression vector (Fig. 3B). To achieve a high level of recombinant RGNNV-CP expression in *Y. lipolytica*, we sub-

sequently constructed rDNA-based integrative vectors in combination with defective auxotrophic selection markers. We constructed a series of vectors using the G-type rDNA unit as a targeting element and various *ura3d* with truncated promoters as a selection marker to direct multiple integrations into the tandem repeated rDNA loci, thus further increasing the expression of recombinant RGNNV-CP in the *Y. lipolytica* transformants (Fig. 4C). The highest integration copy number obtained in the present study was eight copies of the 26SrDNA2-ura3d15-NNV construct, which was lower than that reported in a previous study using rDNA-based integration vectors in combination with defective *ura3* markers (Le Dall *et al.*, 1994). We speculate that this difference in maximum integration copy numbers is because of the different promoters used in the two studies. Considering that the expression of alkaline extracellular protease (AEP) was inducible and that multiple integrations with a high copy number were stable only when AEP synthesis was not induced in a study by Le Dall *et al.* (1994), the Yl*TEF1* promoter used for the expression of RGNNV-CP in our rDNA-based constructs might limit the integrations to eight copies. The decreased growth rate of *Y. lipolytica* transformant Pol1f/ura3d15-15 carrying eight copies of the integrated vector RGNNVmight reflect the metabolic burden of the high level of recombinant RGNNV-CP expression from the strong constitutive Yl*TEF1* promoter.

In the present study, we observed that that deletion of the basic N-terminal arm affected the immunogenicity of RGNNV-CP in an expression analysis of the RGNNV-CP deletion constructs (Fig. 1B). The first 33 residues of the N-terminal arm were recently reported to be disordered with a flexible structure, and this region is thought to play an important role in RNA encapsidation in intact virus particles. The Pro38 amino acid residue, highly conserved across genotypes in the genus *Betanodavirus*, is necessary for stabilization of the β-annulus structure at the N-terminus of CPs, and the S-domain forms a conserved jelly roll structure in canonical viruses (Chen *et al.*, 2015). Thus, the N-terminal deletions in our constructs might have resulted in alterations of the overall structure of RGNNV-CP that affected the antigenicity and stability of the recombinant CP (Fig. 1). On the other hand, the addition of a FLAG tag at the C-terminus appeared not to significantly affect the antigenicity, stability, and VLP-forming capacity of RGNNV-CP expressed in *Y. lipolytica* (Fig. 5). The assembly and structural integrity of VLPs can be affected by chelating agents, unnecessary proteins, and the purification process, which can promote dissociation of the particle (Wu *et al.*, 2008; Wang *et al.*, 2010; Kim *et al.*, 2016). Our sucrose density gradient analysis suggested that a substantial portion of the RGNNV-CP produced in yeasts can undergo intracellular assembly (Fig. 5). Along with our preliminary data, which showed that the whole recombinant *Y. lipolytica* transformant expressing RGNNV-CP conferred protective immunity against virus challenge in orally immunized mice, the VLP formation of the RGNNV-CP expressed in *Y. lipolytica* supports the great potential of this yeast as a host for production of VLP-based vaccines. Moreover, some strains of *Y. lipolytica* have been isolated from marine environments such as fish guts (Zaky *et al.*, 2014), increasing the potential for this yeast to serve as a whole-cell based oral vaccine for fish.

The yeast biomass has been used as single cell protein (SCP) as protein supply for animal feed. As an oleaginous yeast that can accumulate large quantities of lipid in its biomass, *Y. lipolytica* can be exploited not only as SCP but also as single cell oil, which is a superior feature of this yeast over *S. cerevisiae*. The biomass of *Y. lipolytica* has been evaluated as a safe alternative source of the very long chain eicosapentaenoic acid (EPA) to replace fish oil as the major source of EPA in feed for Atlantic salmon (Hatlen *et al.*, 2012). The potential of the whole recombinant *Y. lipolytica* as oral vaccine, presented in this study, highlights the promising features of this yeast to be developed as functional feed additives, simultaneously supplemented with oil-rich nutrients and endowed with vaccination capacity, particularly useful for aquaculture of marine fishes.

Acknowledgements

The work was supported by the grant No NRF-2013M3A6-A8073554 (Global Frontier Program for the Intelligent Synthetic Biology) from the National Research Foundation of Korea (NRF). Van-Trinh Luu is a recipient of CASSY fellowship from Chung-Ang University. We are grateful to Prof. Hong-Jin Kim for his kind guidance in carrying out oral immunization of mice.

References

Bal, J., Lee, H.J., Cheon, S.A., Lee, K.J., Oh, D.B., and Kim, J.Y. 2013. *Ylpex5* mutation partially suppresses the defective hyphal growth of a *Yarrowia lipolytica* ceramide synthase mutant, *Yllac1*, by recovering lipid raft polarization and vacuole morphogenesis. *Fungal Genet. Biol.* **50**, 1–10.

Barth, G. and Gaillardin, C. 1997. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. *FEMS Microbiol. Rev.* **19**, 219–237.

Chen, D.C., Beckerich, J.M., and Gaillardin, C. 1997. One-step transformation of the dimorphic yeast *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol.* **48**, 232–235.

Chen, N.C., Yoshimura, M., Guan, H.H., Wang, T.Y., Misumi, Y., Lin, C.C., Chuankhayan, P., Nakagawa, A., Chan, S.I., and Tsukihara, T. 2015. Crystal structures of a piscine betanodavirus: mechanisms of capsid assembly and viral infection. *PLoS Pathog.* **11**, e1005203.

Cheon, S.A., Han, E.J., Kang, H.A., Ogrydziak, D.M., and Kim, J.Y. 2003. Isolation and characterization of the *TRP1* gene from the yeast *Yarrowia lipolytica* and multiple gene disruption using a TRP blaster. *Yeast* **20**, 677–685.

Choi, Y.R., Kim, H.J., Lee, J.Y., Kang, H.A., and Kim, H.J. 2013. Chromatographically-purified capsid proteins of red-spotted grouper nervous necrosis virus expressed in *Saccharomyces cerevisiae* form virus-like particles. *Protein Expr. Purif.* **89**, 162–168.

Costa, J.Z. and Thompson, K.D. 2016. Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. *Fish Shellfish Immunol.* **53**, 35–49.

Gomes, A., Byregowda, S., Veeregowda, B., and Balamurugan, V. 2016. An overview of heterologous expression host systems for the production of recombinant proteins. *Adv. Anim. Vet. Sci.* **4**, 346–356.

Gomez-Casado, E., Estepa, A., and Coll, J. 2011. A comparative review on European-farmed finfish RNA viruses and their vaccines. *Vaccine* **29**, 2657–2671.

Hatlen, B., Berge, G.M., Odom, J.M., Mundheim, H., and Ruyter, B. 2012. Growth performance, feed utilisation and fatty acid deposition in Atlantic salmon, *Salmo salar* L., fed graded levels of high-lipid/high-EPA *Yarrowia lipolytica* biomass. *Aquaculture* **364**, 39–47.

Kim, H.J. and Kim, H.J. 2016. Yeast as an expression system for producing virus like particles: what factors do we need to consider? *Lett. Appl. Microbiol.* **64**, 111–123.

Kim, H.J., Kwag, H.L., Kim, D.G., Kang, B.K., Han, S.Y., Moon, H., Hwang, J.Y., Kwon, M.G., Kang, H.A., and Kim, H.J. 2016. Assembly of the capsid protein of red-spotted grouper nervous necrosis virus during purification, and role of calcium ions in chromatography. *Biotechnol. Bioprocess. Engineer.* **21**, 373–380.

Kim, H., Lee, J., Kang, H., Lee, Y., Park, E.J., and Kim, H.J. 2014. Oral immunization with whole yeast producing viral capsid antigen provokes a stronger humoral immune response than purified viral capsid antigen. *Lett. Appl. Microbiol.* **58**, 285–291.

Kim, H., Yoo, S.J., and Kang, H.A. 2015. Yeast synthetic biology for the production of recombinant therapeutic proteins. *FEMS Yeast Res.* **15**, 1–16.

Le Dall, M.T., Nicaud, J.M., and Gaillardin, C. 1994. Multiple-copy integration in the yeast *Yarrowia lipolytica*. *Curr. Genet.* **26**, 38–44.

Lin, C.S., Lu, M.W., Tang, L., Liu, W., Chao, C.B., Lin, C.J., Krishna, N.K., Johnson, J.E., and Schneemann, A. 2001. Characterization of virus-like particles assembled in a recombinant baculovirus system expressing the capsid protein of a fish nodavirus. *Virol. J.* **290**, 50–58.

Liu, W., Hsu, C.H., Chang, C.Y., Chen, H.H., and Lin, C.S. 2006. Immune response against grouper nervous necrosis virus by vaccination of virus-like particles. *Vaccine* **24**, 6282–6287.

Lu, M.W., Liu, W., and Lin, C.S. 2003. Infection competition against grouper nervous necrosis virus by virus-like particles produced in *Escherichia coli*. *J. Gen. Virol.* **84**, 1577–1582.

Ludwig, D.L. and Bruschi, C.V. 1991. The 2-μm plasmid as a nonselectable, stable, high copy number yeast vector. *Plasmid* **25**, 81–95.

Madzak, C., Gaillardin, C., and Beckerich, J.M. 2004. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. *J. Biotechnol.* **109**, 63–81.

Madzak, C., Tréton, B., and Blanchin-Roland, S. 2000. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J. Mol. Microbiol. Biotechnol.* **2**, 207–216.

Moon, H.Y., Lee, D.W., Sim, G.H., Kim, H.J., Hwang, J.Y., Kwon, M.G., Kang, B.K., Kim, J.M., and Kang, H.A. 2016. A new set of rDNA-NTS-based multiple integrative cassettes for the development of antibiotic-marker-free recombinant yeasts. *J. Biotechnol.* **233**, 190–199.

Moon, H.Y., Van, T.L., Cheon, S.A., Choo, J., Kim, J.Y., and Kang, H.A. 2013. Cell-surface expression of *Aspergillus saitoi*-derived functional α-1, 2-mannosidase on *Yarrowia lipolytica* for glycan remodeling. *J. Microbiol.* **51**, 506–514.

Müller, S., Sandal, T., Kamp-Hansen, P., and Dalbøge, H. 1998. Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Klyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. *Yeast* **14**, 1267–1283.

Oh, H.J., Moon, H.Y., Cheon, S.A., Hahn, Y., and Kang, H.A. 2016. Functional analysis of recombinant human and *Yarrowia lipolytica O*-GlcNAc transferases expressed in *Saccharomyces cerevisiae*. *J. Microbiol.* **51**, 506–514.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual.Cold spring harbor laboratory press New York.

Schmidt, F. 2004. Recombinant expression systems in the pharmaceutical industry. *Appl. Microbiol. Biotechnol.* **65**, 363–372.

Sohn, J.H., Lee, S.K., Choi, E.S., and Rhee, S.K. 1991. Gene expression and secretion of the anticoagulant hirudin in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **1**, 266–273.

Song, Y., Choi, M.H., Park, J.N., Kim, M.W., Kim, E.J., Kang, H.A., and Kim, J.Y. 2007. Engineering of the yeast *Yarrowia lipolytica* for the production of glycoproteins lacking the outer-chain mannose residues of *N*-glycans. *Appl. Environ. Microbiol.* **73**, 4446–4454.

Tang, L., Lin, C.S., Krishna, N.K., Yeager, M., Schneemann, A., and Johnson, J.E. 2002. Virus-like particles of a fish nodavirus display a capsid subunit domain organization different from that of insect nodaviruses. *J. Virol.* **76**, 6370–6375.

Thiéry, R., Cozien, J., Cabon, J., Lamour, F., Baud, M., and Schneemann, A. 2006. Induction of a protective immune response against viral nervous necrosis in the European sea bass *Dicentrarchus labrax* by using betanodavirus virus-like particles. *J. Virol.* **80**, 10201–10207.

van Heerikhuizen, H., Ykema, A., Klootwijk, J., Gaillardin, C., Ballas, C., and Fournier, P. 1985. Heterogeneity in the ribosomal RNA genes of the yeast *Yarrowia lipolytica*; cloning and analysis of two size classes of repeats. *Gene* **39**, 213–222.

Wang, C.H., Hsu, C.H., Wu, Y.M., Luo, Y.C., Tu, M.H., Chang, W.H., Cheng, R.H., and Lin, C.S. 2010. Roles of cysteines Cys115 and Cys201 in the assembly and thermostability of grouper betanodavirus particles. *Virus Genes* **41**, 73–80.

Wu, Y.M., Hsu, C.H., Wang, C.H., Liu, W., Chang, W.H., and Lin, C.S. 2008. Role of the DxxDxD motif in the assembly and stability of betanodavirus particles. *Arch. Virol.* **153**, 1633–1642.

Yang, J., Kang, H.A., Ko, S.M., Chae, S.K., Ryu, D.D., and Kim, J.Y. 2001. Cloning of the *Aspergillus niger pmrA* gene, a homologue of yeast *PMR1*, and characterization of a *pmrA* null mutant. *FEMS Microbiol. Lett.* **199**, 97–102.

Zaky, A.S., Tucker, G.A., Daw, Z.Y., and Du, C. 2014. Marine yeast isolation and industrial application. *FEMS Yeast Res.* **14**, 813–825.

Zinjarde, S.S. 2014. Food-related applications of *Yarrowia lipolytica*. *Food Chem.* **152**, 1–10.