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Article in *Biotechnology Letters* · November 2020

DOI: 10.1007/s10529-020-02995-x

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A plant-based transient expression system for the rapid production of highly immunogenic Hepatitis E virus-like particles

Eugenia S. Mardanova · Katerina H. Takova · Valentina T. Toneva ·
Gergana G. Zahmanova · Liudmila M. Tsybalova · Nikolai V. Ravin

Received: 4 June 2020 / Accepted: 25 August 2020 / Published online: 1 September 2020
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Abstract

Objective Hepatitis E virus (HEV) infection is a major cause of acute hepatitis worldwide. The aim of the study is the development of plant expression system for the production of virus-like particles formed by HEV capsid and the characterization of their immunogenicity.

Results Open reading frame (ORF) 2 encodes the viral capsid protein and possesses candidate for vaccine production. In this study, we used truncated genotype 3 HEV ORF 2 consisting of aa residues 110 to 610. The recombinant protein was expressed in

Nicotiana benthamiana plants using the self-replicating potato virus X-based vector pEff up to 10% of the soluble protein fraction. The yield of HEV 110-610 after purification was 150–200 µg per 1 g of green leaf biomass. The recombinant protein formed nanosized virus-like particles. The immunization of mice with plant-produced HEV 110-610 protein induced high levels of HEV-specific serum antibodies.

Conclusions HEV ORF 2 (110-610 aa) can be used as candidate for the development of a plant-produced vaccine against Hepatitis E.

Keywords Hepatitis E virus · Plant biofactory · Potato virus X · Vaccine · Viral vector

E. S. Mardanova · N. V. Ravin (✉)
Institute of Bioengineering, Research Center of
Biotechnology of the Russian Academy of Sciences,
Moscow 119071, Russia
e-mail: nravin@biengi.ac.ru

K. H. Takova · V. T. Toneva · G. G. Zahmanova
Department of Plant Physiology and Molecular Biology,
University of Plovdiv, 4000 Plovdiv, Bulgaria

V. T. Toneva
Institute of Molecular Biology and Biotechnologies,
4000 Plovdiv, Bulgaria

G. G. Zahmanova
Center of Plant Systems Biology and Biotechnology,
4000 Plovdiv, Bulgaria

L. M. Tsybalova
Research Institute of Influenza, Russian Ministry of
Health, St. Petersburg 197376, Russia

Abbreviations

HEV Hepatitis E virus
ORF Open reading frame
PVX Potato virus X

Introduction

Hepatitis E virus (HEV) infection is a worldwide disease and the primary cause of acute viral hepatitis in the world with an estimated 20 million cases every year (Larrue et al. 2020). In recent years, increased cases have been reported in developing countries; however sporadic locally acquired cases of HEV

infections have been also reported in Europe, Australia and Japan (Goel and Aggarwal 2020). Prophylactic vaccination is an effective method to protect against HEV infection and control HEV infection-associated diseases (Li et al. 2020).

Hepatitis E virus belongs to the *Hepevirida* family, genus *Orthohepevirus*. The genome of HEV is a single single-stranded positive-sense RNA molecule with a length of about 7.3 kb, containing three open reading frames (ORFs) (Tam et al. 1991). ORF1 encodes a polyprotein responsible for replication of viral RNA, ORF2 encodes a capsid protein of the virus, and the protein product ORF3 is supposed to be involved in virus particle secretion (reviewed by Debing et al. 2016). HEV virions are non-enveloped icosahedral particles of 27–32 nm in size, resulting from the self-assembly of 180 monomers of ORF2 capsid protein (Bradley et al. 1988). The only HEV structural protein, ORF2 capsid, contains epitopes recognized by virus-neutralizing antibodies and therefore it is the basis of all recombinant vaccines (Meng 2013; Emerson et al. 2006). To date, more than a dozen candidate vaccines against HEV have been developed, of which three have reached the stage of clinical trials (Cao et al. 2017; Li et al. 2020). One recombinant vaccine, with a trade name of Hecolin, is already approved for use in China.

Vaccines under development are limited to baculovirus and bacterial expression systems (Shrestha et al. 2007; Yu et al. 2019; Zhang et al. 2015; Zhu et al. 2010; Cao et al. 2018). Plants could become promising biofactories for expression of recombinant proteins due to the low final cost and inherent safety of products resulting from the absence of pathogens common to plants and animals (Lomonosoff and D'Aoust 2016). The self-replicating recombinant viral vectors based on the potato virus X (PVX) were successfully used for the expression of different proteins in *Nicotiana benthamiana* leaves at a high level (Mardanov et al. 2017).

The HEV ORF2 capsid protein consists of 660 aa, but its shorter version consisting of aa residues 112 to 608 was able to form virus-like particles (VLPs) when expressed in insect cells (Li et al. 2005). Previously we expressed different HEV ORF2 variants and fusions of HEV ORF2 with influenza A virus M2e peptide in *N. benthamiana* plants using the pEAQ-HT (Sainsbury and Lomonosoff 2008) and pEff (Mardanov et al. 2017) viral vectors (Zahmanova et al. 2020). Several

proteins were produced but only the HEV ORF2 consisting of aa residues 110 to 610 (HEV 110-610) and chimeric HEV 110-610 with M2e insertion spontaneously assembled into virus-like particles (Zahmanova et al. 2020). Here we report large-scale transient expression of HEV capsid protein, purification of the protein, and testing its immunogenicity in an animal model.

Materials and methods

Vector for expression of HEV 110-610 protein in plants

The target HEV 110-610 gene was amplified by PCR using primers HEV110_Asc-F/ HEV-his_R and cloned into pEff vector at AscI and SmaI restriction sites resulting in pEff_HEV 110-610 recombinant vector as described in the study by Zahmanova et al. (2020).

HEV110_Asc-F: TAGGCGCGCCATGGGTATG
GCTACTTCTCCTG);
HEV-his_R: TACCCGGGCTAATGATGGT
GATGGTGATGAGCAAGAGCAGAGTGAGGA
GCAAG.

The recombinant vector pEff_HEV 110-610 was transferred from *E. coli* to the *A. tumefaciens* GV3101 strain using electroporation.

Agroinfiltration of *Nicotiana benthamiana* plants

Plants were grown in a greenhouse in a 16 h daylight regime with additional illumination with full spectrum phytolamps until 5–6 true leaves appear. Recombinant *A. tumefaciens* GV3101 strain with pEff_HEV 110-610 vector was grown overnight in a shaking incubator at a temperature of 28 °C. The agrobacterial cells (10 mL) were harvested by centrifugation for 5 min at 4000×g, and the pellet was resuspended in 10 mL of 10 mM MES (pH 5.5) and 10 mM MgSO₄. A suspension of agrobacteria (OD₆₀₀ ~ 0.2) was introduced into the *N. benthamiana* plants by extruding from a syringe without a needle into the lower surface of the leaves. After agroinfiltration, plants continued to grow in a greenhouse under the same conditions.

SDS-PAGE and Western blotting of plant-produced proteins

For small-scale expression, pieces of leaves from the agroinfiltrated zones (~ 10 mg) were excised and homogenized in a 50 µL extraction buffer (0.4 M sucrose, 50 mM Tris pH 8.0, 5 mM MgCl₂, 10% glycerol, 5 mM β-mercaptoethanol). The obtained suspension was centrifuged at 14,000×g for 10 min, and the supernatant was taken. An equal volume of 2× SDS-PAGE loading buffer (20% glycerol, 5% SDS, 62.5 mM Tris pH 6.8, 0.5% bromophenol blue, 5% β-mercaptoethanol) was added to the supernatant. Then, 10 µL of the obtained mixture (corresponding to about 1 mg of leaf tissue) was analyzed by SDS-PAGE. After electrophoresis, the gel was stained with One-Step Blue® Protein Gel Stain (BIOTIUM, USA). The relative intensities of the bands in the photograph of stained gel were analyzed using Nonlinear Dynamics. TotalLab. TL120.v2009-NUL software.

For Western blotting the SDS-PAGE gel was used to transfer the proteins onto a Hybond-P membrane (GE Healthcare, USA) by semi-dry transfer using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). Then the membrane was treated with 5% (w/v) solution of dry milk in TBS-T (150 mM NaCl, 20 mM Tris, 0.1% Tween 20, pH 8.0) buffer and probed with mouse anti-HEV ORF2 antigen primary antibody followed by washing with TBS-T. The bound antibodies were detected with secondary rabbit anti-mouse antibodies conjugated with peroxidase and visualized using a Western Blot ECL Plus kit (GE Healthcare).

Isolation and purification of recombinant proteins

Agroinfiltrated *N. benthamiana* leaves were ground with a mortar and pestle to prepare a homogenous suspension in PBS buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl). The resulting mixture was centrifuged at 14,000×g for 10 min, and the supernatant was used as a soluble protein extract at a concentration of 0.2 g of fresh leaf tissue per mL. The soluble protein extract was applied to Ni-NTA resin equilibrated with the same buffer and incubated for 60 min. Then, the resin was washed twice with the same buffer containing 10 mM imidazole and then 20 mM imidazole. The recombinant protein HEV 110-610 was eluted with buffer containing 500 mM

imidazole. After elution, the protein was dialyzed against PBS (1:100, four changes of buffer) using Slide-A-Lyzer Mini dialysis devices (Thermo Fisher Scientific, USA). Dialyzed protein preparation was passed through 0.22 µm Sprintzen/Syringe-Filter (TPP Techno Plastic Products AG, Switzerland). Proteins were quantitated by a Qubit Protein Assay Kit using Qubit Fluorometer (Invitrogen, USA), following the manufacturer's instructions.

Electron and atomic force microscopy

Virus-like particles were examined in transmission electron microscope JEOL LEM 1011. For atomic force microscopy an Integra Prima microscope and Nova SPM software (NT-MDT, Russia) were used. The scanning was performed in semi contact mode using gold cantilever NSG01 (NT-MDT). The protein sample was applied to a sapphire substrate coated with mica and dried at room temperature. PBS was used as a negative control.

Mouse immunization

Female BALB/c mice (16–18 g) were immunized intramuscularly three times at 2 weeks intervals with 50 µg of protein with a sodium desoxyribonucleate (Derinat) as an adjuvant. Derinat is a 1.5% solution of a sodium salt of native DNA (270–500 kDa) diluted with a 0.1% solution of sodium chloride. Derinat is licensed for the human use in Russian Federation as an immunomodulator (certificate RU No. 002916 from 08.08.2008). Control group was intramuscularly injected with PBS. Five mice for each group participated in the experiment. The study was carried out in accordance with the Russian Guidelines for the Care and Use of Laboratory Animals, and the protocol approved by the Committee for Ethics of Animal Experimentation at the Research Institute of Influenza.

Antibody detection by ELISA

Antigen-specific levels of antibodies were determined by ELISA in 96-well microtiter plates coated with the HEV 110-610 peptide (2 µg/mL) in PBS (pH 7.2) overnight at 4 °C. After blocking with PBS containing 5% fetal calf serum the plates were washed thrice with PBS containing Tween. The diluted serum samples were then added to the wells (100 µL per well), after

which the plates were incubated for 1 h. HRP-labelled goat anti-mouse IgG (ab97040, Abcam) was used as a conjugate at 1:5000 dilution. After adding tetramethylbenzidine substrate (BD Bioscience) and monitoring the color development, the reaction was stopped with H_2SO_4 , and the OD_{450} was measured on a microplate spectrophotometer. The differences between antibody titers were evaluated by the Mann–Whitney U-test.

Results and discussion

Viral vector for the expression of capsid protein of HEV consisting of aa residues 110 to 610 (HEV 110-610) in *Nicotiana benthamiana*.

The pEff viral vector (Mardanov et al. 2017) was used to express the target proteins in *N. benthamiana* plants. This vector includes the 5'-untranslated region of the PVX genome, the RNA-dependent RNA polymerase gene, the first promoter of the subgenomic RNA, the 5'-untranslated region of the alfalfa mosaic virus RNA 4, acting as a translational enhancer, the green fluorescent protein gene (*gfp*), and the 3'-untranslated region of the PVX genome (Fig. 1). These genetic elements are placed between the 35S promoter and the NosT terminator within the tDNA region of a binary vector that can replicate in *E. coli* and *A. tumefaciens* cells. Capsid protein of HEV consisting of amino acid residues 110 to 610 was cloned into pEff in place of *gfp* to make a recombinant viral vector pEff_HEV 110-610_his. A 6-histidine tag was added at the C-terminus of the hybrid protein to facilitate its purification by metal affinity chromatography (Fig. 1).

Expression of his-tagged HEV 110-610 in plants using pEff vector and protein purification

Recombinant binary vector pEff_HEV 110-610_his was introduced into *A. tumefaciens* strain GV3101 by transformation. The obtained strain was used for infiltration of the leaves of *N. benthamiana* plants. After agroinfiltration, the t-DNA region of the vector became transferred to the plant cell, followed by the synthesis of the RNA of modified PVX from the 35S promoter, its replication, the synthesis of subgenomic RNA for HEV 110-610 gene, and its translation. The level of expression of the target protein reached a

Fig. 1 Structure of the expression vector pEff_HEV 110-610_his. RDRP, RNA-dependent RNA polymerase of PVX; Sgp1, subgenomic promoter of the PVX protein 25K; AMV, the leader sequence of RNA 4 of the alfalfa mosaic virus; 6his, sequence that encodes the C-terminal 6-histidine tag; HEV CP, capsid protein of HEV consisting of aa residues 110 to 610; 35S, promoter of the cauliflower mosaic virus RNA; NosT, terminator of the nopaline synthase gene from *Agrobacterium tumefaciens*; P24, gene of silencing suppressor P24 from grapevine leafroll-associated virus-2; LB and RB are the left and right borders of T-DNA

Fig. 2 Transient expression of HEV 110-610 protein analysed by SDS-PAGE. Molecular weight marker (sizes are shown in kDa). Lane 1, total proteins from empty leaf. Lanes 2–6, total protein samples obtained from leaves infiltrated with vector pEff_HEV 110-610 at 2, 3, 4, 5, and 6 days after agroinfiltration, respectively

maximum on day 4 after agroinfiltration (Fig. 2). With a longer cultivation of agroinfiltrated plants, tissue necrotization in the infiltration zones was observed.

To isolate the recombinant protein, the biomass of agroinfiltrated leaves was collected on the 4th day. Protein samples were analyzed using SDS-PAGE and Western blotting with HEV-specific antibodies. The results presented in Fig. 3 show that the HEV 110-610 protein was successfully expressed and accounted for about 10% of total soluble protein, corresponding to about 400 $\mu\text{g/g}$ of fresh leaf tissue.

Since the target protein was present in the soluble fraction, its isolation using metal-affinity chromatography was performed under native conditions. After purification the protein samples were dialyzed against phosphate-buffered saline (PBS). The final yield after purification was 150–200 μg per 1 g of green leaf

Fig. 3 Coomassie brilliant blue stained gel (a) and Western blot (b) of proteins isolated from *N. benthamiana* plants and separated by SDS-PAGE. M, molecular weight marker (sizes are shown in kD); lane 1, total soluble proteins from empty leaves; lane 2, total protein from leaf infiltrated with vector pEff_HEV 110-610; lane 3, HEV 110-610 protein purified on Ni-NTA column (position is shown by an arrow)

biomass, about 10 mg of HEV 110-610 protein with a purity higher than 95% was obtained.

Analysis of virus-like particles

The assembly of the HEV 110-610 protein into nanosized virus-like particles was analyzed by electron and atomic force microscopy (Fig. 4). Particulate structures were observed by both methods. According to transmission electron microscopy the particles had a diameter of about 100 nm. At higher magnification the particles appeared as spheres with presumably “empty” central cavity.

Immunogenicity of plant-produced HEV capsid VLPs

To characterize the immunogenicity of the candidate vaccine, mice were immunized thrice with the plant-produced HEV 110-610 and PBS as a control. Mice were immunized intramuscularly three times at two-

Fig. 5 Titers of IgG antibodies to HEV 110-610 in sera of mice immunized with HEV 110-610 and PBS after the third immunization. Antibody titers in individual mice are shown in circles, geometric mean antibody levels in groups are shown by horizontal lines

week intervals with 50 µg of protein with Derinat as an adjuvant. Sera of mice immunized with PBS were used as a negative control. Blood samples were taken 2 weeks after the third immunization. Sera were analyzed by ELISA to identify IgG antibodies directed against HEV 110-610.

It was found that immunization with HEV 110-610 induced high titers of the specific IgG in sera compared to negative control (Fig. 5). The difference between titers of IgG for test and control groups was significant ($p < 0.01$).

Conclusions

Overall, this work shows that expression of recombinant capsid protein of Hepatitis E virus consisting of aa residues 110 to 610 (HEV 110-610) in *Nicotiana benthamiana* plants is feasible. The use of self-

Fig. 4 Analysis of the HEV 110-610 particles by transmission electron microscopy (a, b) and atomic force microscopy (c)

replicating viral vector based on PVX genome allowed achieving very high level of expression, about 10% of the total soluble protein. We have developed a simple protocol for protein purification based on metal chelate affinity chromatography. The yield of HEV 110–610 after purification was 150–200 µg per 1 g of green leaf biomass, and the purity was greater than 95%. The intramuscular immunization of mice with purified recombinant protein induced high levels of HEV-specific serum antibodies. The reported transient expression system could become a promising approach for the development of a plant-produced vaccine against hepatitis E.

Acknowledgements This research was funded by the Russian Foundation for Basic Research (Grant 18-54-18005), the Bulgarian National Science Fund (Grant DNTC/Russia 02-6) and the European Union's Horizon 2020 research and innovation program project PlantaSYST (SGA-CSA No. 739582 under FPA No. 664620).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Bradley D, Andjaparidze A, Cook EH, McCaustland K, Balayan M, Stetler H, Velazquez O, Robertson B, Humphrey C, Kane M (1988) Aetiological agent of enterically transmitted non-A, non-B hepatitis. *J Gen Virol* 69:731–738
- Cao YF, Tao H, Hu YM, Shi CB, Wu X, Liang Q, Chi CP, Li L, Liang ZL, Meng JH, Zhu FC, Liu ZH, Wang XP (2017) A phase I randomized open-label clinical study to evaluate the safety and tolerability of a novel recombinant Hepatitis E vaccine. *Vaccine* 35:5073–5080
- Cao Y, Bing Z, Guan S, Zhang Z, Wang X (2018) Development of new Hepatitis E vaccines. *Hum Vaccin Immunother* 14:2254–2262
- Debing Y, Moradpour D, Neyts J, Gouttenoire J (2016) Update on Hepatitis E virology: implications for clinical practice. *J Hepatol* 65(1):200–212
- Emerson SU, Clemente-Casares P, Moiduddin N, Arankalle VA, Torian U, Purcell RH (2006) Putative neutralization epitopes and broad cross-genotype neutralization of Hepatitis E virus confirmed by a quantitative cell-culture assay. *J Gen Virol* 87:697–704
- Goel A, Aggarwal R (2020) Hepatitis E: epidemiology, clinical course, prevention, and treatment. *Gastroenterol Clin North Am* 49(2):315–330
- Larrue H, Abravanel F, Péron JM (2020) Hepatitis E, what's the real issue? *Liver Int* 40(Suppl 1):43–47
- Li TC, Takeda N, Miyamura T, Matsuura Y, Wang JC, Engvall H, Hammar L, Xing L, Cheng RH (2005) Essential elements of the capsid protein for self-assembly into empty virus-like particles of Hepatitis E virus. *J Virol* 79(20):12999–13006
- Li Y, Huang X, Zhang Z, Li S, Zhang J, Xia N, Zhao Q (2020) Prophylactic Hepatitis E vaccines: antigenic analysis and serological evaluation. *Viruses* 12(1):109
- Lomonosoff GP, D'Aoust MA (2016) Plant-produced biopharmaceuticals: a case of technical developments driving clinical deployment. *Science* 353(6305):1237–1240
- Mardanov EA, Blokhina EA, Tsybalova LM, Peyret H, Lomonosoff GP, Ravin NV (2017) Efficient transient expression of recombinant proteins in plants by the novel pEff vector based on the genome of potato virus X. *Front Plant Sci* 8:247
- Meng X-J (2013) Zoonotic and foodborne transmission of Hepatitis E virus. *Semin Liver Dis* 33:41–49
- Sainsbury F, Lomonosoff GP (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol* 148:1212–1218
- Shrestha MP, Scott RM, Joshi DM, Mammen MP Jr, Thapa GB, Thapa N, Myint KS, Fournneau M, Kuschner RA, Shrestha SK et al (2007) Safety and efficacy of a recombinant Hepatitis E vaccine. *N Engl J Med* 356:895–903
- Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* 185:120–131
- Yu XY, Chen ZP, Wang SY, Pan HR, Wang ZF, Zhang QF, Shen LZ, Zheng XP, Yan CF, Lu M et al (2019) Safety and immunogenicity of Hepatitis E vaccine in elderly people older than 65 years. *Vaccine* 37:4581–4586
- Zahmanova GG, Mazalovska M, Takova KH, Toneva VT, Minkov IN, Mardanov EA, Ravin NV, Lomonosoff GP (2020) Rapid high-yield transient expression of Swine Hepatitis E ORF2 capsid proteins in *Nicotiana benthamiana* plants and production of chimeric Hepatitis E virus-like particles bearing the M2e influenza epitope. *Plants* 9:29
- Zhang J, Zhang XF, Huang SJ, Wu T, Hu YM, Wang ZZ, Wang H, Jiang HM, Wang YJ, Yan Q et al (2015) Long-term efficacy of a Hepatitis E vaccine. *N Engl J Med* 372:914–922
- Zhu FC, Zhang J, Zhang XF, Zhou C, Wang ZZ, Huang SJ, Wang H, Yang CL, Jiang HM, Cai JP et al (2010) Efficacy and safety of a recombinant Hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. *Lancet* 376:895–902

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