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Efficient cloning of tilapia lake virus complementary DNAs using an in vivo strategy in baker's yeast

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Abstract

Cloning and protein expression in heterologous systems are very useful tools for the study of viral proteins. In this work, an *in vivo* cloning strategy was applied using the yeast *Saccharomyces cerevisiae*, as an efficient and low-cost method to clone several cDNAs from the tilapia lake virus (TiLV). Samples of infected tilapia *Oreochromis niloticus* tissues were taken and used to isolate their RNA and to obtain and clone the ten viral cDNAs in a shuttle plasmid. The cloning efficiencies range from 5 to 100% but for seven of the cDNAs the values were above 40%, demonstrating the high efficiency of the method.

KEYWORDS

plasmid construction, *Saccharomyces cerevisiae* in vivo cloning, viral recombinant proteins

1 | INTRODUCTION

In aquaculture, the most common causes of infectious diseases are bacteria (55%) followed by viruses (23%), parasites (19%), and fungi (3%) (Asencios et al., 2016; Kibenge & Godoy, 2016). Due to the high susceptibility of the aquatic animals and the limited availability of therapeutics, viruses are the main pathogens worldwide. In the case of tilapia *O. niloticus*, only a few viruses have been reported to cause severe damage (Surachetpong et al., 2017). Accordingly, it is of great interest to study the tilapia lake virus (TiLV), a virus associated with massive mortality in almost 40 countries (Dong et al., 2017; Jansen & Mohan, 2017) since 2009, and was formally described for the first time in 2014 (Eyngor et al., 2014).

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TiLV was preliminarily identified as an Orthomyxo-like virus (Bacharach et al., 2016) but has been classified as the sole species of the new genus Tilapinevirus, and thus named *Tilapia tilapinevirus* (Adams et al., 2017). Its genome is a single-stranded RNA of negative sense, composed of 10 segments or open-reading frames (ORFs), each of which is flanked by 13-base sequences that are repeated as shown in Figure 1 (Bacharach et al., 2016). The lengths of the segments vary from 465 to 1,641 bases, for the first and tenth, respectively. Interestingly, this viral genome lacks homology in almost all its segments except for the first, for which a weak homology with the polymerase gene from the influenza virus has been reported (Bacharach et al., 2016; Eyngor et al., 2014).

Currently, TiLV infections have seriously affected the global tilapia production causing great economic losses. Accordingly, there is an urgent need to advance the investigations regarding this virus and to search for preventive measures such as the use of vaccines. Due to its relative novelty, previous research regarding TiLV has mainly relied on diagnostic methods based on polymerase chain reaction (PCR) (Dong et al., 2017; Eyngor et al., 2014; Tattiyapong, Sirikanchana, & Surachetpong, 2018; Yin et al., 2019).

Production of biologically active proteins is an useful and key tool in immunological research and product development because of their many potential uses as antigens and immune modulators (Balamurugan et al., 2010). Recently, studies using an indirect ELISA method showed that some tilapia specimens produced antibodies that recognized the recombinant protein encoded by the eighth segment (Hu et al., 2020). No other report has been published thus far and therefore more research on the other TiLV proteins is needed as well. This knowledge will allow the identification of all possible viral targets and the validation of their immunogenic activities.

Different expression systems (Andersen & Krummen, 2002; Chen, 2012; Rosano & Ceccarelli, 2014) have been developed in bacteria and yeasts (Mattanovich et al., 2012) that collectively allow the efficient production of large amount of recombinant viral proteins (Ferrer-Miralles, Saccardo, Corchero, Xu, & García-Fruitós, 2015). On the other hand, unlike conventional cloning techniques that are inefficient and tedious, it is possible to take advantage of the DNA repair system in yeast for the construction of recombinant plasmids. This so called *in vivo* cloning is widely recognized as one of the most efficient means for error-free construction of plasmids (Kitazono, 2009). Furthermore, this strategy also allows the cloning of one or more different fragments in a single plasmid, without using *in vitro* digestion or ligation reactions (Eckert-Boulet, Rothstein, & Lisby, 2011; Kitazono, 2011). The simplicity and versatility of this cloning method is based on the availability of homologous flanking sequences (15–60 bp) shared between the ends of the fragment(s) to be cloned and preferably, a linearized plasmid (Kevin, Vo, Michaelis, &

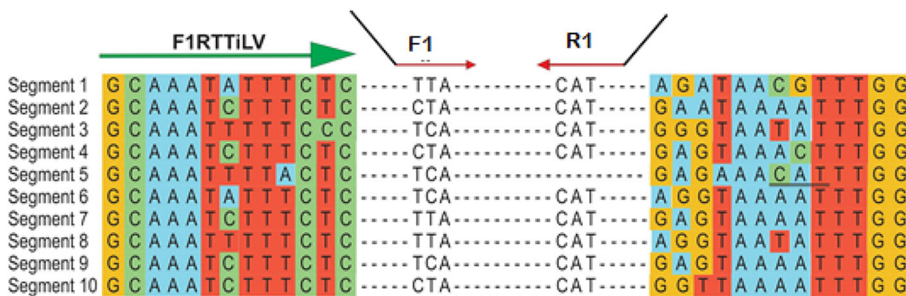

FIGURE 1 Alignment of the cDNA sequences flanking the 10 segments identified in the TiLV genome and schematics of the designed primers. The aligned sequences correspond to the GenBank accession numbers KU751814 to KU751823. The presence of the repeated sequences allowed the use of the same degenerate primers for the reverse transcription polymerase chain reactions (RT-PCRs) (F1RTTiLV for the obtention of all cDNAs except for that of Segment 5, which required the use of primer F2RTTiLV5). A second set of primers was also designed for the cloning of the cDNAs and their analysis. These primers are represented with the F1 and R1 arrows, which included the sequences of the respective ends of the plasmid and were required for the *in vivo* cloning (F1: internal forward primer; R1: internal reverse primer). The sequences of all primers are listed in Table 1. In each case, the CAT triplet corresponds to the ATG start codon in the coding strand of the respective cDNA

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Paddon, 1997; Kitazono, 2009). This strategy can also be easily adapted for its application to any type of plasmid (Iizasa & Nagano, 2006; Joska, Mashruwala, Boyd, & Belden, 2014). Homologous recombination in yeast has been successfully used in the cloning of both natural and synthetic DNA fragments (van Leeuwen, Andrews, Boone, & Tan, 2015; Zhang, Yamanaka, Tang, & Moore, 2019).

Currently, *Saccharomyces cerevisiae* and *Picchia pastoris* are the most widely used system as antigen factories (Geels & Ye, 2010), and in the production of several proteins including virus-like particles (VLPs) (Cho et al., 2017; Galao et al., 2007). These yeasts are preferred for their rapid growth, low production costs, the advantage of providing eukaryotic post-translational modifications (PTMs), their well-developed and easily accessible genetic tools, their designation as generally recognized as safe (GRAS) organisms, and others. Importantly, these yeasts have been successfully used for the generation of antigens and third generation vaccines (Cho et al., 2017; Kumar & Kumar, 2019) for different types of bacterial (Toranzo, Romalde, Magariños, & Barja, 2009) and viral pathogens (Bal et al., 2018; Lei, Jin, Karlsson, Schultz-Cherry, & Ye, 2016).

In this study, we report an *in vivo* cloning strategy in the baker's yeast *S. cerevisiae* for the construction of expression plasmids that will allow the production of recombinant TiLV proteins. It is important to note that with this strategy it is possible to simultaneously achieve both the cloning of the cDNAs or genes of interest and their expression, thus allowing their immediate application in studies such as the development of diagnostic assays and vaccines.

2 | MATERIALS AND METHODS

2.1 | Biological materials

Tilapia specimens that exhibited viral symptoms such as lethargy, erratic swimming, darkening of the body, exophthalmia, skin lesions, eye clouding, and abdominal swelling were collected from two regions in Peru with TiLV outbreaks (Piura and San Martin regions). Different tissues (kidney, liver, spleen) were immediately extracted and kept immersed in preservative solution (25 mM sodium citrate, 10 mM EDTA, 70% ammonium sulfate, pH 5.2) until their arrival at the laboratory. Additionally, tissues of healthy specimens were obtained from the Fish Research and Production Center (CINPIS) at the National Agrarian University La Molina. These samples were used as negative controls.

The *Saccharomyces cerevisiae* and *Escherichia coli* strains used were from our laboratory stocks. The yeast strain had the W303 genetic background (*MATa leu2-3,112 his3-11,15 ura3-1 ade2-1 can1-100 trp1-1*) (Thomas & Rothstein, 1989). The *E. coli* strain used was TOP10 (*F– mcrA Δ(mrr-hsdRMS-mcrBC)Φ80lacZ ΔM15 ΔlacX74 recA1 araD139 Δ (ara leu) 7697 galU galK rpsL (StrR) endA1 nupG*).

2.2 | Plasmid and primer design

The pEGH plasmid (Figure 2) was used for the cloning of the cDNAs. To improve the cloning efficiency, the plasmid was first linearized using the restriction enzyme *Sal*I (Promega) and then dephosphorylated (Antarctic phosphatase, NEB), following the protocols suggested by the respective manufacturers.

All primers used for both the synthesis of the cDNAs and the PCR amplifications were designed on the basis of the published TiLV sequences with GenBank accession numbers KU751814 to KU751823, MH319378.1 to MH319387.1, and KX631921 to KX631936 (Al-Hussinee, Subramaniam, Ahasan, Keleher, & Waltzek, 2018; Bacharach et al., 2016; Eyngor et al., 2014; Surachetpong et al., 2017) (Table 1). The primers used for the generation of each fragment to be cloned included 44–48 bases that were identical to the plasmid ends (Figures 1 and 2, and Table 1).

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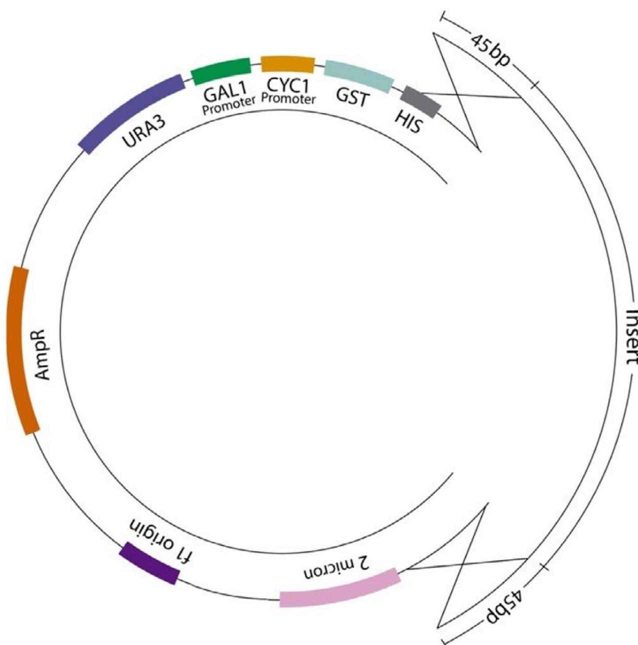

FIGURE 2 Schematic representation of the pEGH plasmid used for the *in vivo* cloning in yeast. Each viral cDNA was cloned as a fragment with flanking sequences (44 - 48 bp) that are homologous to the plasmid ends generated via restriction-enzyme digestion. This plasmid includes the 2 μ multicopy replication origin and a phage replication origin (f1 origin), which allow for its replication in yeast and *E. coli*, respectively; the *URA3* gene that allows for its selection in yeast *ura3* mutant cells, the galactose-inducible promoter (*GAL1*), the gene that codes for the glutathione-S-transferase (GST), and six tandem histidine codons (HIS). With the cloning of a cDNA in this plasmid, it is possible to produce the respective viral protein in yeast, fused to both the poly-histidine and GST tags

2.3 | RNA extraction and RT-PCR

Each of the obtained kidney, liver, and spleen samples were pooled and directly used for RNA extraction using the Quick-RNA MiniPrep Plus kit (Zymo Research) according to the manufacturer's instructions. The synthesis of the first strand cDNA was achieved by RT-PCR using the RevertAid Reverse Transcriptase kit (Thermo Scientific), the primer F1RTTiLV or F2RTTiLV5 (Figure 1 and Table 1), and treatments at 42°C for 60 min and 70°C for 10 min. The reaction products were stored at −20°C.

2.4 | cDNA amplification

All cDNA fragments used for cloning were amplified by PCR, using 2 μL of each cDNA as a template with their overlapping specific primers (Table 1), and the Phusion High-Fidelity DNA Polymerase (ThermoFisher). For the amplification of the cDNAs of segments 1 to 3, the following PCR protocol was used: A denaturation step at 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 58°C for 40 s, 72°C for 50 s, and a final extension of 72°C for 5 min. In cases in which the first reaction produced a reduced yield, a secondary amplification protocol was applied, based on a two-step program (a first denaturation step of 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 72°C for 60 s, and a final extension of 72°C for 5 min). For the segments 4 to 10, the protocol was changed as follows: A first denaturation step of 98°C for 30 s followed by 25 cycles of 98°C for 10 s, an annealing gradient from 58°C to 72°C (+0.56°C per cycle) for 30 s, 72°C for 40 s, followed by 10 cycles of 98°C for 30 s, 72°C for 50 s, and a final extension of 72°C for 5 min. The length and quality of the amplified products were verified by 0.8% agarose gel electrophoresis.

2.5 | Cloning and yeast transformation

For the *in vivo* cloning, a previously described protocol was optimized, as summarized in Figure 3 (Kitazono, 2009). Briefly, the previously linearized and dephosphorylated plasmid was mixed with each PCR product in proportions 1:1

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TABLE 1 Sequences and purpose of the oligonucleotides used in this study

Primer	Sequence (5′- > 3′)[a]	Segment	Purpose
F1RTTiLV	GCAAATITTTCYC	All	RT-PCR
F2RTTiLV5	GCAAATITTACYC	5	RT-PCR
Fw2TiLV1IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**ATAGTTAAAGTTTGTGGGTTGTT	1	Cloning
RvTiLV1IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATGTGGGCATTTCAAGAAGGA		
FwTiLV2IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**ATCTACTGATTTAGATCCATATTTTCC	2	Cloning
Rv2TiLV2IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC** ATGAGTCARTTTGRGAARTCAT		
F1TiLV3IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**TYATCTCGCAAATGGGTGTACT	3	Cloning
R1TiLV3IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATGGACTCGCGGTTYGCA		
F1TiLV4IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**ACT TCGTTGCCTATCTYCCAA	4	Cloning
R1TiLV4IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATG GTGAGAACTACAAAGACTAGT		Cloning
F1TiLV5IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGAA**AG GATCAAGATAATGGAAGCA	5	Cloning
R1TiLV5IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATG TTTCTCTTATCTCAGACTCCA		
F1TiLV6IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**TCACATGTATTTATTGATTTTACAGCA	6	Cloning
R1TiLV6IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATG CAT TTT TAT CTA CAG GAT TYT CC		
F1TiLV7IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**TTAGAGTTCAAAHGTGATTCCT	7	Cloning
R1TiLV7IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATG TCCTACAAGATTGGTGAG		
F1TiLV8IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**TTATTTAAGCATTTCACGGAA	8	Cloning
R1TiLV8IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATGGCTCAAATRCCAACA		
F1TiLV9IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**CCTCATAAAGTTYTATCGCC	9	Cloning
R1TiLV9IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**T TTT TCCGCTTGGTGATG		
F1TiLV10IVC	**GATCGTCAGTCAGTCACGATGAATTAAGCTTGAGCTCGAGTCGA**CTAAGACTGCACGTCRAGAGA	10	Cloning
R1TiLV10IVC	**AGAGGATCGCATCACCATCACCATCACGGTGGTGGTCTAGACTCC**ATGAGTGTGGCAGATTATTTGTC		
pEGH9145FW	GACCCAATGTGCCTGGATGCGTTC	pEGH	Sequencing
3bRVpEGH	ATCGTCAGTCAGTCACGATG		

[a]The bolded sequences correspond to those that are homologous to the plasmid ends at the point of insertion. The inclusion of degenerate bases is indicated using the conventional nomenclature: R = A, G; Y = C, T; H = A, C, T; I = inosine.

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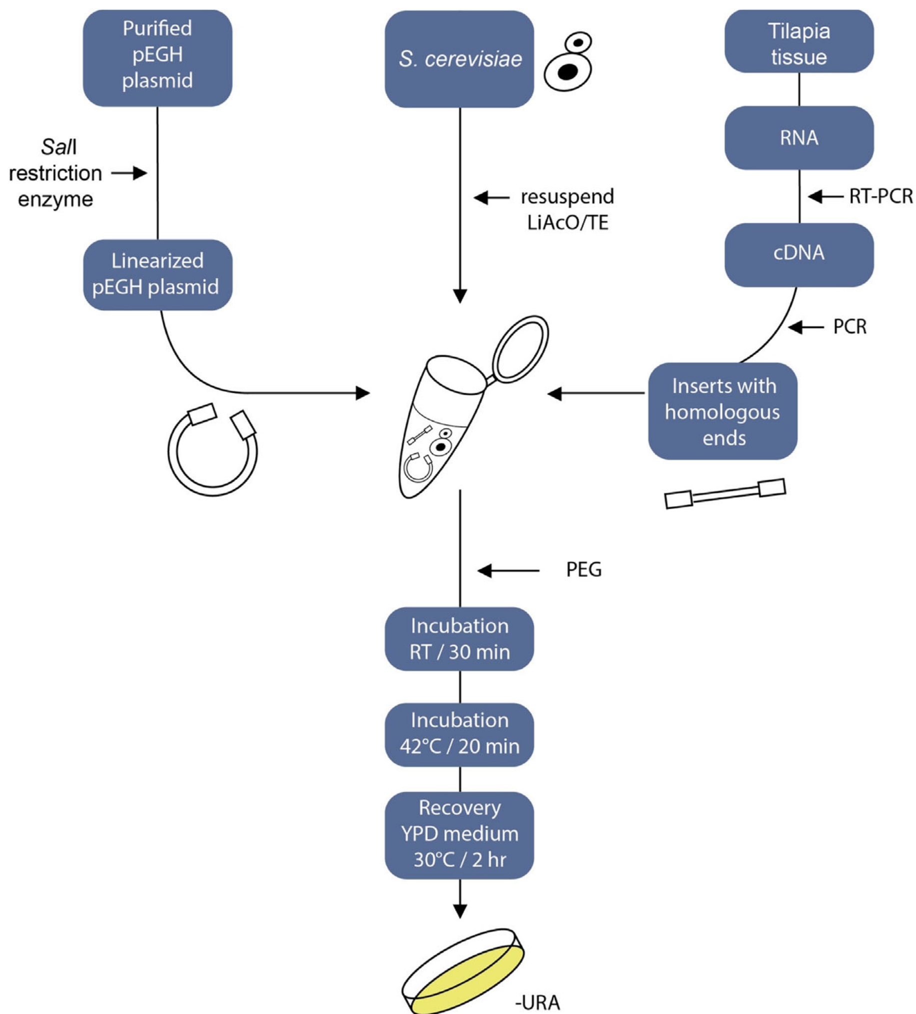

FIGURE 3 Schematics of the strategy for the *in vivo* cloning in yeast. Each amplified cDNA was mixed with the linearized pEGH plasmid and used for the co-transformation of yeast cells. The transformants were selected on synthetic medium lacking uracil

to 1:6 (plasmid:insert) for a total amount of 200 ng. These mixes were used for the transformation of *S. cerevisiae* cells using lithium acetate and heat shock as follows: Overnight cultures (5 mL) were diluted 5–10-fold and incubated at 30°C until the cell suspension reached an $OD_{600\ nm}$ of 0.6–0.8. At this point, the cells were collected by centrifugation, washed and resuspended in lithium acetate/TE buffer. Aliquots of this cell suspension were then mixed with boiled salmon sperm DNA, lithium acetate/TE buffer containing PEG3350, the digested plasmid and one of the amplified cDNAs; and the mixes were incubated for 30 min at room temperature with gentle shaking. The mixes were then treated at 42°C for 20 min, allowed to cool to room temperature and centrifuged to collect the cells. The PEG-containing supernatants were discarded, and the cell pellets resuspended in rich medium (YPD). The cell suspensions were incubated with gentle shaking for 2 hr at 30°C. Additionally, a cloning control (only linearized plasmid) and a transformation control (non-linearized, intact plasmid) were also included. The transformant selection was carried out by growth on synthetic solid medium lacking uracil for 3 days at 30°C. The colonies obtained on each plate were counted to estimate the transformation efficiencies. Random colonies were selected for further studies.

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2.6 | Extraction of total DNA from yeast transformants and analytical PCR

The transformed yeast cells were grown in synthetic liquid medium lacking uracil. The DNA extraction was carried out using a well-known protocol (Guthrie & Fink, 2002). Briefly, the cell pellet was resuspended in 200 μL of lysis buffer (1.2% SDS, 0.1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), 1 mm-glass beads and 200 μL of phenol:chloroform mix (24:1) were added, and the mixes were subjected to vigorous mixing for 5 min. The released nucleic acids were recovered in the supernatant after centrifugation and precipitated after the addition of 1/5 volume of 10 M ammonium acetate and 2 volumes of cold ethanol. The nucleic acid pellet was recovered by centrifugation, washed three times with 90% ethanol, and re-suspended in 50 μL of TE buffer.

For the verification of the presence of the desired cloned fragment, 1 μL of the total yeast DNA solution was used for the PCR reactions. These used the same specific primers used to amplify each cDNA and Taq Polymerase (NEB). A typical PCR program included: An initial denaturation step of 95°C for 4 min, followed by 35 cycles of 58°C for 40 s, 72°C for 90 s, and a final extension step of 72°C for 5 min. The lengths of the amplified products were verified by 0.8% agarose gel electrophoresis.

2.7 | Recovering and amplification of plasmids in *E. coli* and analysis

The yeast transformants whose extracted total DNA showed presence of the desired cloned fragment were selected and subjected to the following procedure, to recover and amplify the respective plasmids: 10 μL of the total DNA solutions was used to transform *E. coli* by heat shock (42°C for 90 s) (Kitazono, 2009). After this treatment, the *E. coli* cell suspensions were incubated for 2 hr at 37°C in LB broth and then the collected cells were plated on LB agar supplemented with ampicillin (100 μg/mL). A transformation control (empty vector) was included. A few of these transformants were selected for plasmid isolation, purification and analysis.

Plasmid purification was performed with Zyppy Plasmid MiniPrep Kit (Zymo Research) according to the manufacturer's instructions. Then, the plasmids were subjected to restriction enzyme digestion (*BamH*I and *Hind*III, Promega) and analyzed via 1% agarose gel electrophoresis. Finally, in order to confirm that the sequences corresponded to the TiLV cDNAs, two plasmids for each viral ORF were sequenced using the primers pEGH9145Fw and 3bRVpEGH (Macrogen Korea) and analyzed by BLASTn (NCBI).

3 | RESULTS

3.1 | Cloning of the TiLV cDNAs in pEGH

The cloning strategy was designed to achieve the efficient construction of the pEGH plasmids harboring the TiLV cDNAs, taking advantage of the repeated sequences flanking each ORF or segment (Figure 1). After the yeast cells are transformed, each colony might carry an empty plasmid or a plasmid with the expected insert and once this is confirmed, those cells could be immediately used for the production of the recombinant protein. As shown in Figures 2 and 3, the pEGH plasmid was selected because it allows the production of fusion proteins with both the glutathione-S-transferase (GST) and polyhistidine tags. The presence of these tags greatly facilitates the detection and purification of the recombinant proteins using simple affinity chromatography protocols.

For all the transformation assays and as shown in Figure 4, between 1,300 and 24,920 colonies were obtained per microgram of plasmid DNA. From these, 10–40 colonies were randomly selected for further characterization. For each yeast colony, the total DNA was extracted and used for a PCR assay that allows detection of the expected cloned cDNA. These assays determined that roughly 50% of the colonies tested (range 5–100%) included the expected cDNA and therefore, had successfully accomplished the plasmid construction (Table 2). These results

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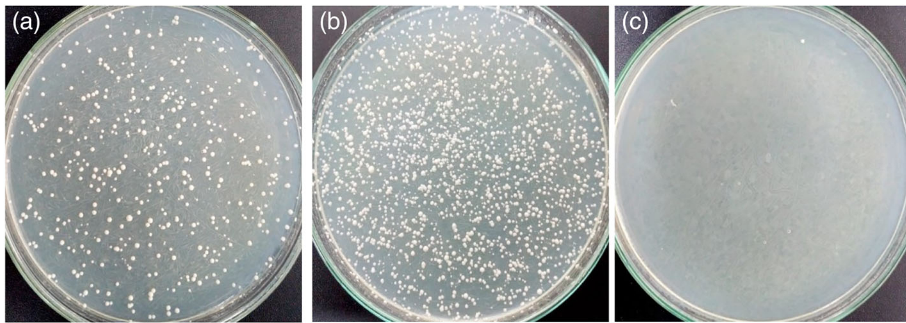

FIGURE 4 Representative results of yeast transformations performed for each *in vivo* cloning procedure. The figures show yeast colonies that have grown on synthetic media lacking uracil, after 2–3 day incubations at 30°C. (a) Co-transformation of yeast cells with the linearized plasmid and the amplified cDNA (Segment 5). (b) Transformation of yeast cells with a similar amount of non-linearized plasmid (control). (c) Transformation of yeast cells with a similar amount of the linearized plasmid, without the amplified cDNA (negative control)

TABLE 2 Cloning efficiencies obtained for all 10 viral segments

Segment number	Cloning efficiency (%)[a]
1	43
2	20
3	50
4	5
5	82
6	80
7	100
8	83
9	5
10	100

[a]Estimated by PCR analysis of total DNA isolated from random yeast transformants, with primers that allow amplification of the insert present in the pEGH plasmid.

demonstrate the high cloning efficiency that this strategy can achieve, even when starting with viral RNA isolated from infected tilapia tissues.

3.2 | Recovery of the constructed plasmids using *E. coli* and their analysis

The yeast transformants that gave a positive result in the preliminary PCR assay were selected for further studies. In these, each total DNA solution was used to transform *E. coli*. This step allows for amplification of the plasmid, which is possible because the plasmid pEGH is able to replicate and be maintained both in *S. cerevisiae* and in *E. coli*. For each type of plasmid, 6–10 *E. coli* transformants were selected and grown to allow the purification plasmid DNA. The purified plasmids were then submitted to restriction analysis, to verify the presence of the inserts (Figure 5). This analysis further confirmed that all plasmids carried inserts of the expected sizes. Finally, with the nucleotide sequencing analyses, it was possible to unequivocally demonstrate that the strategy had allowed the precise cloning of nine out of the 10 cDNAs (Figure 6). Unfortunately, and despite the confirmation of the presence of the insert of the expected length in the plasmid, it was not possible to obtain readable sequences for the ninth ORF. The completion of the sequencing analysis for this plasmid will require several more

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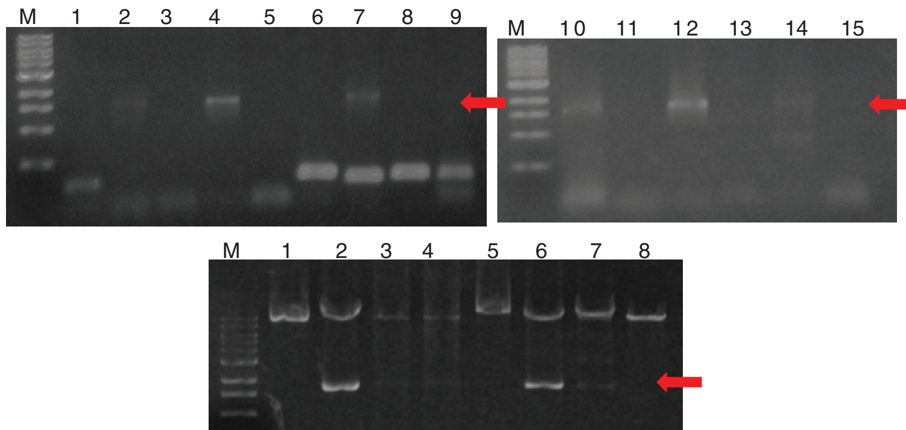

FIGURE 5 Representative results of the procedures followed for the analysis of the yeast transformants and the isolated plasmids. The red arrows indicate the positions of the bands of the expected sizes. Upper panel: polymerase chain reaction-based assay for the presence of the cDNA of ORF1 in the total DNA isolated from randomly picked yeast transformants. Fourteen yeast colonies were evaluated (Lanes 1–14) and from these, six (Lanes 2, 4, 7, 10, 12, and 14) exhibited bands of the expected size. The negative control (Lane 15) consisted of total DNA isolated from yeast cells that had not been transformed. Bottom panel: Two types of plasmids recovered from two independent yeast transformants were recovered and amplified in *E. coli*. The purified plasmids were digested with restriction enzymes *Hind*III and *BamH*I (Lanes 2–7). The original pEGH plasmid was similarly digested as a control (Lane 1) or run without such treatment (Lane 8). M: 1 kb DNA Marker (NEB)

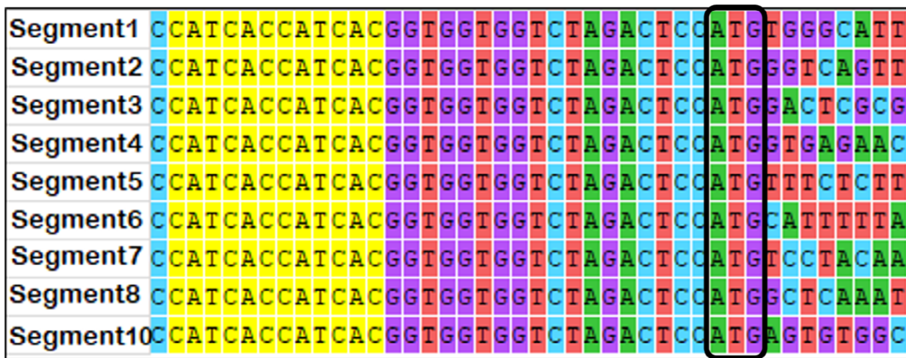

FIGURE 6 Summary of the results of the nucleotide sequencing analyses. The plasmids were purified from two independent transformants and submitted to nucleotide sequencing analysis, using primers pEGH9145FW and 3bRVpEGH. For each type of plasmid, identical sequences were obtained for the two samples analyzed. The alignment shows the correct cloning of all inserts, with the correct positioning of the ATG codon and the expected sequences in each case. The sequences in yellow correspond to the region coding for the polyhistidine tag

attempts with different primers, and their design is currently under process. The analysis of all sequences obtained for the TiLV isolated in Peru will allow comparison with those already reported and will be a matter of a separate communication.

4 | DISCUSSION

The described *in vivo* cloning strategy is a more simple and efficient method than the traditional one that uses restriction and ligase enzymes. This report described its efficient application in the cloning of several viral cDNAs in a single

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reaction, starting from a linearized plasmid and a PCR product. The process is not only efficient but highly economical and requires only minimal equipment and reagents.

Previously, an optimized a protocol for *in vivo* cloning in *S. cerevisiae* was reported in which fragments with 45 bp homologous sequences at the ends were cloned (Kitazono, 2011). The same protocol was applied here except that several plasmid: insert ratios were tested, finding that those in the range 1:1 to 1:6 were adequate. Importantly, as shown in Table 2, among the yeast transformants tested, 5–100% of them had successfully constructed the plasmids. As expected, the best results corresponded to the smaller fragments (segments 5–10), possibly due to the facilitated assembly of the recombinant plasmids, as had been reported previously for *in vitro* strategies (Gibson et al., 2009) and others using bacterial cells (Jacobus & Gross, 2015).

Interestingly, several yeast-based vaccines (Allnutt et al., 2007; Bal et al., 2018; Cho et al., 2017; Choi, Kim, Lee, Kang, & Kim, 2013) have also been explored with increasing interest. Currently, there are no validated vaccines for TiLV, although a patent has been granted (Bacharach et al., 2016) for a potential one that uses an attenuated virus. Otherwise, a recombinant TiLV antigen expressed in *E. coli* was detected by specific antibodies with an indirect ELISA test (Hu et al., 2020) and therefore, it is also a potential candidate for future vaccine development. Given the need to find measures to prevent further TiLV damages in the tilapia industry, further research is urgently needed.

5 | CONCLUSION

The proposed *in vivo* cloning method was successfully applied for the construction of several plasmids carrying TiLV cDNAs, allowing at the same time, expression of the cloned ORFs in yeast. Importantly, this strategy could be easily adapted to clone any other type of DNA fragment or fragments, to facilitate the study of the recombinant proteins.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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