PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B, Vol. 71 (2017), No. 4 (709), pp. 266–274.
DOI: 10.1515/prolas-2017-0045

IN VIVO PACKAGING OF mRNA IN YEAST-PRODUCED BACTERIOPHAGE GA DERIVED VIRUS-LIKE PARTICLES

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Communicated by Tatjana Kozlovskaja

Bacteriophage GA coat protein formed self-assembly competent virus-like particles (VLPs) have been expressed previously in bacterial and yeast cells. On the basis of our previous experiments on the yeast vector pESC-URA / S. cerevisiae *system containing two oppositely oriented promoters, new constructions were created with point-mutations in coat protein to mimic phage MS2-like RNA binding characteristics. Simultaneously, the MS2 operator sequence was added to mRNA desired for packaging. After the introduction of single-point mutations (S87N, K55N, R43K) and double-point mutations (S87N + K55N and S87N + R43K), the coat protein's ability to form VLPs was retained, but yield from cells was decreased. Exchange of the 87th Ser to Asn in coat protein sequence in combination with bacteriophage MS2 translational operator provided specific packaging of the gene of interest (GFP). Although non-specific nucleic acid sequences were packaged, the remarkable specificity for packaging of the gene of interest can be achieved using the described approach.*

Key words: *virus-like particles, GA, MS2, operator.*

INTRODUCTION

There have been recent studies on the production of recombinant bacteriophage GA coat protein formed nanoparticles with *in vivo* packaged mRNAs. It was found that high-yield production of GA coat protein formed virus-like particles (GA CP VLPs) occurred by expression of the appropriate coding gene in yeast *Pichia pastoris* (Freivalds *et al*., 2008), whereas gene constructions based on vector pESC-URA from Stratagene in *Saccharomyces cerevisiae* gave much lower production of VLPs, which allowed to produce mosaic particles (Rūmnieks *et al*., 2008) and to pack mRNAs into particles during growth (Rūmnieks *et al*., 2008; Strods *et al*., 2013).

It is well-known that the coat proteins of single-strand RNA bacteriophages are bifunctional. They form icosahedral shells for protection of viral nucleic acids and also switch off viral replicase synthesis by binding to the specific RNA hairpin that contains the replicase ribosome binding site. This hairpin structure is placed just downstream of the coat protein coding sequence, before the next replicase-coding sequence. The MS2 genome was the first genome to be completely sequenced by Walter Fiers and his team in 1976 (Fiers *et al*., 1976). The above mentioned hairpin or stem-loop structure designated as a translational operator (Bernardi and Spahr, 1972) is also bifunctional. It is involved not only in the effective repressing of the synthesis of replicase, but also is believed to be a packaging signal that initiates the assembly of the capsid and ensures recognition and selective encapsidation of the phage RNA.

Recombinant VLPs derived from bacteriophage coat proteins retain the ability for packaging of RNA (unspecific, host source). Nevertheless, translational operator sequences are involved in the target RNA sequences to improve specificity of packaging.

RNA binding properties of the coat protein from bacteriophage GA was investigated by Uhlenbeck with co-workers (Gott *et al*., 1991). Filter binding assays *in vitro* showed that despite 46 of 129 amino acid differences between GA (serological group II) and R17 (serological group I) coat proteins, the binding sites are fairly similar and GA coat protein binds RNA with „considerable specificity".

We have shown previously that the operator had rather small effect on the specificity of capsid contents for recombinant capsids, which are produced with and without GA-operator sequences in mRNAs (Strods *et al*., 2013), although many successful examples of use of MS2 operator have been elaborated (Pickett and Peabody, 1993; Pasloske *et al*., 1998; Legendre and Fastrez, 2005; Wei *et al*., 2008).

Altering RNA binding specificities of translational repressors and coat protein mutants that influence this binding were studied by Dr Peabody's group (Lim and Peabody,

1994; Lim *et al*., 1994). The first strategy of this study involves investigation of the affinity of the coat protein variants for RNA *in vitro*, and the second one includes measurements of translational repression *in vivo*. Taken together, the introduction of specific GA-like substitutions into MS2 coat protein sequence have been performed, which in one case resulted in six amino acid substitutions (positions 43, 55, 59, 83, 87, 89) that markedly influenced the RNA binding sites of MS2 and GA coat proteins (Lim *et al*., 1994). In the next series the role of two additional substitutions (positions 29 and 66) and deletion of FG-loop was established (Lim and Peabody, 1994). Three of the most important mutations, K43R, N55K and R83K, were called by authors as „super-repressor mutations", because they bound both operator RNAs more tightly than wild type protein (Lim *et al*., 1994). The main determinant of the difference in GA and MS2 specificity seems to be the N87S substitution (Lim *et al*., 1994). Other codon-directed mutagenesis experiments confirmed earlier results showing that the identity of Asn-87 was for specific binding of MS2 RNA and for discrimination of Qb RNA binding (Spingola and Peabody, 1997). Intensively were studied also crystal structures of complexes between recombinant MS2 capsids with mutations and RNA operator sequences (Valegård *et al*., 1997; Johansson *et al*., 1998; Helgstrand *et al*., 2002; Horn *et al*., 2004). The conversion of Asn-87 to Ala improved the ability of purine-RNAs (GA operator type) to bind MS2 coat protein, but binding to pyrimidine-RNAs (MS2 operator type) still remained tighter (Johansson *et al*., 1998). The authors also appointed the possible role of some other amino acids in the complex formation and particularly of Ser-52, Asn-55 and Lys-57. Lys-57 is present in all types of coat protein and therefore might not be responsible for specificity of interaction. Gly occurs in place of Ser-52 in GA, and Lys in place of Asn-55 in GA (Tars *et al*., 1997).

It is assumed that, in spite of large differences in binding affinities, the structures of the variant complexes are very similar to the wild-type operator complexes, and the interaction of wild-type MS2 operator with native MS2 coat protein seems to be if not the strongest one, but at least more functionally specific. Based on wild-type MS2 protein-MS2 operator interaction, two new techniques have been developed recently: MS2 tagging (Chubb *et al*., 2006) and tethering (Keryer-Bibens *et al*., 2008) techniques. MS2 tagging and tethering are based on the highly specific interaction of the MS2 bacteriophage coat protein with the stem-loop structure of the MS2 operator sequence. The mRNA packaging into VLPs in eukaryotes during growth has one very distinguishing advantage — it goes through 3'-end processing, which includes post-translational cleavage of mRNA precursor with subsequent 3'-end processing and polyadenylation (Colgan and Manley, 1997; Keller and Minvielle-Sebastia, 1997; Zhao *et al*., 1999; Dheur *et al*., 2005), becoming functionally matured. Most eukaryotic mRNAs, with a very few exceptions, acquire poly(A) tracts at their 3'-ends. Entity of the poly(A) tail in translation processes has high importance for mRNA function (Preiss and Hentze, 1999). A Poly(A) tail behaves as a stabilising factor and in the absence or removal of its, mRNA is rapidly decapped and degraded. If we have plans to package functionally active mRNAs into virus like particles for delivery and "work" in a certain kind of eukaryotic cells, we must pack functionally active mRNAs, and the direct packaging *in vivo* during VLP growth could solve this problem. It is known that in yeast poly(A) tails have average length of about 70 adenosine residues, whereas in other (higher) mammals such tails are synthesised to an average length up to 250 adenosine residues (Dheur *et al*., 2005).

It was very important for us to combine our rather good results in production of GA CP VLPs with more specific packaging of desired mRNAs *in vivo*. Therefore, our goal was directly opposite to the Peabody's work (Lim *et al*., 1994) — to introduce into GA coat protein amino acids that would make them like MS2 coat protein (mimic them as MS2) for strong and specific interaction with MS2 operator sequence joined to the packaging GFP mRNA. Single substitutions as well as combinations of these mutations in pairs (Table 1) were chosen. Even though our main task is to improve packaging, we need to determine under which promoter, GAL1 or GAL10, higher yield of VLPs is obtained. In addition, we wanted to show the first cases of mutated GA coat proteins expressed in yeasts and successfully formed capsids.

This study had several objectives. The first objective was to test GA capsid formation by expression of GA coat protein in yeast with three single amino acid mutations (amino acids 43, 55, 87) and with two double mutations (amino acids 43+87 or 55+87). Mutations for the introduction into GA coat protein sequence were chosen based on the analysis of already published data (Gott *et al*., 1991; Lim and Peabody, 1994; Lim *et al*., 1994; Ni *et al*., 1996). It was also planned

Table 1

CONSTRUCTIONS FOR TRANSCRIPTION AND EXPRESSION OF GA CP AND/OR GFP IN YEASTS

Introduced mutation(s)	Construction number	Construction	
		GAL1	GAL10
	pIC921	GFP	GA CP
	pIC984	GA CP	GFP
S87→N	*pIC994*	GFP	GA CP (S87→N)
	pIC995	GA CP (S87→N)	GFP
	pIC1067	GA CP (S87→N)	GFP + MS2op
K55→N	*pIC989*	GFP	GA CP (K55→N)
	pIC991	GA CP (K55→N)	GFP
R43→K	*pIC996*	GFP	GA CP (R43→K)
	pIC997	GA CP (R43→K)	GFP
S87→N; K55→N	*pIC904*	GFP	GA CP (S87→N; K55→N)
	pIC906	GA CP (S87→N; K55→N)	GFP
S87→N; R43→K	*pIC905*	GFP	GA CP (S87→N; R43→K)
	pIC907	GA CP (S87→N; R43→K)	GFP

to compare dependency of VLPs yield on the construction with a GA CP sequence under the promoter. The next task was to analyse the amount of packaged RNA in relation to the obstacle mentioned above. And finally, to ascertain wherever the introduction of the MS2 operator sequence into packaged GFP mRNA raised specificity of packaging, also resulting in the best quality particles for further investigation. This final task was solved to prognosticate the more important mutation of 87^{th} amino acid serine to asparagine.

MATERIALS AND METHODS

Plasmid constructions. All constructions were based on plasmid pESC-URA (Agilent Technologies) for protein expression in yeast. This plasmid contains two promoters (GAL1 and GAL10) for expression of GA coat protein — unmodified and modified — and for synthesis of mRNA. New constructions are based on plasmids pIC921 and pIC984 (Strods *et al*., 2013) by introducing a single amino acid change following standard site-directed mutagenesis protocol. PCR reaction mixture consisted from 2x PCR Master Mix (Thermo Scientific), pair of degenerate primers and template DNA as described in the manufacturer's protocol, and summarised in Table 2 — constructions pIC994, pIC995, pIC989, pIC991, pIC996, and pIC997 are based on either pIC921 or pIC984 and constructions pIC904, pIC906, pIC905, and pIC907 are based either on pIC994 or pIC995. In case of construction pIC1067, firstly, the vector was obtained by digestion of plasmid pIC995 with restriction endonucleases EcoRI and NotI and, secondly, the GFP sequence containing the fragment from plasmid pIC921 was cloned out by using primers pINC-525 (5'-TCG AAT TCC ATG GTG AGC AAG GGC GAG GA-3') and pINC-526 (5'-AAG CGG CCG CGA CAT GGG TAA TCC TCA TGT TTT GCT TAC TTG TAC AGC TCG TCC ATG CC-3') and digested with the abovementioned restriction endonucleases and thirdly, the vector and fragment were ligated together. All constructions were produced through transformation in *E. coli* competent cells and purification with a GeneJET Plasmid Miniprep Kit (Thermo Scientific), followed by sequence verification using sequencing method.

Expression of constructions. Yeast *Saccharomyces cerevisiae* strain YPH499 (ura3-*52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-Δ63 his3-Δ200 leu2-Δ1*, haplotype a) was transformed with all plasmid constructions according to the standard lithium acetate/polyethylene glycol procedure using a Sigma-Aldrich Yeast Transformation Kit according to the procedure described by Gietz *et al*., 1992. Transformants were selected and grown in uracil-free synthetic dextrose minimal medium (SDU-) for maximum protein expression as described previously by Strods *et al*., 2013.

Purification of VLPs. Yeast cells were suspended in working buffer (20 mM Tris-HCl, 5 mM EDTA, 0.65 M NaCl, pH7.8, supplemented with 0.03 mM PMSF) and subjected through French press (three strokes, 20 000 psi), after which cell lysate was stirred with equal volume of glass beads (30 sec stirring and 1 min rest on ice, repeated 15 times) and sonicated at 22 kHz (14 sec sonicate and 1 min rest on ice, repeated 15 times). Cell lysate was centrifuged (30 min at 12 000 rpm), debris was additionally washed with working buffer and volume of joined supernatants was reduced by dialysis against solution consisting from mixture of working buffer and glycerol (1:1 by volume). Afterwards, VLPs were subsequently purified through a Sepharose CL-4B gel filtration column (2 × 63 cm), eluting them with working buffer (at flow rate of 2 ml per hour). The next step was purification through a DEAE Sephadex A-50 column (1 × 5 cm) — samples were applied on column, then flow-through and additional washing with 3 ml of TEN buffer (20 mM Tris-HCl, 5 mM EDTA, 0.15 M NaCl, pH7.8) were combined in the dialysis tube and concentrated using dry Sephadex G-100 powder (GE Healthcare).

Products were purified by sucrose density gradient centrifugation, using a preformed stepwise sucrose gradient from 36% to 5% sucrose concentration in working buffer (total volume of each tube — 12 ml, centrifugation was performed at 25 000 rpm for 13 h in a Beckman Coulter Optima L-100XP ultracentrifuge (rotor SW32 Ti)). After piercing the bottom of the tube, fractions were collected (1 ml each) and those containing VLPs were joined together, dialysed against working buffer and, if necessary, concentrated using dry Sephadex G-100 powder.

VLPs preparations from constructions IC991, IC989, IC995, and IC996 were subjected to a two equal layer CsCl gradient, where the bottom 6 millilitre was from ready solution (44 g CsCl + 60 ml working buffer), and the upper 6 millilitre consisted of VLPs solution, 2.2 g of CsCl and working buffer. Ultracentrifugation and fractionation were performed similarly as with the sucrose gradient, with only the exception that 20 500 rpm for 13 h regime was used.

Quantification of VLPs was done by using spectrophotometric analysis. Based on Freivalds *et al*., 2008 measurements and personal observations it was estimated that OD_{260} = 5 corresponds to 1 mg of pure GA VLPs.

Extraction of VLPs inner content. To the solution of VLPs, a mixture of equal amounts of phenol and chloroform (1 : 1) was added, thoroughly vortexed and centrifuged (8000 rpm for 5 min). The upper (aqueous) phase was subtracted, washed triple with diethyl ether and nucleic acids were precipitated by adding ethanol. After centrifugation (13 000 rpm for 15 min), the debris was washed with 70% ethanol and dissolved in DEPC-water. Amount of RNA was estimated using a ND-1000 spectrophotometer (NanoDrop).

Purification of poly-A tail containing RNA. In order to measure mRNA content in whole RNA extract from VLPs, affinity chromatography using an oligo(dT)-cellulose (Sigma-Aldrich) column was used. Oligo(dT) cellulose was incubated with RNA in binding buffer (10 mM Tris-HCl, 1 mM EDTA, 400 mM NaCl, 0.1% SDS) at 65 °C 10 min, followed by washing with binding buffer, washing buffer

(10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 0.1% SDS) and finally eluted with elution buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS). Eluted fractions containing mRNA were combined and total amount of RNA was determined using optical density measurements.

Reverse transcription qPCR. Reverse transcription real-time PCR (RT-qPCR) was done in two steps. Firstly, cDNA with random hexamer or oligo(dT) primers were synthesised from equal amounts of each sample by using a First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's recommendations, using total RNA extracted from VLPs as a template. Secondly, qPCR reaction mixture for each sample was prepared in duplicate as follows — 6 µl Power SYBR Green PCR Master Mix (Life Technologies), 2 µl H_2O, 2 µl primer mix (2 pmol/µl), and 2 µl cDNA (diluted two-fold). qPCR reaction was done on a ViiA™ 7 Real-Time PCR System (Life Technologies) according to pre-set protocol for SYBR Green qPCR reaction. Primer sets for qPCR were as follows: coat protein primers — pARS-24 (5'-TTG GAT CCA CAA TGG CTA CTT TGA GAT CAT TTG TTT TGG T-3') and pINC-438 (see Table 2), GFP primers — pJAR-18 (5'-TCG AAT TCC ATG GTG AGC AAG GGC GAG GA-3') and pINC-446 (5'-CAC TGC ACG CCG TAG GAG AAG GTG GTC ACG AG-3') and MS2 operator primers — pINC-447 (5'-GAC CCC AAC GAG GCT GCT GAT CAC ATG GTC-3') and pINC-590 (5'-GCG ACA TGG GTA ATC CTC ATG-3'). Ct values were used to calculate $2^{deltaCt}$ to represent the amount of PCR product.

Table 2

SUMMARY OF CONSTRUCTIONS MADE WITH SITE-DIRECTED MUTAGENESIS METHOD

Construction	Primers	Template DNA
pIC994	pINC-439 (5'-TGG AAG GCA TAT GCT AAT ATT GAT TTG AC-3') and pINC-440 (5'-GTC AAA TCA ATA TTA GCA TAT GCC TTC CA-3')	pIC921
pIC995	pINC-439 and pINC-440	pIC984
pIC989	pINC-437 (5'-GCT TCT GGT GCT GAT AAT AGA AAA TAT AC-3) and pINC-438 (5'-GTA TAT TTT CTA TTA TCA GCA CCA GAA GC-3)	pIC921
pIC991	pINC-437 and pINC-438	pIC984
pIC996	pINC-451 (5'-CAA GCA TAT AAA GTT ACT GCT TC-3') and pINC-452 (5'-GAA GCA GTA ACT TTA TAT GCT TG-3')	pIC921
pIC997	pINC-451 and pINC-452	pIC984
pIC904	pINC-437 and pINC-438	pIC994
pIC906	pINC-437 and pINC-438	pIC995
pIC905	pINC-451 and pINC-452	pIC994
pIC907	pINC-451 and pINC-452	pIC995

RESULTS

Synthesis and purification of virus-like particles. Plasmids pIC921 and pIC984 were used for all constructions from pIC904 till pIC1067 (listed in Table 1). The plasmids were based on commercial plasmid pESC-URA (Stratagene) and contained bacteriophage GA coat protein sequence with optimised codons that were described previously (Strods *et al*., 2013). In the synthesis process (as described in Methods and Table 2), in total 11 constructions were generated, which included single-point mutations S87N, K55N, R43K and double-point mutations S87N + K55N and S87N + R43K.

Fig. 1 shows the construction of main interest — pIC1067, which is based on pIC984 and contains bacteriophage MS2 operator sequence added to the 3' end of the GFP gene in order to provide more specific packaging of GFP gene through binding of the operator's stem-loop structure (Fig. 2) with modified bacteriophage GA coat protein. After transformation and protein expression in yeast *S. cerevisiae*, all of the above mentioned constructions (with the exception of construction IC997) produced virus-like particles.

A typical example from the purification process is shown in the case of IC984 VLPs (Fig. 3), where cell lysate was ap-

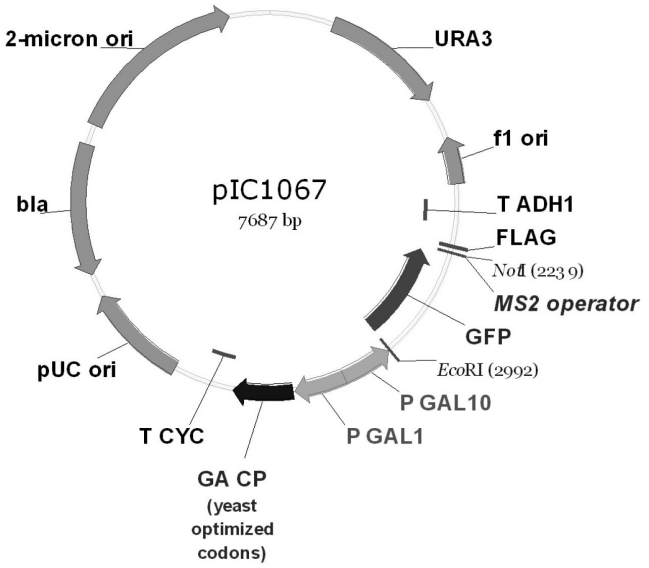

Fig. 1. Plasmid map of the construction pIC1067 (created with Vector NTI software package (Invitrogen)).

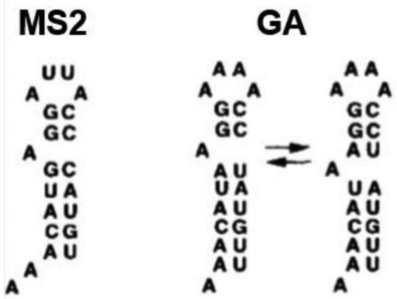

Fig. 2. Secondary structure of MS2 RNA operator stem-loop, compared with GA RNA operator stem-loop (Lim *et al*., 1994).

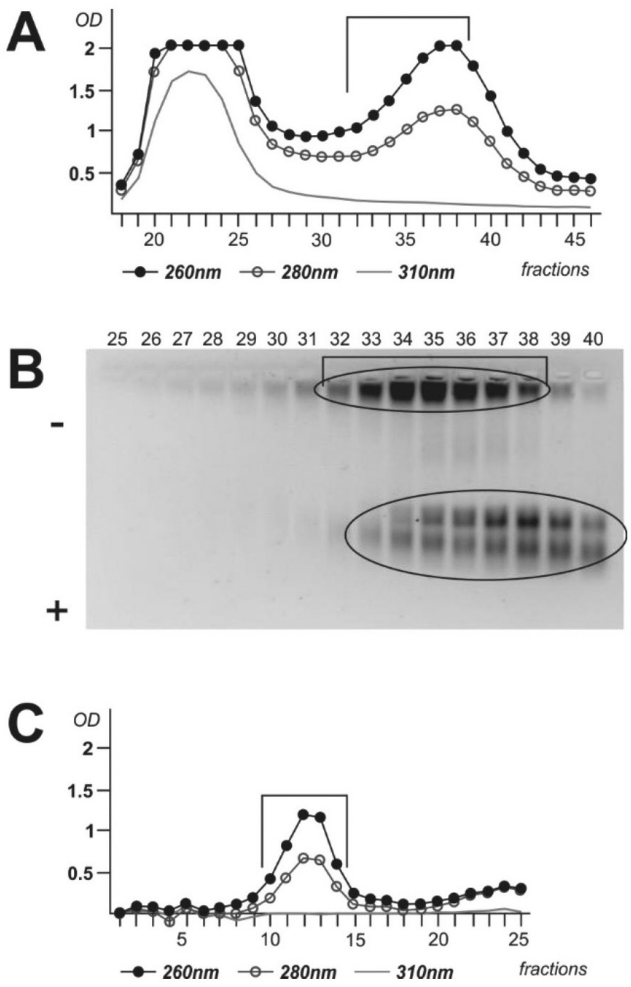

Fig. 3. Purification steps of IC984 VLPs: optical density (**A**) and native agarose gel electrophoresis (**B**) profile after column chromatography on Sepharose CL-4B; (**C**) optical density profile of sucrose gradient (fraction size 0.5 ml). Brackets show collected fractions, in agarose gel electrophoresis upper ellipse and lower ellipse show VLPs and nucleic acids, respectively.

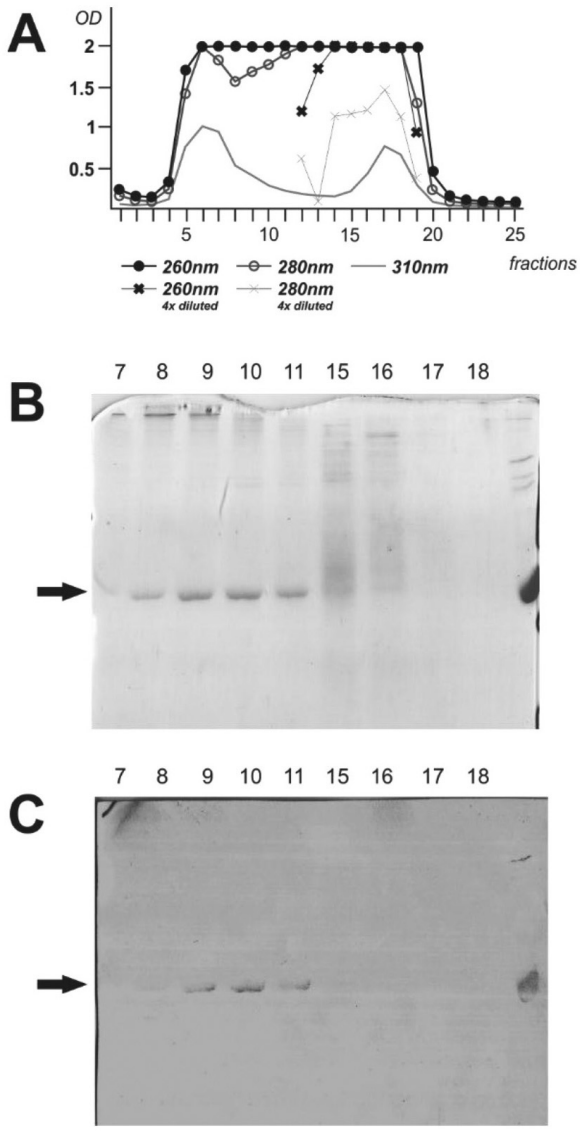

Fig. 4. Purification steps of IC989 VLPs: optical density (**A**), Coomassie stained SDS-PAGE (**B**) and Western blot (**C**) profile after column chromatography on Sepharose CL-4B. Arrows points to the coat protein monomer.

plied to a Sepharose CL-4B gel filtration column and optical density of the fractions measured at A_{260}, A_{280} and A_{310} (Fig. 3A). Fractions were also analysed with agarose gel electrophoresis (Fig. 3B) and due to the presence of nucleic acids a further purification on DEAE ion-exchange column was introduced. Due to the overall positive charge of GA VLPs, they were eluted as unbound material free from nucleic acid contamination. As a final step, additional purification of VLPs on a sucrose density gradient ultracentrifugation was done (Fig. 3C). Similarly, IC989 VLPs were purified using another Sepharose CL-4B column (Fig. 4A); Coomassie stained SDS-PAGE (Figure 4B) and Western blot (Fig. 4C) analysis were used to identify fractions that contain the coat protein. In a similar manner, VLPs from all constructions were obtained and the yields were calculated and is summarised in Table 3. The presence of VLPs was confirmed via electron microscopy, showing two variants as an example (Fig. 5).

As shown in Table 3, the best yield after VLPs purification was observed in the case of constructions where no modifications were made to coat protein, in constructions IC921 and IC984 respectively, and these data roughly corresponded to the yield of the VLPs formed from GA coat protein expressed from the native coat protein sequence (Freivalds *et al*., 2008). However, the yield was lower than in the case of construction A65 (Strods *et al*., 2013), which can probably be explained by additional stress for the yeast cells to transcribe and synthesise both coat protein and GFP gene. Comparing gene placements under promoters, the yield of the VLPs differed almost twice in favour for the coat protein placed under GAL1 promoter (i.e., construction IC984).

All other constructions bearing point-mutations in coat protein amino acid sequences showed noticeably lower yields of VLPs per gram of cell mass. Yields of VLPs according to the placement of the coat protein under GAL1 or GAL10 promoter were different and showed no obvious regularity.

Table 3

YIELD OF PURIFIED VLPS PER G OF CELLS AND YIELD OF TOTAL RNA AND mRNA EXTRACTED FROM PURIFIED VLPS

	Nr	Construction		yield, mg VLPs per gram cells	µg of RNA from 1 mg of VLPs		% of mRNA from total RNA
		GAL1	GAL10		total RNA	mRNA	
	IC921	GFP	GA CP	0.872	104.2	8.6	8.3%
	IC984	GA CP	GFP	1.525	168.6	29.8	17.7%
S87N	IC994	GFP	GA CP (S87→N)	0.184	32.4	2.4	7.4%
	IC995	GA CP (S87→N)	GFP	0.271	42	4.2	10.0%
	IC1067	GA CP (S87→N)	GFP + MS2op	0.943	104.4	14	13.4%
K55N	IC989	GFP	GA CP (K55→N)	0.312	56.2	12.6	22.4%
	IC991	GA CP (K55→N)	GFP	0.183	38.2	2.4	6.3%
R43K	IC996	GFP	GA CP (R43→K)	0.11	30.4	2.2	7.2%
	IC997	GA CP (R43→K)	GFP	-	165.2	0	-
S87N; K55N	IC904	GFP	GA CP (S87→N; K55→N)	0.163	16.2	2	12.3%
	IC906	GA CP (S87→N; K55→N)	GFP	0.24	12.4	2.6	21.0%
S87N; R43K	IC905	GFP	GA CP (S87→N; R43→K)	0.25	24.2	2.2	9.1%
	IC907	GA CP (S87→N; R43→K)	GFP	0.233	52	2.6	5.0%

Also, no coherencies between VLPs yields and different mutations — either single or double amino acid exchanges — were found, suggesting that our mutations introduced in different places of the coat protein sequence did not significantly interfere with the protein production or particle assembly process. The only exception in this series was one construction — IC1067 with mutation (S87→N) in coat protein and the MS2 operator sequence added to the GFP sequence. The construction without the MS2 operator sequence (IC995) showed almost four times less yield of VLPs, indicating the important role of the MS2 operator in the process of particle formation.

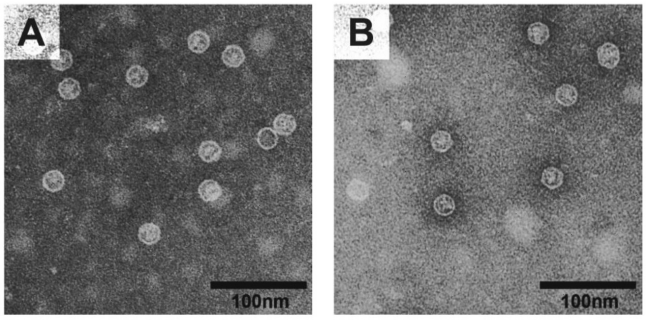

Fig. 5. Electron microscopy images with purified IC995 (**A**) and IC996 (**B**) VLPs.

Analysis of VLPs inner content. Comparing VLPs yield and RNA yield from purified VLPs (Table 3), direct correlation can be seen, suggesting an important role of RNA packaging ability and assembly of VLPs. The lowest RNA packaging capacity was observed in construction IC904 and IC906 bearing S87→N and K55→N double-mutation. Otherwise, no prevalence of RNA packaging level in comparison with the location of the coat protein under GAL1 or GAL10 promoter was found.

Constructions that each possess different point-mutation were compared in CsCl density gradient (Fig. 6), and also this method revealed divergent properties of those VLPs. VLPs with mutations S87N (IC995) and R43K (IC996) showed similar densities, but mutation K55N (constructions IC991 and IC989) bearing VLPs showed markedly lower density on CsCl density gradient. These disparities in VLPs densities can also explain differences in yield and inner filling of the VLPs.

To assess the amount of coding capable mRNAs, they were thoroughly separated from total RNA of all samples and analysed as shown for an example in Fig. 7). However, no

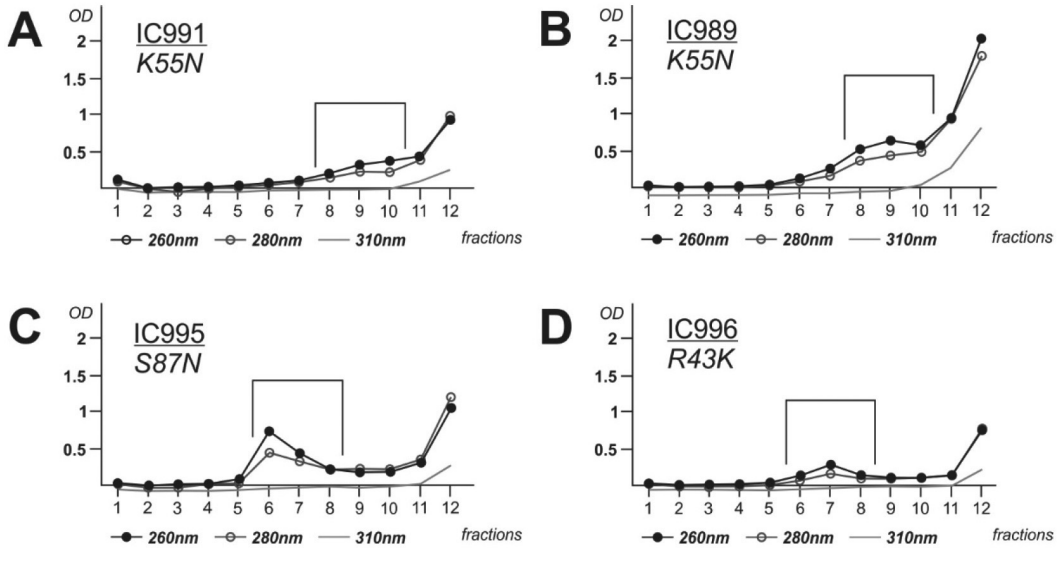

Fig. 6. Optical density profiles of CsCl density gradients for IC991 (**A**), IC995 (**B**), IC996 (**C**) and IC989 (**D**) VLPs. Brackets show collected fractions.

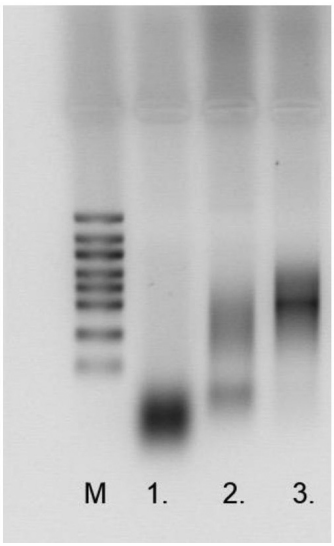

Fig. 7. Oligo(dT) cellulose affinity chromatography of the RNA, extracted from construction IC984 and analysed in a denaturing 1% FA agarose gel. Samples were: M – RiboRuler High Range RNA Ladder; 1. – total extracted RNA from VLPs; 2. – washing buffer fraction from oligo(dT) column; 3. – eluted mRNA from oligo(dT) column.

prevalence of mRNA packaging in the case of construction IC1067 was observed (Table 3). Despite the variation of mRNA content in different constructions, their average amount tentatively corresponded to the relative amount of mRNA from total RNA found in yeast cells (Table 3).

Total RNA from two constructions — IC984 containing GFP gene and IC1067 containing GFP gene supplemented with MS2 operator — were used as a template for quantitative PCR. cDNAs from the total RNA pool and mRNA pool were obtained using random hexamer and oligo(dT) primers, respectively. Despite the fact that extracted RNA does not contain any reference genes, we assumed that real time PCR data are comparable, since initial RNA was taken in equal amounts between both samples (Fig. 8). Real time PCR analysis with the GFP primer set revealed the presence of the GFP gene in both VLPs. However, when the GFP gene was prolonged with MS2 operator sequence (IC1067), the packaging level of GFP gene was at least one and a half times higher (Figure 8, random hexamer cDNA with GFP primers). Regarding the packaging level of the GFP specific mRNA, even greater differences were observed — at least three times higher (Figure 8, oligo(dT) cDNA with GFP primers). Using the MS2 operator specific primer set, the prolongation of GFP mRNA sequence with MS2 operator sequence was confirmed (Figure 8, MS2 operator primers).

DISCUSSION

Exploration of the above mentioned constructions was carried out to examine the possibility to form VLPs, the yield of VLPs and its dependency on coat protein placement under one of the two promoters, the RNA filling level and also the specificity of its packaging. Formation of VLPs was observed in all except one construction, and thus the objectives could be carried out. Our previous observations in the case of VLPs with unmodified coat protein showed overall better VLP yield in comparison with mutant constructions. However, in all mutations containing constructions, yields of the VLPs were lower independently from promoters. Our idea of duplicate constructions with opposite orientation of genes (regarding placement under GAL1 or GAL10 promoter) was to find the best combination for increasing VLPs production and for ensuring specific packaging. Minor variations in the yield of VLPs in mutant constructions were observed, but no clear preference in favour of coat protein alignment under one of the promoters was identified. The same was observed when total RNA was extracted and mRNA was purified, suggesting that gene placement under a promoter influenced the yield of VLPs and inner content, but it is not the only factor and for optimal results, and therefore different construction strategies should be used. When comparing the yield of VLPs and total RNA among the constructions, direct correlation was observed, allowing us to hypothesise that wholesome nucleic acid filling is necessary for optimal self-assembly of the capsids.

Observed higher yield of VLPs due to the coat protein placement under GAL1 promoter in S87N mutant encour-

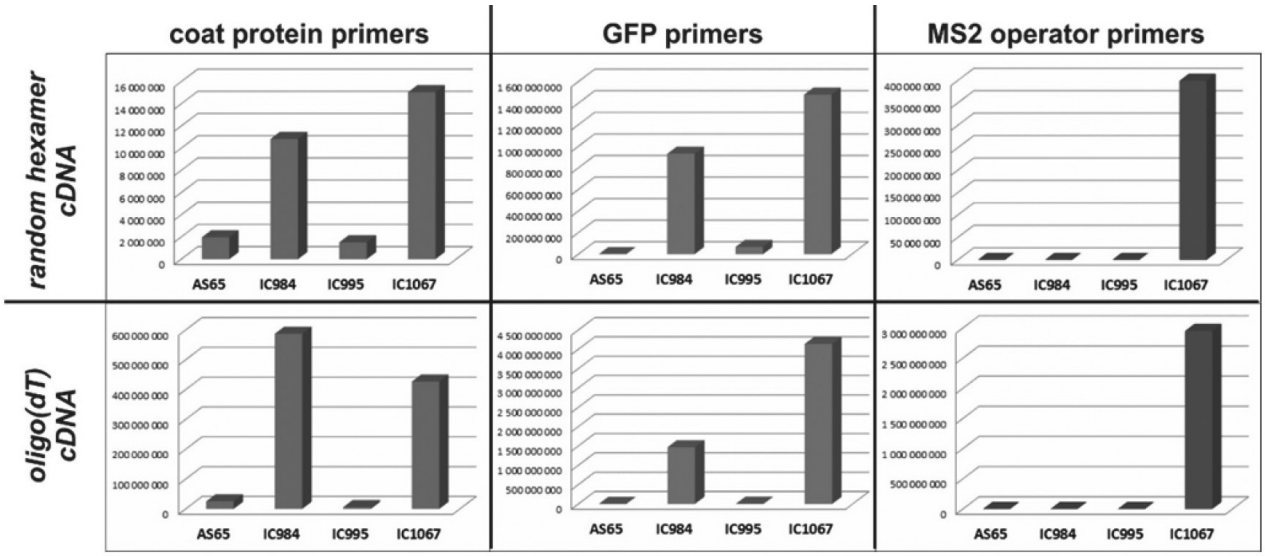

Fig. 8. Real-time qPCR, using appropriate (gene specific) primers and cDNA amplified with random hexamer or oligo(dT) primers from extracted VLPs total RNA as a template. Graphs show $2^{deltaCt}$ values.

aged us to add MS2 operator exactly to this construction. This resulted in the construction of our interest — IC1067, which was examined in more detail. Introduction of the point mutation S87→N in GA coat protein resulted in more specific interaction with the MS2 operator sequence, especially in comparison with the GA operator (interaction) with GA coat protein (Rūmnieks *et al*., 2008). Packaging of the mRNA of interest (in our case — GFP gene supplemented with MS2 operator) was also higher, proving our hypothesis that specific attachment of the MS2 operator sequence to the modified coat protein can provide specific packaging of RNA. These results are in accordance with previously studies (Lim *et al*., 1994) that found the main determinant for the specificity of MS2 operator binding to be S87N substitution in coat protein. More specific packaging of RNA is also strong enough to partially compensate negative effects of amino acid exchange in coat protein, therefore raising the overall VLPs production level. Nevertheless, the positive tendency for specific packaging is realised, and VLPs still contain a broad spectra of internal filling. Sample analysis with coat protein specific primers showed that full specificity of GFP packaging was not achieved and that VLPs contained significant amounts of coat protein gene mRNA (Fig. 8). Some previous packaging experiments on the basis of the GA operator indicated the same tendency; while even some specificity of the gene of interest was achieved, full exclusion of unnecessary amino acid sequences could not be done (Rūmnieks *et al*., 2008). Mimicking of GA virus like particles toward MS2-like particles gave relatively more specific packaging of the sequence of interest — it was found that GFP mRNA with MS2 operator (Fig. 8, oligo(dT) cDNA with GFP primers) was packaged approximately three times better than without the operator.

ACKNOWLEDGEMENTS

We thank Dr. Velta Ose for electron microscopy. This research was supported by the European Social Foundation (ESF), Project No. 2013/0002/1DP/1.1.1.2.0/13/APIA/VIAA/005.

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Received 11 June 2015
Accepted in the final form 26 May 2017

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mRNS *IN VIVO* IEPAKOŠANA RAUGOS PRODUCĒTĀS NO BAKTERIOFĀGA GA ATVASINĀTĀS VĪRUSIEM-LĪDZĪGĀS DAĻIŅĀS

Bakteriofāga GA apvalka proteīna veidotās, pašsavākties spējīgās vīrusiem-līdzīgās daļiņas (VLD) ir ekspresētas baktērijās un raugos. Uz iepriekšējo eksperimentu bāzes divus pretēji vērstus promoterus saturošā rauga vektora pESC-URA / *S.cerevisiae* sistēmā tika izveidotas jaunas konstrukcijas ar punktveida mutācijām apvalka proteīna gēnā, lai iegūtu MS2 fāgam līdzīgu RNS saistību. Vienlaicīgi pie iepakojamā mRNS tika pievienota MS2 operatora sekvence. Pēc punktveida (S87N, K55N, R43K) un dubultmutācijām (S87N + K55N un S87N + R43K) apvalka proteīns saglabāja spēju veidot VLD, lai arī ar mazāku iznākumu no šūnām. Serīna nomaiņa uz asparagīnu 87. apvalka proteīna sekvences pozīcijā kombinācijā ar bakteriofāga MS2 translācijas operatoru nodrošināja interesējošā gēna (GFP) specifisku iepakošanos. Lai arī tika iepakotas nespecifiskas nukleīnskābju sekvences, interesējošā gēna ievērojamu specifisku iepakošanos var panākt, izmantojot iepriekšminēto pieeju.