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Madhusudhan R
Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India

Neha Guleria
Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India

Satish Kumar K
Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India

Ramanjini Gowda PH
Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India

Correspondence
Neha Guleria
Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India

Effect of gene copy number, methanol and time period on production of CVS rabies glycoprotein expressed in *Pichia pastoris*

Madhusudhan R, Neha Guleria, Satish Kumar K and Ramanjini Gowda PH

Abstract
Rabies is a viral disease that causes acute encephalitis in warm blooded animals. Rabies is almost invariably fatal if post-exposure prophylaxis is not administered prior to the onset of severe symptoms. Glycoprotein is the major surface protein of rabies virus, responsible for the production of neutralizing antibodies. Currently, available mammalian cell culture based rabies vaccine is highly expensive because of cell line maintenance and downstream processes. Large quantity of this vaccine is required to meet the clinical requirements at economical level. Therefore, the present investigation was conducted to study the effect of multicopy gene insertion, methanol concentration and incubation time interval on the protein expression of challenge virus standard (CVS) rabies glycoprotein (RGP) in *Pichia pastoris*. The optimal methanol concentration and incubation time for induced CVS rabies glycoprotein were 1 % (w/v) and 96 h respectively. Clone 13 (single copy number) produced 0.4g/l whereas Clone 14 (5 copies) produced 1.5 g/L of recombinant protein. The clone with five copy number expressed CVS_RGP at a higher level than single copy number clone. Therefore, the gene copy number is one of the major factor involved in heterologous protein expression in *P. pastoris*.

Keywords: Gene copy number, *Pichia pastoris*, Rabies glycoprotein, Western blot

1. Introduction
Rabies is one of the oldest zoonotic diseases known to man, but its successful control has remained elusive. The rhabdovirus causes acute encephalitis in warm-blooded animals. The World Health Organization (WHO) estimates 55,000 human deaths due to rabies every year [1]. The worldwide incidence of rabies and the inability of currently used vaccination strategies to provide highly potent and cost-effective therapy indicate the need for an improved rabies vaccine. Glycoprotein (G) is the major surface protein of rabies virus, responsible for the production of neutralizing antibodies and hence, the subunit vaccines that contain G could provide complete protection against RV challenge [2]. One of the most important elements in the effective control of rabies is through the use of efficacious vaccines. *Pichia pastoris*, an eukaryotic methylotrophic yeast, has emerged as one of the powerful host systems for the production of biopharmaceuticals. It has many advantages as an expression system including a tightly controlled and strong AOX1 promoter, a cell capable of post-translational processes, the ability to grow to high cell densities and high expression rates [3]. There are many factors which influence the expression yield of foreign proteins in the multistep, tightly regulated protein biosynthesis pathway of yeast. Generally, a gene copy number have been identified as one of the most important factors influencing the heterologous protein expression in *Pichia pastoris* [4]. However, the number of integrated expression cassettes does not always correlate with the yield of recombinant proteins, particularly in the case of secreted proteins [5]. Earlier we have reported recombinant and stable expression of *CVS_RGP* in pPICZαA *P. pastoris* vector at single copy insertion [6]. Hence, in the present study, we report the multimerization and molecular characterization of *CVS_RGP* produced in *P. pastoris*.

2. Materials and Methods
Construction of recombinant *P. pastoris* expression plasmid (pPIC9K CVS_RGP)
The *P. pastoris* strain GS115 cells and plasmid pPIC9Kwas used to construct the multicopy *CVS_RGP* expression vector The *CVS_RGP* gene was amplified using Forward primer sequence with *Eco*RI site GCAGCAGAATTCATGGTTCCTCAGCCTGCTCTCCT and Reverse primer sequence with *Not*I site TAATGCGGCCGCCAGTCTGGTCTGACCC CCACCACT. The PCR production was digested and inserted at *Eco*RI and *Not*I sites in the

pPIC9K vector (Invitrogen) (Fig 1). The constructed expression plasmid *(pPIC9K CVS_RGP*) was confirmed by PCR and sequencing. All cloning steps were performed in *E. coli* DH5α.

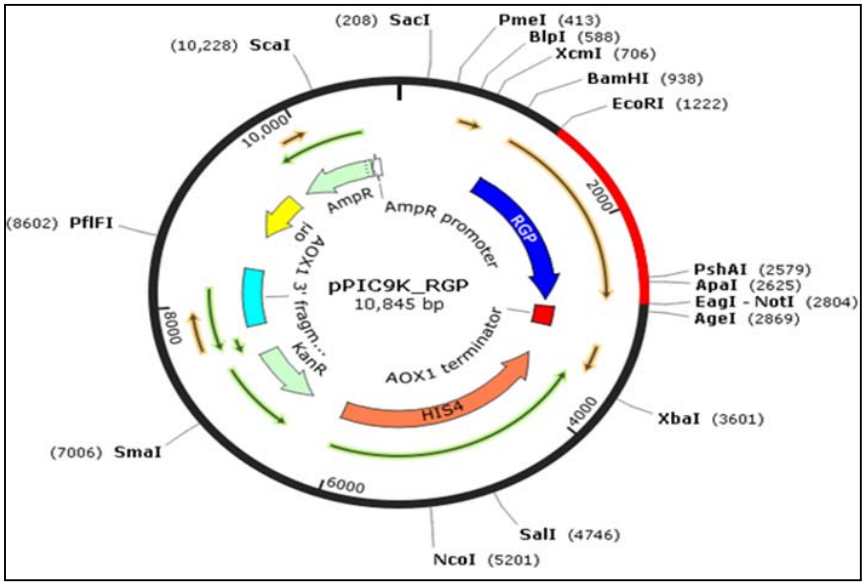


Fig 1: Construct map of pPIC9K*CVS_RGP* containing full length *CVS rabies glycoprotein* gene

P. pastoris Transformation

The plasmid pPIC9K containing *CVS_RGP* gene was linearized by digesting with *Sac I* enzyme and transformed into *P. pastoris* GS115 cells by electroporation. Briefly, 80 µl of electro competent GS115 cells were mixed with 5 µg of linearized pPIC9K *CVS_RGP* expression plasmid in a 0.2-cm cuvette and pushed on the GenePulser (Biorad). The push parameters were 7.5 KV/cm, 50 µF, and 400Ω. Initially, cells were plated on YPDSA plates (1% Yeast extract, 2% Peptone, 2% Dextrose, 1M Sorbitol and 2% Agar) with 0.25 mg/mL concentration of geneticin and incubated at 30°C for 3 days until colonies appeared. The pPIC9K without insert, linearized with *SacI* was also transformed as a negative control. The colonies obtained were restreaked on fresh YPDSA plates.

Screening of Transformants using PCR

Transformants containing *CVS_RGP* gene were detected by a genomic PCR using the gene-specific primers. *CVS_RGP* gene was amplified using Forward primer sequence with *Eco*RI site GCAGCA**GAATTC**ATGGTTCCTCAGCCTGCT CTCCT and Reverse primer sequence with *Not*I site TAAT**GCGGCCGC**CAGTCTGGTCTGACCCCCACCACT . PCR was carried out under the following conditions: 98 °C for 15 s, 60 °C for 1 min, 72 °C for 1 min, for 35 cycles, and finally 72 °C for 10 min.

Screening of Multicopy transformants of *P. pastoris*

In vivo screening

After transformation, simultaneously cells were plated on YPDSA plates with 0.25, 0.5, 1, 2 and 3 mg/mL concentration of geneticin and incubated at 30°C for 3 days until colonies appeared. The different concentrations of geneticin were used to select for clones with high resistance level. The colonies obtained were restreaked on fresh YPDSA plates. The colonies appeared within 3 days after the second plating event were considered to be true positives.

Real-Time (RT) PCR

The *CVS_RGP* gene insertion was analyzed by RT-PCR for the genomic DNA of *P. pastoris* clones screened on geneticin. The RT-PCR was carried out for colonies grown on a higher concentration of geneticin to know the copy number by considering 0.25 mg/mL geneticin-resistant colony. The SYBR Green was used for Real-Time PCR. The forward primer sequence 5'-GCATTTCCGCCCAACACCAGATG-3' reverse primer sequence 5'-CTCGAGTGAAGGGATCT GTC-3'was specifically designed for 200 bp amplicon size which could suit for RT-PCR chemistry. The genomic DNA (100 ng) of recombinant *P. pastoris* clones screened *in vivo* were used as template in RT-PCR. Each reaction was kept in triplicates along with No Template Control (NTC). It was carried out in 2 step reaction under following conditions 95 ºC for 1 min, 55 ºC for 30 s, for 40 cycles.

Production of Recombinant Rabies Glycoprotein

Induction of protein expression was done by standard procedure with modification. The positive recombinant clones were maintained in YPD medium. A single colony was inoculated into 25 ml of BMGY medium (1M phosphate buffer, 10X yeast nitrogen base, 10X glycerol, 500 X Biotin and 100 mg/L geneticin) and incubated at 30 °C overnight at 250 rpm. The growth was allowed until OD_{600} (2-6). The cells were harvested by centrifugation at 3000 rpm for 5 min at RT. The pellet was then resuspended in BMMY medium. The induction started by adding methanol once in 24 h. Different concentrations of methanol was used (0.5 %, 1 % and 1.5 %). Incubation was continued for additional 72 h, 96 h and 120 h at 30 °C. The supernatant was harvested at every interval with different concentrations of methanol. Each expression experiment had 5 replications.

Purification of Rabies Virus Glycoprotein in *P. pastoris*

The CVS_RGP produced by *P. pastoris* clones was precipitated with ammonium sulfate. The polyhistidine containing recombinant protein was purified from the secreted media by affinity column containing the ProBond™ resin. The final concentration of the protein in the purified solution was estimated by using Bradford's method.

SDS-PAGE and Western blot analysis

Protein expressed by *P. pastoris* clones carrying rabies virus glycoprotein gene was detected by SDS-PAGE using discontinuous buffer system followed by Western blot. The protein produced by clone without the insert in the expression cassette was used as a negative control.

3. Results and Discussion

Cloning and generation of recombinant *P. pastoris* clones (pPIC9K *CVS_RGP*)

Before transformation into *P. pastoris* x-33 cells, the recombinant clones (pPIC9K *CVS_RGP*) was sequenced using *CVS_RGP* gene-specific primers by employing primer walking technique. The nucleotide sequences were analyzed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. Sequenced insert had 100 % similarity with reported Glycoprotein of CVS rabies virus. The linearized recombinant construct was inserted at *AOXI* region in *P. pastoris* GS115 strain to create Mut^{+} recombinants which can express the full-length *CVS_RGP* gene when induced with methanol. Colonies appeared on the plate in which *P. pastoris* transformed with pPIC9K *CVS_RGP* whereas there were no colonies with untransformed *P. pastoris*.

Confirmation of gene integration into *P. pastoris* genome by PCR

The PCR analysis showed a single and intense DNA band of size 1,575 bp in case of recombinant colonies carrying full-length *CVS_RGP* gene, indicating the presence of *CVS_RGP* gene insert in *P. pastoris* genome. While there was no amplification in *P. pastoris* clone transformed with vector without insert (Fig 2). Amplification of the *CVS_RGP* gene in the *P. pastoris* recombinant clones confirmed the integration of *CVS_RGP* gene. When a gene is transformed into plant cells/organisms, PCR is a quick and simple technique for amplification of the gene so transformed.

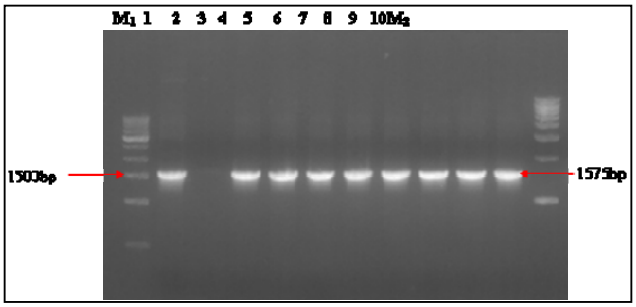


Fig 2: PCR confirmation of the transformed *P.pastoris* GS115 clones with *CVS_RGP* gene specific primers: M_1 - 500bp ladder; M_2 – 1kb ladder; lane 1 – pPIC9K *CVS_RGP* construct (Positive control); lane 2 – *P. pastoris* GS115 clone with pPIC9K (Negative control); lanes 3 to 10 – *P. pastoris* GS115 clones transformed with pPIC9K *CVS_RGP*

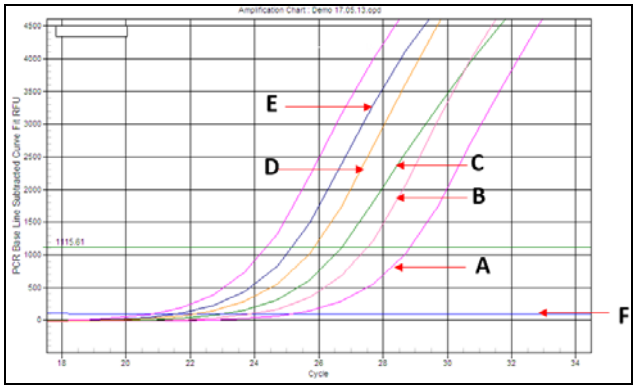
Screening and confirmation of multicopy gene integration

***In vivo* screening of multiple inserts**

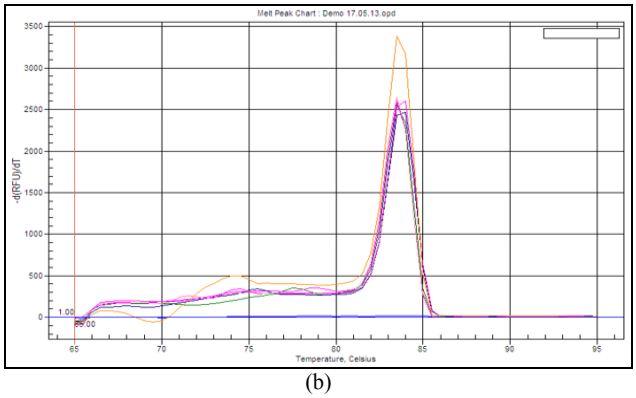
A single copy of pPIC9K integrated into the *P. pastoris* genome confers resistance to geneticin to a level of ~ 0.25 mg/mL. Multiple integrated copies of pPIC9K can increase the genetic resistance level from 0.5 mg/mL (1-2 copies) up to 3 mg/mL (7-12 copies). The presence of the *kanamycin* gene on pPIC9K has been used as a tool to detect pPIC9K transformants that harbor multiple copies of the gene. Thus, the ability of the transformants to tolerate increasing concentrations should correlate with enhanced copy number. Our results go in line with the study done by Inan *et al.*, (2006) [7]. who screened *P. pastoris* clones on a high concentration of geneticin had high gene copy number and produced more amount of recombinant protein compared to clones having less copy number which were screened on a low concentration of geneticin.

Confirmation of multicopy gene integration by Real-Time PCR

The RT-PCR was carried out for colonies grown on a higher concentration of geneticin to know the copy number by comparing with 0.25 mg/mL geneticin-resistant clone (Fig 3a). Clones screened on 3 mg/mL of geneticin reached threshold Cycle (Ct) values earlier than clones screened on 0.25 mg/mL of geneticin. Clone 13, 6, 16, 10 and 14 had a Ct value 28.42, 26.92, 26.45, 26.48 and 25.15 respectively. Further details are shown in Fig 5a. Hence, the threshold cycle is inversely proportional to the original relative expression level of the gene of interest. The melting curve of the RT- PCR reaction was also recorded to confirm the specific amplification of the PCR product. A single peak in the melt curve analysis graph indicated specific amplification of the PCR product, ruling out nonspecific amplification (Fig 3b). Lin-Cereghino *et al.*, (2008) [8] performed RT-PCR and Southern blotting to confirm the presence of multi copies of *CVS_RGP* gene.



(a)



(b)

Fig 3: a. Confirmation of multicopy gene integration by Real-Time PCR analysis for *Pichia* clones screened on increasing concentration of geneticin**. A** – GS115 clone grown on 0.25 mg/ml of geneticin (Clone 13)**; B** - GS115 clone grown on 0.50mg/ml of geneticin (Clone 6)**; C** - GS115 clone grown on 1.00mg/ml of geneticin (Clone 16)**; D** - GS115 clone grown on 2 mg/ml of geneticin (Clone 10)**; E** - GS115 clone grown on 3 mg/ml of geneticin (Clone 14)**; F** – No Template Control (NTC)**. b.** Melt curve analysis graph of Real-Time PCR for the PCR amplification done for *P. pastoris* clones having multicopy *RGP* inserts

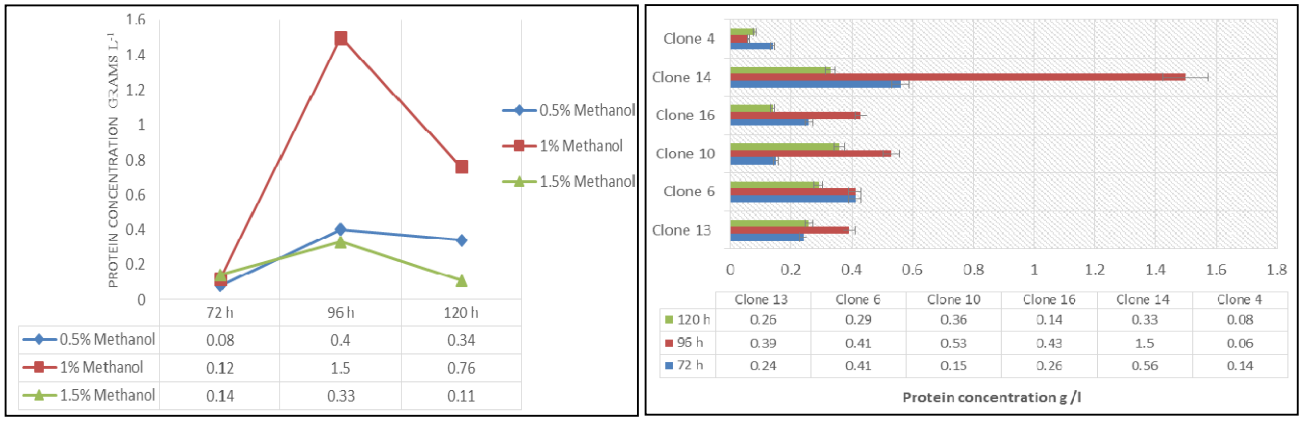
Effect of methanol and time interval on protein expression

The methylotrophic *P. pastoris* use methanol the sole carbon source. Methanol gets oxidized into formaldehyde and hydrogen peroxide using molecular oxygen by the enzyme alcohol oxidase. To avoid the toxic effect of hydrogen peroxide, the methanol metabolism takes place in within specialized cell organelle, peroxisome which sequesters toxic by-products away from the rest of the cell. In the present study, the *CVS_RGP* gene was cloned under the control of alcohol oxidase 1 (AOX1) promoter. This promoter is strongly regulated and induced by methanol. Therefore, the optimized concentration of methanol for the induction plays a major role to have a balance between slightest toxic effect and highest protein expression. When the induction was done at 0.5 % of methanol the protein expression was found to be 0.34 g/L as compared to the 1% methanol 1.5 g/L (Fig 4a). This may be due to the fact that low concentration of methanol is not enough for the initiation of transcription. At a concentration of 1.5 % of methanol, the protein expression level was decreased which shows the toxic effect of methanol on the cells. Santoso *et al.,* (2012) [9] showed that at 0.5 % methanol concentration the expression was low and as concentration was increased expression level was increased. In rather other studies, expression of SAG2 [10] and GST-CB [11] in *P. pastoris* reported that the 0.5-1 % and 1 % of methanol induction gave optimal expression result respectively.

The higher expression level was observed when induction time period was 96 h at 1% of methanol, a further increase in time period resulted in decrease in the protein level. The longer the incubation time the higher the number of the cells until it peaked at 96 h incubation time. Wang *et al.,* (2008) [12] showed that there was a progressive expression of the recombinant protein of Zbtb7A from 24 h up to 96 h while the expression level presented no significant increase after the time point of 96 h. Our result correlates with this study in which *CVS_RGP* expression also reached maximum at 96 h and started decreasing thereafter (Figure 4a). The longer incubation period possibly leads to the proteolytic lysis. Therefore, long incubation time will eventually result in complete degradation of the protein. The studies had found that recombinant proteins secreted by *P. pastoris* are often subjected to proteolytic degradation in the high cell density environment of bioreactor cultivation [13]. Intracellular vacuolar proteases from dead lysed cells are believed to be the major source of proteolytic activity in the culture medium because *P.pastoris* secretes small amounts of endogenous proteins. Hence, controlling cell viability is important for controlling proteolytic degradation, most likely by increasing the viability of cells and reducing the activity of the proteases [9].

Effect of copy number and time interval on expression of rabies virus glycoprotein

The generation of multicopy expression strains is a technique routinely used to increase the yield of recombinant proteins in *P. pastoris.* All the 6 clones screened at the different concentration of geneticin were induced by 1 % methanol at 72, 96 and 120 h at 30 ºC. Clone 14 screened on 3 mg/ml and clone 13 screened on 0.25 mg/mL produced 0.4 g/L of protein at 96 h respectively (Fig 4b). Thus, the increase in copy number enhance the *CVS_RGP* expression level, indicating the direct co-relation between multicopy and enhance protein expression level. The multiple insertions of a gene would result in the higher production of heterologous mRNA which increases the expression levels of protein. Several studies have shown that this parameter is critical for heterologous protein expression in *P. pastoris* [14-18].



(a) (b)

Fig 4: a. Effect of different concentration of methanol and time interval on production of rabies glycoprotein (Clone 14 was used to optimize the methanol and time period of induction) **b.** Effect of multicopy gene insertion and time interval on production of rabies glycoprotein

Detection and Characterization of Expressed Proteins

The SDS-PAGE indicated that all the three clones (13, 10, 14) seen as a single band with an increase in the band intensity w.r.t. increasing gene copy number, the band size was found to be approximately 78 kDa which was not corresponding to Rabipur vaccine (66 kDa, positive control) (Fig 5a). In contrast, Sakamoto *et al.,* (1999) [19] has reported the CVS_RGP protein size of 66 kDa. Ben Azoun *et al.,* (2016) [20], also reported the 66 kDa band size of expressed RGP recombinant protein. The difference of increased size in our study was may be due to the addition of 6X His tag and alpha secretory signal, which adds approximately 12 kDa. Further, the band intensity of expressed CVS_RGP protein on western blot confirms the expression and specificity of protein (Fig 5b). A typical immunoblot relies upon protein samples containing target protein that can be detected by antibodies. Therefore, antibodies specific to rabies glycoprotein made a complex via specific binding of antibodies to proteins immobilized on a membrane and confirms the integrity of expressed protein.

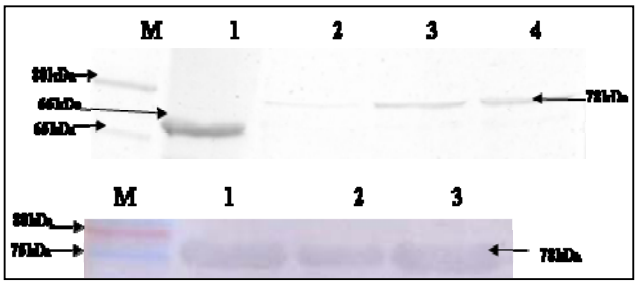


Fig 5: SDS PAGE and western blot analysis of purified rabies glycoprotein protein of *P. pastoris* clones **a.** M – Protein ladder, lane 1: Rabipur (Positive control), lane 2,3 and 4 – purified rabies glycoprotein produced by clone 13, 10 and 14 respectively screened on increasing concentration of geneticin **b.** The purified rabies glycoprotein protein was analysed on western blot. lane 1-3: purified rabies glycoprotein produced by clone13, 14, and 10

4. Conclusion

The observations, therefore, of the present study suggested that the multicopy gene number, optimization of methanol concentration and induction time interval contribute to the higher recombinant rabies glycoprotein expression level. The expressed protein found to be biologically active when characterized using western blot. Therefore, the pPIC9K with rabies glycoprotein recombinant vector is suitable for the multicopy expression of the target protein and can be used for further clinical studies for the development of vaccine and also for the diagnostic kit.

5. Acknowledgements

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