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ГЕНОМИКА.
ТРАНСКРИПТОМИКА

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EXPRESSION OF ANTI-NEUROEXCITATION PEPTIDE III OF SCORPION *Buthus martensii* Karsch BmK ANEP III IN PLANTS

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Anti-neuroexcitation peptide III of *Buthus martensii* Karsch (BmK ANEP III) has better anti-epileptic and anticonvulsive effects in the test animal models. The present study is aimed at developing transgenic tomato and tobacco lines overproducing the ANEP III protein. Using the molecular cloning technique, the plant expression vector pBI-*ANEP III* was constructed successfully. The *ANEP III* expression cassette included a double CaMV 35S promoter with Ω enhancers, the *ANEP III* gene with the Kozak sequence, the ER retention signal and the NOS terminator. Recombinant plasmids were transferred into *Agrobacterium tumefaciens* EHA105 by freeze–thaw transformation methods. By the *Agrobacterium*-mediated leaf disc transformation method, tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicum esculentum*) lines were transformed. Transformants were screened and confirmed by PCR, RT-PCR and western blotting analysis. It was demonstrated that the *ANEP III* gene was successfully expressed in the genomic DNA of transgenic plants. The ANEP III protein was detected by immunofluorescence analysis, and the results confirmed the high amount of ANEP III protein, being 0.81 and 1.08% of total soluble proteins in transgenic tobacco and tomato. The study of plants with high expression levels of ANEP III has an important theoretical and practical significance and provides valuable information for establishing a new, economical and effective system for industrial protein production.

Keywords: **anti-neuroexcitation peptide, *Agrobacterium tumefaciens*, *Buthus martensii* Karsch, ER retention signal, plant expression vector, plant transformation.**

Scorpions are used in traditional Chinese medicine for thousands of years [1]. And the neurotoxins of *Buthus martensii* Karsch have been widely studied in recent years [2–6]. Anti-neuroexcitation peptide III (ANEP III) [7] was puried from the venom of *B. martensii* Karsch (BmK), which is widely distributed in China. Previous studies suggested that ANEP III, a depressant insect-selective toxin, has good anti-epileptic and anticonvulsive effects in animal models *in vivo* and *in vitro* [8].The results of conventional pharmacological and acute toxicity tests also indicated that this polypeptide could be the first peptide used in the prophylactic-therapeutic pharmaceutics for brain neuroexcitopathy [9].

In previous reports, recombinant *ANEP III* was expressed in an *Escherichia coli* expression system. However, the protein expressed by prokaryotic organisms usually exists as an inclusion body and shows relatively low activity [7]. Using transgenic plants to express recombinant proteins is an emerging strategy for obtaining natural products with correct three-dimensional structure. However, one factor that has limited the development of this technology is the relatively modest expression levels of some recombinant proteins in plant tissues. In a former study, a transgenic plant expression system was used to obtain a complete modified and soluble ANEP III protein by a plant expression vector in *Agrobacterium* LBA4404, but the ANEP III protein concentration in transgenic plants was low and could not be detected by ELISA analysis [10]. Therefore, in this study, we have developed a new transgenic plant expression system to stably and economically attain the high expression levels of ANEP III.

EXPERIMENTAL

Construction of the plant transformation vectors. According to the full sequence of the *ANEP III* gene in the GenBank (Acc. no. AF242737.1), the DNA sequence encoding *ANEP III* was modified by C-terminus extension with the hexapeptide ER retrieval signal, SEKDEL [11]. The coding sequences were amplified with the forward primer F1 (5'-CGGGATCCGCCACCATGGATGGATATATAAGAGGAAGTAACGGATG-3'), which added a BamHI site, Kozak sequence [12] and reverse primer R1 (5'-ACGCGTCGACTTATAGCTCATCTTTCTCAGACTTTTTGCCACCGCATGTATTACTT-3'), which added a SalI site and a SEKDEL sequence. The PCR product was

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cloned into a pMD19-T vector and analyzed by sequencing. The recombinant plasmid was named as pT-*ANEP III*. The expression cassettes from BamHI to SalI in pT-*ANEP III* were ligated and subcloned into the binary vector pBin438, and the recombinant plasmid was named as pBin-*ANEP III*. The expression cassette for the *ANEP III* gene along with the CaMV DE35S promoter and the Nos terminator was excised from pBin-*ANEP III* using HindIII/XbaI and subcloned into the plant binary vector pBI121-Gusint-D. The resulting vector was designated as pBI-*ANEP III*. The exogenous *ANEP III* gene has been integrated into pBI-*ANEP III* and the analysis of double-strand DNA sequencing showed that the sequences were right.

Plant transformation. Plant expression vectors were transformed into *Agrobacterium tumefaciens* EHA105 by the freeze-thaw transformation method [13]. The *A. tumefaciens* EHA105 containing Ti plasmid was cultivated for 48 h, and then mixed with 20 mmol/L $CaCl_2$ and the recombinant pBI-*ANEP III* plasmid. Chilled in liquid nitrogen for 10 s, and then placed in 37°C for 90 s. Then chilled on ice for 2 min. The *Agrobacterium* clone containing the plant expression construct was confirmed by PCR and propagated in YEB resistance medium containing 100 mg/L rifampicin, 50 mg/L kanamycin at 28°C and 250 rpm, until OD_{600} of 0.5–0.6, and then the culture was used to infect tobacco and tomato explants. *Agrobacterium*-mediated transformation protocols of tobacco and tomato were derived from methods described by Linlin Lai [14].

Detection of transgene in transformed plants. PCR and RT-PCR analysis of transgenic tobacco and tomato plants. Genomic DNA was isolated from about 0.5 g of fresh leaves of the transgenic tomato and tobacco plants using the CTAB-method [13]. The transgenic plants were detected by amplifying the *ANEP III* gene sequence by PCR using the *ANEP III* forward primer F1, reverse primer R1, and plant genomic DNA as a template. Reaction was performed according to the following protocol: incubation for 5 min at 94°C, then 24 cycles of incubation at 94°C for 20 s, followed by 50°C for 30 s and 72°C for 40 s per cycle, after this a 10 min final step at 72°C.

RNA was extracted from about 0.1 g of fresh leaves of transgenic tomato and tobacco plants using the TRNzol Reagent ("TIANGEN BIOTECH", Beijing) according to the manufacturer's protocols. RNA was detected by electrophoresis in a 1% agarose gel and quantitated by UV spectrophotometry. Total RNA and $Oligo(dT)_{18}$, as a primer, were mixed at 70°C and incubated for 5 min, then chilled on ice, followed by addition of the reaction buffer, dNTP and Ribonuclease Inhibitor to the mixture, and incubation at 37°C for 5 min. After adding the Mo-MuLV Reverse Transcriptase ("TAKARA") the mixture was incubated at 42°C for 60 min, and then the reaction was terminated by heating to 70°C for 10 min. Fraction of RT products was directly used for PCR with gene-specific primers.

Detection of target proteins in transgenic tomato and tobacco. Transgenic tomato and tobacco leaves were screened for the presence of the ANEP III protein by western blotting. The total protein was extracted with the improved acetone sedimentation method [15] from 0.5 g of fresh or frozen transgenic plant leaves. The contents of soluble protein samples were detected by the UV absorption method. The standard recombinant ANEP III protein was purified from the soluble prokaryotic expression products of the engineered *E. coli* which contained the target gene. Loading: 1 μg standard protein as a positive control, 30 μl protein extract of non-transgenic tomato or tobacco plants as a negative control and 30 μl protein extract of transgenic tomato or tobacco plants separated by 15% SDS-PAGE. Subsequent transfer to a NC membrane at 9 V for 1 h by the semi-dry electrophoretic transfer method. The membrane was blocked for 1.5 h with 5% BSA in TBST, and then incubated with rabbit anti-ANEP III polyclonal antibody (1 : 100 in TBST) at 4°C overnight. After washing, the membrane was incubated with alkaline phosphatase (AKP) conjugated goat anti-rabbit IgG (1 : 1.000 in TBST) for 1.5 h. After washing, BCIP/NBT was added as a substrate. When protein bands were exposed by the coloration reaction, sterile water was used for washing.

The standard ANEP III protein was diluted to 0, 0.1, 0.125, 0.4, 0.8 and 1 μg/ml, and the soluble protein samples were diluted with the coating buffer. 100 μl of solution were used to coat each well of the 96-well microtiter plate, and the plates were incubated at 4°C overnight. The plates were washed three times with PBST (PBS plus 0.1% Tween-20). The background was blocked with 1% (w/v) BSA solution in PBS at 37°C for 2 h, and then the plates were washed three times with PBST. The plates were then incubated with rabbit anti-ANEP III polyclonal antibody that was diluted 1 : 100 in 0.01 M PBS containing 0.5% BSA for 2 h at 37°C, following by three washes with the PBST buffer. Secondary labeling was done using fluorescein-conjugated goat anti-rabbit IgG ("Santa Cruz Biotechnology") with 1 : 200 dilution in 0.01 M PBS containing 0.5% BSA for 2 h at 37°C, followed by three washes with the PBST buffer. After washing with the PBST buffer, the plates were read at 485/535 nm using the enzyme-labeling instrument (VICTOR 1420), and the amount of plant-expressed ANEP III was estimated basing on the known amount of standard purified ANEP III.

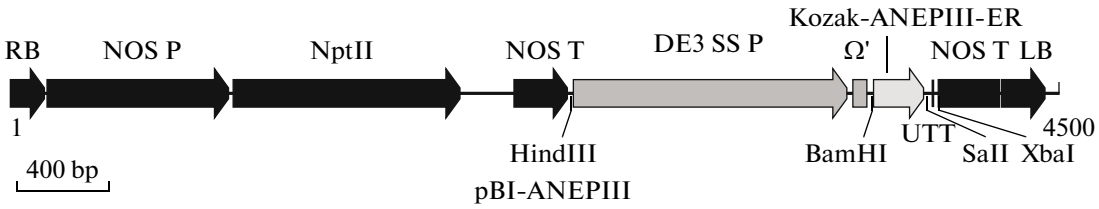

Fig. 1. Verification of plant expression vector pBI-*ANEPIII*. T-DNA of plant transformation vector pBI-*ANEPIII*. NptII – neomycin phosphotransferase II; LB – Left border; RB – Right border; DE35SP– double cauliflower mosaic virus 35S promoter; NOSP – the nopaline synthase promoter; NOST – the nopaline synthase terminator; *ANEPIII*-ER – the *ANEP III* and SEKDEL gene; Ω – Ω enhancer.

RESULTS

Construction of the recombinant plant expression vector

The targeting gene sequence that encodes ANEP III and SEKDEL was about 240 bp. It was obtained by PCR using the pNJU- *ANEP III* plasmid with F1 and R1 as primers, and cloned into the plasmid pMD19-T to obtain the pT-*ANEP III* vector, in which the targeting gene sequence had been confirmed to be correct by sequence analysis in "TAKARA Biotechnology CO". The targeting gene sequence excised from pT-*ANEP III* using BamHI and SalI were subcloned into the pBin438 vector to construct the pBin-*ANEP III* vector. The preliminarily results of the PCR of pBin-*ANEP III* were analyzed by 1% agarose gel electrophoresis and indicated that the pBin-*ANEP III* construction was correct. The resulting vector pBI-*ANEP III* was obtained using HindIII and XbaI double digestion of vectors pBin-*ANEP III* and pBI121-Gusint-D, and then a small (about 750 kb) fragment of Bin-*ANEP III* and a big fragment of pBI121-Gusint-D were ligated using the T4 ligase. PCR analysis revealed that the targeting gene sequence was successfully subcloned into the pBI121-Gusint-D. In pBI-*ANEP III,* the *ANEP III* expression cassette included a double CaMV 35S promoter with Ω' enhancers, the *ANEP III* gene with the Kozak sequence, the ER retention signal and the NOS terminator (fig. 1).

Identification of Agrobacterium tumefaciens EHA105 containing the pBI-ANEP III vector

A. tumefaciens EHA105 transformed by the freeze-thaw method was screened in YEB resistance medium containing rifampicin and kanamycin, and then were detected by colony PCR. Plasmids pBI-*ANEPIII* were purified from the pBI-*ANEPIII* EHA105 positive strains and amplified by PCR. The results of 1% agarose gel electrophoresis indicated that the band place of the PCR product from pBI-*ANEP III*/EHA105 plasmid DNA was the same as that from the pBin-*ANEP III* (fig. 2), and thus *A. tumefaciens* EHA105 containing the pBI-*ANEP III* vector was obtained.

Agrobacterium-mediated plant transformation and plant system

Transformation of tobacco and tomato plants was carried out by the leaf-disc method. Tomato and tobacco cotyledons were transformed by co-cultivation with the *A. tumefaciens* EHA105 strain containing the pBI-*ANEP III*. Shoots and roots were formed on different resistance medium. Subsequently the plantlets were replanted in sterilized soil for growth in a temperature-controlled greenhouse. When they were strong enough, the plantlets were replanted into natural environment (fig. 3).

Molecular detection of the exogenous ANEP III gene in transgenic tomato plants

PCR analysis. A total of 26 independent transgenic tobacco and 18 transgenic tomato plants were regenerated after *Agrobacterium*-mediated transformation. The PCR analysis of transgenic plants was used to amplify the *ANEP III* gene sequence by the *ANEP III* forward primer F1 and reverse primer R1, and plant genomic DNA as a template. Genomic DNA from 14 tomato and 18 tobacco plants had the exogenous *ANEP III* gene of expected size of 240 bp (fig. 4).

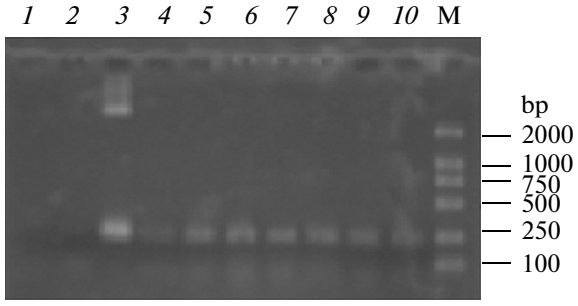

Fig. 2. Identification of pBI-*ANEP III*/EHA105 by PCR. Lane M – Marker DL2000; Lane *1* – PCR amplification of ddH_2O as a negative control; Lane *2* – PCR product of untransformed EHA105 Plasmid DNA as a negative control; Lane *3* – PCR amplification of pBin-*ANEP III* as a positive control; Lane *4–10* – PCR products of the plasmid DNA from pBI-*ANEP III*/EHA105.

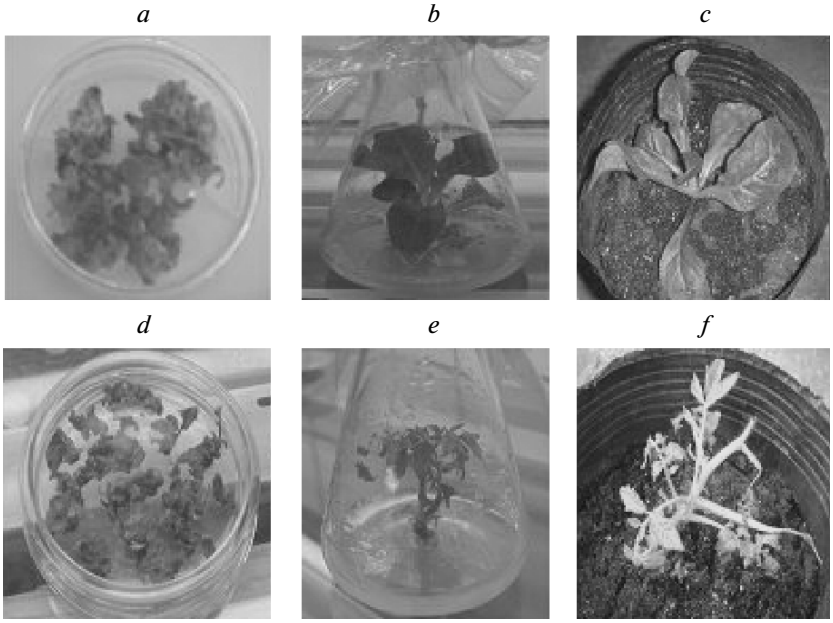

Fig. 3. Transformation and regeneration of transgenic tobacco and tomato plants. Pictures *a* and *d* – shoots formed by transformed tobacco and tomato leaf disks, respectively; pictures *b* and *e* – roots formed by young transgenic tobacco and tomato plants, respectively; pictures *c* and *f* – transgenic tobacco and tomato growing in nature.

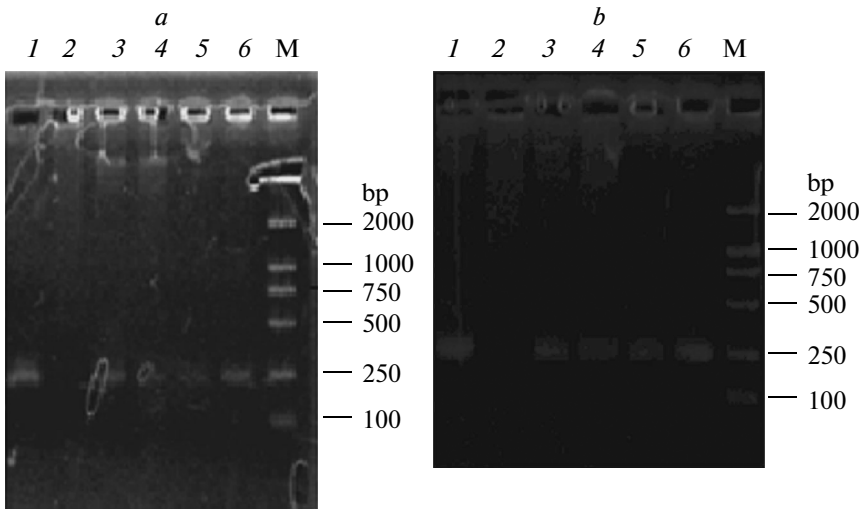

Fig. 4. Partial PCR analysis of transgenic tobacco (*a*) and transgenic tomato (*b*) plants. Lane *1* – PCR product of pBin-*ANEP III* as a positive control; Lane *2* – PCR product of non-transgenic tobacco/tomato as a negative control; Lane *3–6* – PCR products of transgenic tobacco/tomato plants; Lane M – Marker DL2000.

RT-PCR analysis. Total RNA was isolated from fresh transgenic plant tissue. Then first cDNA chains were synthesized using Oligo$(dT)_{18}$ or R1 as a primer and total RNA as the template with Mo-MuLV Reverse Transcriptase. PCR was performed with F1 and R1 primers, and cDNA as a template. PCR products about 240 bp in size were detected using pBin-*ANEP III* products as a positive control and those from non-transgenic plants as a negative control. The results suggested that the *ANEP III* gene was transcribed in transgenic tobacco and tomato plants (fig. 5).

Targeting protein analysis

Total protein was extracted from transgenic plants with an improved acetone sedimentation method. Western blotting was performed with the *ANEP III* standard protein as a positive control and the total protein extracted from non-transgenic plants as a negative

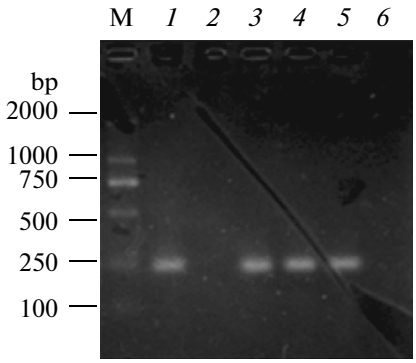

Fig. 5. Partial RT-PCR analysis of transgenic tobacco and transgenic tomato plants. Lane M – Marker DL2000; Lane *1* – PCR product of pBin-*ANEP III* as a positive control; Lanes *2* and *6* – RT-PCR products of non-transformed tobacco/tomato RNA as a negative control; Lanes *3* and *4* – RT-PCR products of transgenic tomato RNA; Lane *5* – RT-PCR products of transgenic tobacco RNA.

control. The 8.3 kDa standard protein band was at the same place as the transgenic tobacco and tomato protein sample bands (fig. 6). It confirmed that *ANEP III* was expressed in transgenic tobacco and tomato plants.

In transgenic tobacco and tomato, the expression level of the ANEP III protein was respectively detected by the immunofluorescence analysis technique. The standard curve was drawn with the following series of concentrations: 0, 0.1, 0.125, 0.4, 0.8 and 1 μg/ml of the standard ANEP III protein, and standard curve equation was $y = 49.993X + 73.778$ ($R^2 = 0.991$). Total protein extracted from transgenic plants was diluted with 0.01 M PBS in the ratio of 1 : 10, and then incubated with the first and second antibodies. The ANEP III protein content in transgenic plants was then calculated from the fluorescence values detected at 485/535 nm. The result showed that the estimated expression level of the ANEP III protein was up to 0.81 and 1.08% of the total soluble protein in transgenic tobacco and tomato plants respectively (table).

DISCUSSION

Transgenic plants are a promising alternative to the conventional recombinant protein production systems, such as bacteria, yeast, animal and insect cell cultures [16]. One of the most important problems in this field is how to increase the yield of recombinant proteins in plants. At present, some strategies are being developed, including new expression cassettes, improvement of protein stability and accumulation by using specific subcellular targeting signals and development of downstream processing technologies [17, 18]. In our work, it was demonstrated that ANEP III with a C-terminal SEKDEL tag can accumulate to a very high level in transgenic plants. The SEKDEL sequence has a strong effect on reducing the rate of protein exit from the ER without generating absolute retention [11], and the SEKDEL modification of the C-terminus can enhance protein expression in transgenic tobacco and tomato [19]. In this study, we examined the effects of an ER retention signal (SEKDEL) on the ANEP III accumulation in transgenic tobacco and tomato plants and also got the same results. In addition in order to improve the expression of ANEP III the tobacco mosaic virus omega sequences (Ω enhancers), known for promoting efficient initiation of translation and as an mRNA leader [20], were also introduced into the *ANEP III* expression cassette.

Since the commercial antibodies to ANEP III protein are unavailable, we prepared polyclonal antibody from immunized rabbit serum which was purified by Protein A Sepharose 4FF. The first antibody obtained by this way didn't have the same specificity as a monoclonal antibody, and the exogenous protein of transgenic plants was expressed in a low level. All of this caused difficulties in detecting the trace targeting proteins in the transgenic plants in our earlier studies. We used the alkaline phosphatase (AKP) conjugated goat anti-rabbit IgG as the second antibody, and expected to calculate the ANEP III protein content in transgenic plant total protein by ELISA analysis. But the results were not satisfactory. Thus, we tried to use fluorescein-conjugated second antibodies to increase the detection limit, and results were better than before. Our calculations showed that we obtained the ANEP III

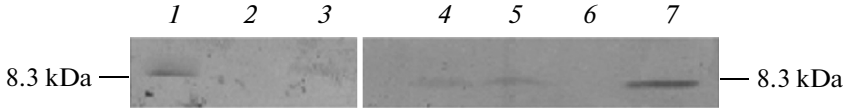

Fig. 6. Western blot analysis of transgenic tomato and tobacco plants. Lanes *1* and *7* – 1 μg of *ANEP III* standard proteins as a positive control; Lanes *2* and *6* – protein extracted from non-transgenic tomato and tobacco plants, respectively, as a negative control; Lane *3* – protein extracted from transgenic tobacco plants; Lane *4* and *5* – proteins extracted from transgenic tomato plants.

Assay of the total soluble protein and ANEP III in transgenic tobacco and tomato by the immunofluorescence technique ($n = 3$)

Sample	Average fluorescence value, 485/535 nm	Total soluble protein, μg/ml	ANEP III, μg/ml	Dilution factor	ANEP III protein, %
Transgenic tobacco	87.0 ± 3.4	487.29 ± 5.86	0.264 ± 0.068	15	0.81
Transgenic tomato	96.8 ± 4.2	426.46 ± 7.12	0.461 ± 0.084	10	1.08
Non-transgenic tobacco	62.5 ± 3.7	516 ± 4.24	NA	15	–
Non-transgenic tomato	69.1 ± 3.5	489 ± 6.10	NA	15	–

Percentage of the ANEP III protein (%) was calculated as follows: concentration of ANEP III × dilution factor/concentration of the total soluble protein.

protein at up to 0.81 and 1.08% of the total soluble protein in transgenic tobacco and tomato respectively. So we conclude that the detection sensitivity of the alkaline phosphatase (AKP) approach is lower than that of immunofluorescence technique.

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