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Expression of peptide nanoparticles containing a porcine reproductive and respiratory syndrome (PRRS) virus epitope in plants

Laura Uribe-Campero[1], Héctor G. Núñez-Palenius[2] and Miguel A. Gómez-Lim[1]*

[1]Departamento de Ingeniería Genética, CINVESTAV-IPN, Km 9.6 Carretera Irapuato-León, C.P. 36821 Irapuato, Guanajuato, México.
[2]División de Ciencias de la Vida. Campus Irapuato-Salamanca. Universidad de Guanajuato, Exhacienda El Copal s/n, A.P. 311, Irapuato, Gto. C.P. 36500., Mexico.

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Porcine reproductive and respiratory syndrome is one of the most devastating diseases affecting the pig industry. The licensed vaccines available present several shortcomings and consequently many groups around the world are actively working towards developing an efficacious vaccine. In this work, we have fused the epitope B of the GP5 protein from the PRRRS virus to peptide nanoparticles and expressed the construct in plants in a transient manner. It was shown by transmission electron microscopy that the chimeric protein nanoparticles can be efficiently synthesized and self-assembled inside plant cells. By real-time polymerase chain reaction (PCR), it was also demonstrated that the chimeric constructs are efficiently transcribed. There exists a high potential for these nanoparticles to serve as platforms for vaccines. In the next phase of the project, we will immunize mice to show immunogenicity and pigs, which will be later challenged with a circulating strain of the virus.

Key words: Peptide nanoparticles, epitopes, porcine reproductive and respiratory syndrome virus (PRRSV), GP5, vaccines.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important viral diseases affecting the global pork industry, with annual losses of billions of dollars per year worldwide (Neumann et al., 2005; Holtkamp et al., 2013; Zhang et al., 2014). The syndrome is characterized by reproductive and respiratory failure and it is associated with abortions and infertility, respiratory distress in nursing pigs, poor growth and increased mortality (Rowland and Morrison, 2012; Hu and Zhang, 2014). It is caused by PRRS virus (PRRSV), which is a member of the *Arteriviridae* family in the *Nidovirales* order (Lunney et al., 2010; Snijder et al., 2013). The genome of PRRSV, a single-stranded positive-sense RNA of about 15 Kb in length with a methyl capped 5' and a 3'-polyadenylated tail, expresses a wide range of accessory and structural proteins via two distinct transcription mechanisms (Han and Yoo, 2014; Kappes and Faaberg, 2015). The PRRSV genome is

*Corresponding author. E-mail: mgomez@ira.cinvestav.mx. Tel: +52-462-623-9679. Fax: +52 (462) 624 58 49.

polycistronic and originates 10 overlapping open reading frames (ORFs) (ORF 1a, 1b, 2, 2b, 3-7) (Kappes and Faaberg, 2015). ORFs 2-5 encode glycosylated membrane proteins GP2a, GP3–GP5 plus GP5a. GP2, GP3 and GP4 form a trimeric complex resulting in the minor glycoprotein complex which enhances viral entry and is heavily N-glycosylated (Kappes and Faaberg, 2015). The nucleocapsid protein (N) is encoded by ORF7. N is the major structural element within the PRRSV virion and forms disulfide-linked homodimers; It functions to package the viral genomic RNA and is the only known structural protein, which does not encode a transmembrane domain (Dokland, 2010).

The original work suggested that only GP5, GP6 (M) and N made up the majority of the protein content of PRRSV (Mardassi et al., 1996; Dokland, 2010). Recently, the nsp2 protein, which is coded for by the most variable region of the genome, was demonstrated to be incorporated into virions of several PRRSV strains as a set of differently sized protein isomers (Kappes et al., 2013, 2015b). This unexpected result increases the number of viral proteins to at least 10 (full-length nsp2 and its isomers, nsp2TF, GPs2–5, E, M, N, ORF5a), that are exposed to the porcine immune system on entry of PRRSV into swine alveolar macrophages (Fang et al., 2012; Veit et al., 2014). The enormous genetic and protein variation of all of these structural proteins, from the least conserved nsp2 to the most conserved M protein shows the complexity and plasticity of the PRRSV genome and virion structure (Rascón-Castelo et al., 2015; Kappes and Faaberg, 2015a).

GP5 is a glycosylated protein of approximately 25 kDa and although it carries the major neutralizing epitope (epitope B) (Mardassi et al., 1996; Ostrowski et al., 2002; Plagemann et al., 2002), it is also the most variable viral protein (Dokland, 2010; Kappes et al., 2015a).

PRRSV mutates at an extremely rapid rate, even for a RNA virus (order of 10^{-2} changes/amino acid site/year, as compared to 10^{-3} to 10^{-5} for HIV or hepatitis C virus) than that seen in other RNA viruses (Hanada et al., 2005) and this is probably due to more rapid replication, rather than to a higher error rate (Murtaugh et al., 2010).

Control of PRRSV is primarily focused on prophylactic vaccines. The vaccines currently licensed consist of modified-live and killed-virus, but live PRRS vaccines may revert to virulent virus under farm conditions (Botner et al., 1997) and the efficacy of both vaccines may vary upon heterologous challenge (Diaz et al., 2006; Cano et al., 2007; Rebeaud and Bachmann, 2012). None of the available vaccines is able to prevent respiratory infection or pig-to-pig transmission (Kimman et al., 2009).

The unsatisfactory efficacy and safety of current PRRSV vaccines has driven the continuous efforts for developing better and safer vaccines. However, development of new PRRSV vaccines faces many challenges because of the high variability and the diverse mechanisms that the virus employs to evade the immune response (Huang et al., 2015; Salguero et al., 2015). A number of strategies including recombinant production of different PRRSV antigens in various systems have been tested (Qiu et al., 2005; Jiang et al., 2006; Zheng et al., 2007; Li et al., 2009a, b; Cruz et al., 2010; Chen and Liu, 2011; Chia et al., 2011; Vimolmangkang et al., 2012; Chan et al., 2013; Nam et al., 2013).

The epitope B of GP5 protein has been suggested as a promising vaccine candidate (Wissink et al., 2003; Snijder et al., 2013). It is well known that small, single peptides are poorly immunogenic and therefore their use as individual peptides is not recommended (Bae et al., 2009). However, they could be presented to the immune system on the surface of nanoparticles, which are highly repetitive. These repetitive patterns are efficiently recognized by the mammal immune system. Epitopes that are capable of inducing an immune response can be displayed repetitively at high density on different types of nanoparticles (Pimentel et al., 2009). The key role of epitope density was illustrated in a study showing that nanoparticles decorated with a peptide at different density produce high IgG responses only at the highest density (Jegerlehner et al., 2002) and that only highly repetitive antigens could break B cell unresponsiveness and induce self-specific antibody responses (Bachmann et al., 1993; Chackerian et al., 2002).

We decided to employ peptide nanoparticles as a vehicle of presentation of the GP5 epitope B of PRRSV to the immune system. The nanoparticles are formed by pentameric and trimeric motifs derived respectively from coiled coil domains of a monomer taken from the cartilage oligomeric matrix protein and a *de novo* designed oligomer (Babapoor et al., 2011; Raman et al., 2006). These pentameric and trimeric coiled coils oligomers, use the threefold and fivefold symmetry of an icosahedron to yield a self-assembling nanoparticle (Raman et al., 2006). Self-assembly into a predicted regular icosahedral nanoparticle of about 16 nm of diameter and 473 KDa in size occurs when the coiled-coil domains of different monomers associate to form the icosahedral nanoparticle. A nanoparticle with this type of architecture can be used as a vaccine by extending the ends of the monomer with an epitope sequence (Babapoor et al., 2011). Nanoparticles of this type have only been expressed in bacterial systems and employed to express epitopes of *Plasmodium berghei* (Kaba et al., 2009), avian influenza (Babapoor et al., 2011) and severe acute respiratory syndrome virus (Pimentel et al., 2009).

In this work, as a first step towards using the peptide nanoparticles decorated with the GP5 epitope B of PRRSV as a candidate vaccine, our aim was to demonstrate that peptide nanoparticles, expressing the epitope B of GP5, were able to assemble adequately in a plant expression system. Why use plants? Over the last 10 years, the use of plants as platform has won relevancy because of its many advantages (Kumar et al., 2013). Plants have become a very attractive system for

Table 1. Peptide nanoparticle sequences.

Construction	Sequence
M-GP5	MGHHHHHHASWKWDGGLVPRGSDEMLRELQETNAALQDVRELLRQQVKQITFLRALLMGGRLLARLEELERRLEELERLEELERAINTVDLELAALRRRLEELARGGSGSHLQLIYNL
T-GP5	MGHHHHHHASLVPRGSHLQLIYNLSSGSLYRLTVIIDDRYESLKNLITLRADRLEMIINDNVSTLRALLMGGRLLARLEELERRLEELERRLEELERAINTVDLELAALRRRLEELAR

Pentameric coiled coil domain (green), trimeric de novo designed coiled coil domain (blue), tetrameric coiled coil (pink) and PRRS GP5 Epitope B in orange. Linkers other amino acid residues and his-tags are shown in black (Babapoor et al., 2011).

expression of many antigens and the recent approval of the first plant-made biologics by the U.S. Food and Drug Administration (FDA) for plant production and commercialization has opened the door for more products to be developed (Chen and Lai, 2013).

MATERIALS AND METHODS

Constructions

The peptide nanoparticles M-GP5 and T-GP5 sequences were synthesized by GenScript from the nanoparticles sequences previously reported by Babapoor et al. (2011). These nanoparticles will display the antigen of interest in a repetitive manner in high density in both monomeric (M-GP5) or tetrameric form (T-GP5) which are different conformations of the structure (Babapoor et al., 2011).

The conserved epitope B (SHLQLIYNL) of GP5 PRRSv protein (Ostrowski et al., 2002) was genetically fused to the nanoparticle sequence (Table 1). *E. coli* DH5α cells were transformed with M-GP5 or T-GP5 and incubated overnight at 37°C on LB medium plates in the presence of carbeniciline (50 mg/L). DNA plasmid was extracted from a single colony. The 365 or 361 pb fragments corresponding to M-GP5 or T-GP5 were digested with BsaI and subcloned in the BsaI site of pICH31070 to obtain pICH31070-M-GP5 or pICH31070-T-GP5. Vectors pICH15879, pICH14011, pICH-GFP, pICH31070-M-GP5 and pICH31070-T-GP5 were then transformed in *Agrobacterium tumefaciems* GV3101. Expression vectors were all kindly provided by Dr. Yuri Gleba (Icon Genetics).

Agroinfiltration

Infiltrations were done as described by our laboratory before (Coconi-Linares et al., 2013) Briefly, the GV3101 cells were incubated in YEB medium at 28°C until the OD_{600}nm = 1.5. Then acetosyringone (200 µM) was added and the cultures were incubated at room temperature for two hours more. Bacteria were harvested by centrifugation at 3000 xg and the pellet of each culture was resuspended in agroinfiltration buffer (10 mM MES pH 5.5, 10 mM MgSO4) to a final OD_{600}nm = 0.15. Equal volumes of each culture were mixed and the bacteria suspension used for agroinfiltration. Leaves of six to seven weeks-old *Nicotiana silvestris* plants grown in a controlled ambient chamber were infiltrated with bacterial suspension using a syringe without needle. Infiltrated plants were incubated at 25°C (16/8 ligth/darkness) for 6-10 days.

Extraction of total RNA and cDNA preparation

Total RNA was extracted from 100-200 mg of frozen infiltrated leaves with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All RNA samples were analyzed by agarose gel electrophoresis and visual inspection of the ribosomal RNA bands upon ethidium bromide staining. Total RNA samples (5 µg) were reverse-transcribed to generate single-stranded cDNA using an oligo dT18 primer and 200 units of SuperScript III reverse transcriptase as described by the manufacturer (Invitrogen, Carlsbad, CA, USA).

Table 2. Sequence of primers for quantitative real time PCR.

Primer	Forward (5'-3')	Reverse (5'-3')
M-GP5	agaaacaaacgcagcactacag	gccagtaatcttcctcccataag
T-GP5	accgacttactgtgataattgacg	tcgtgccaataatcttcctccc
L23	aaggatgccgtgaagaagatgt	gcatcgtagtcaggagtcaacc
PP2A	gaccctgatgttgatgttcgct	gagggatttgaagagagatttc

Gene expression analysis by quantitative real-time PCR (qRT-PCR)

The single-stranded cDNA was diluted five-fold in sterile milli-Q water prior to qRT-PCR. Amplifications were performed using SYBR Green and run in triplicate in 96-well reaction plates with the real-time PCR System CFX96 (BioRad Laboratories Incorporated, USA). Amplification reactions were prepared in a total volume of 20 µl as indicated by the manufacturer: 2 µL of cDNA, 2 µL of each amplification primer (2 µM), 10 µL of iQ SYBR® Green Supermix (BioRad Laboratories Incorporated Hercules, CA) and 4 µL of sterile milli-Q water. qRT-PCR was performed in triplicate for each sample. The primers for the endogenous genes used as reference for normalization were designed based on expression stability data in *Nicotiana tabacum* and *Nicotiana benthamiana* previously reported (Liu et al., 2012; Schmidt and Delaney, 2010). The primers for amplification of M-GP5 and T-GP5 were design from the sequence of each peptide nanoparticle sequence (Table 2). Primer design was performed using Primer-Blast (Ye et al., 2012), and Oligo Evaluator™ software (Sigma-Aldrich). The relative expression was determined by evaluating the expression by the 2–ΔΔCT method (Livak and Schmittgen, 2001).

Total soluble protein extraction and SDS-PAGE

Infiltrated leaves were cut and immediately frozen in liquid nitrogen. Frozen tissue was macerated with mortar and pestle and mixed with extraction buffer (50 mM sodium phosphate pH 7.4, 100 mM NaCl, 10 mM EDTA pH 8.0, 5 mg/mL ascorbic acid, 1mM PMSF, inhibitor cocktail, 0.1% triton X-100, 1% sodium bisulphite) using 1

mL of extraction buffer for each gram of fresh tissue. The mix was incubated for 10 min at room temperature and then centrifuged at 14,000 x *g* for 20 min at 4°C. The supernatant was filtered with three layers of miracloth and then with 0.8, 0.45 and 0.22 µm cellulose acetate filters. Protein quantification in total soluble extracts was estimated by the Bradford (1976) assay with bovine serum albumin as a protein standard. 30 µg of total soluble protein were separated by 12% SDS-PAGE. The samples were mixed with 5X loading buffer with β mercaptoethanol (Sigma) and then were boiled for 5 min at 95°C. After migration, the gels were fixed in water containing 10% (v/v) acetic acid and 40% (v/v) methanol, and then washed three times in water. The proteins were visualized by staining with the colloidal Coomassie blue (Sigma).

Peptide nanoparticles purification

The purification protocol employed was based on those used by Hu et al. (2008) and Rao et al. (1994). Briefly, total soluble extracts were mixed with an equal volume of chloroform, stirred for 60 min at 25°C and then centrifuged at 12,000 xg for 20 minu at 4°C. The supernatant was recovered, loaded on a 10% sucrose cushion and centrifuged at 130,000 xg for 2.5 h at 4°C. The pellet was recovered and mixed in resuspension buffer (0.05 M sodium acetate, 0.008 M magnesium acetate, pH 4.5) and then separated on a 10-40% sucrose gradient at 130,000 xg for 1 h at 4°C. Bottom fraction was collected and centrifuged again at 245,400 xg for 2 h at 4°C. Peptide nanoparticles were then purified with PrepEase® Histidine Tagged protein purificaion kit (USB Corporation, Cleveland Ohio USA).

Electron microscopy

Fresh samples were adsorbed on formvar/carbon-coated cupper grids and negative stained with 2% phosphotungstic acid, pH 5.5 for 10 min. The stained peptide nanoparticles were observed by transmission electron microscopy (Morgagni series 5005, Phillips).

RESULTS

In this work, we aimed to confirm whether the peptide nanoparticles containing the GP5 epitope B of PRRSV could be obtained using the plant transient expression platform. To this end, two chimeric constructs were prepared and transiently expressed in leaves of *N. sylvestris*. The sequences had previously been codon-optimized for expression in plants. We designed two constructs, M-GP5 which yields icosahedral nano-particles whereas T-GP5 yields octahedral nanoparticles. According to Babapoor et al. (2011), M-GP5 seems to contain the highest density of the epitope. We will be testing them separately in future immunological challenges in mice and pigs. As a first step towards determining whether nanoparticles could assemble in plants, we purified the particles and performed an analysis by TEM. As shown in Figure 1, we were able to obtain particles in both cases with sizes between 16-25 nm. The size and morphology of the nanoparticles closely resembled those obtained in bacteria (Babapoor et al., 2011; Kaba et al., 2009*;* Pimentel et al., 2009*).* This data demonstrated that nanoparticles were successfully formed in the plant cytoplasm.

In order to confirm the expression of the particles in plants, we obtained the total soluble protein of plant extracts and separated them by SDS-PAGE. We observed that a protein with size about 15 KDa, corresponding to the monomer size of peptide nanoparticles in both versions, was clearly detectable in samples expressing the constructs M-GP5 or T- GP5 and it was not detectable in extracts of non-infiltrated plants (Figure 2). This band was similar in relative molecular mass to the purified protein.

To quantify the relative expression of the chimeric particles M-GP5 and T-GP5, we employed quantitative real time PCR using RNA from infiltrated and non-infiltrated plants. We observed that both constructs were detectable by this technique (Figure 3). Expression levels for infiltrated and non-infiltrated plants were normalized using values of the 18S (18S ribosomal RNA) and eEF1a (eucaryotic elongation factor 1α) constitutive, endogenous genes.

DISCUSSION

Many attempts have been made at developing an efficacious vaccine against PRRS. Current vaccines present several shortcomings but the main one is that the efficacy may vary upon heterologous challenge. This may be due to the high variability and the evasion mechanisms of the virus. Even though a number of viral antigens have been expressed in various systems, including plants, the efficacy of such an approach is still in doubt.

In this sense, it has been suggested that the mammalian immune system has evolved to detect repetitive patterns present in pathogens but absent in mammal, pathogen-associated molecular patterns (PAMP) (Bachmann and Jennings, 2010). The plant-based nanoparticles obtained in this work resemble those obtained by Raman et al. (2006) in *E. coli* in size and appearance and present a repetitive pattern.

When the sequence of these nanoparticles was expressed in *E. coli* and purified, no particles were obtained, they had to be subjected to several folding treatments *in vitro* for the particles to assemble (Raman et al., 2006; Pimentel et al. 2009). However, in our case, the nanoparticles were able to self-assemble spon-taneously in the plant cytoplasm without any further treatment. In this case, our platform might be superior to *E. coli* for nanoparticles expression and folding.

We were interested to determine if the protein making up the particles was detectable by SDS-PAGE. A band of about 15 kDa, which is the expected size of the monomer, was detectable in crude extracts from infiltrated plants but not in crude extracts from non-infiltrated plants. The fact that the unpurified protein was easily detectable in crude extracts by SDS-PAGE gels means that it is been synthesized at significant levels

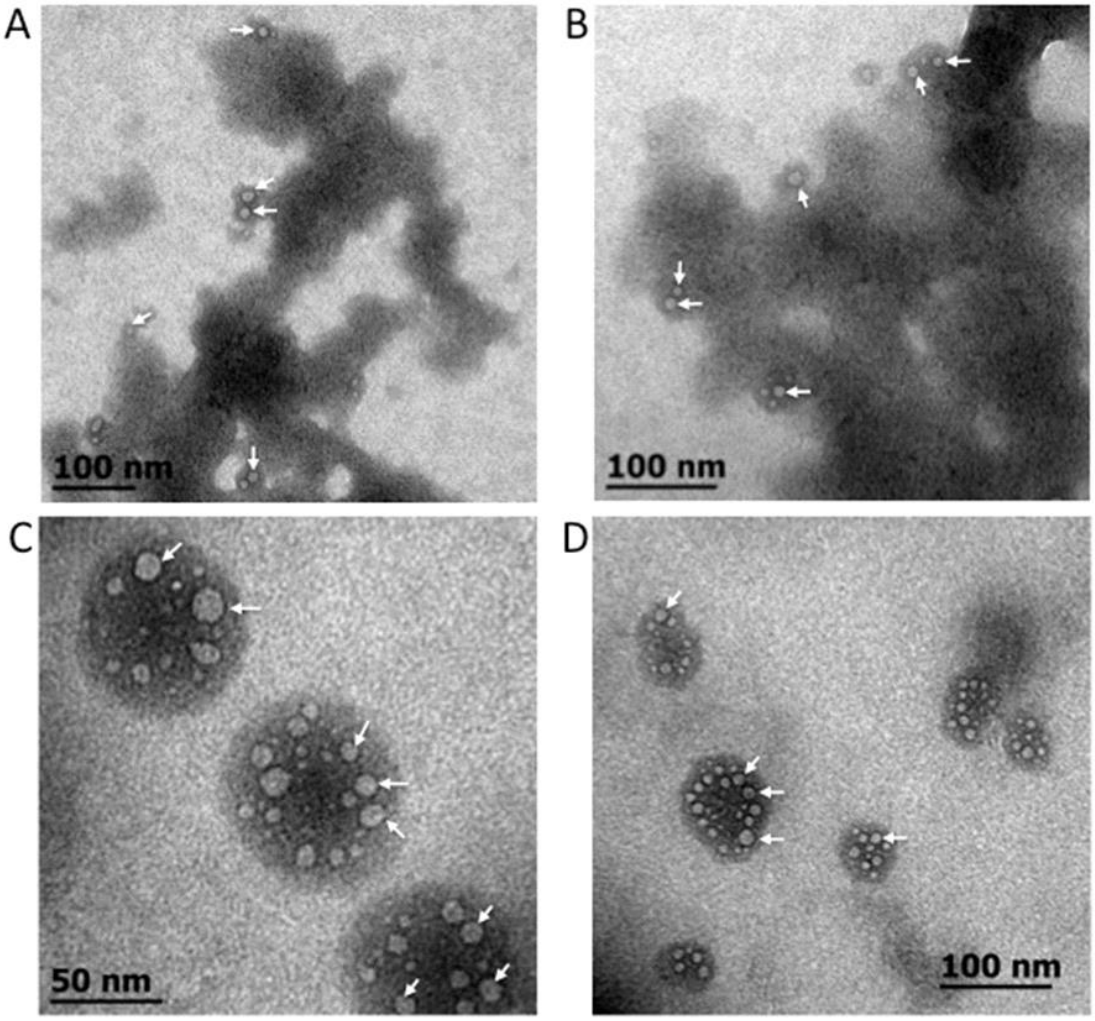

Figure 1. Electron micrographs of the peptide nanoparticles produced in plants. The particles were purified as described in the text and observed by TEM. A-B correspond to M-GP5 and C-D correspond to T-GP5. Particles were stained with 2% phosphotungstic acid at pH 5.5. The white arrows indicate 15-25 nm particles.

because it is known that only abundant proteins are detected by this technique (Virgen-Ortiz et al., 2013). We are in the process of obtaining specific antibodies against the nanoparticle protein and in the next step of the project, we will quantify the nanoparticle protein by ELISA.

In order to confirm transcription of the transgenes we employed qRT-PCR. Interestingly, T-GP5 seemed to express at a much higher level than M-GP5. This result indicates that both transgenes are being expressed in the plant cells but we have no explanation why one of them expresses much better that the other one.

In this work, we have shown that protein nanoparticles containing the epitope B from PRRSV can be synthesized and efficiently self-assembled inside plant cells. The potential for these nanoparticles to serve as platforms for vaccines is quite obvious. Recombinant antigens can be prepared in a matter of weeks to new variants of the antigen which makes this approach a powerful tool for a fast and effective response when new virus strains arise. Nevertheless, the platform has some limitations such as plant glycosylation, which is different from that of animal cells and the fact that antigen purification from plant cells is still a somewhat cumbersome process. In the next phase of the project, we will immunize mice to show immunogenicity with pigs, which will be later challenged with a circulating strain of the virus.

Conclusion

Peptide nanoparticles containing a PRRSV epitope can be efficiently expressed and self-assembled in plant cells.

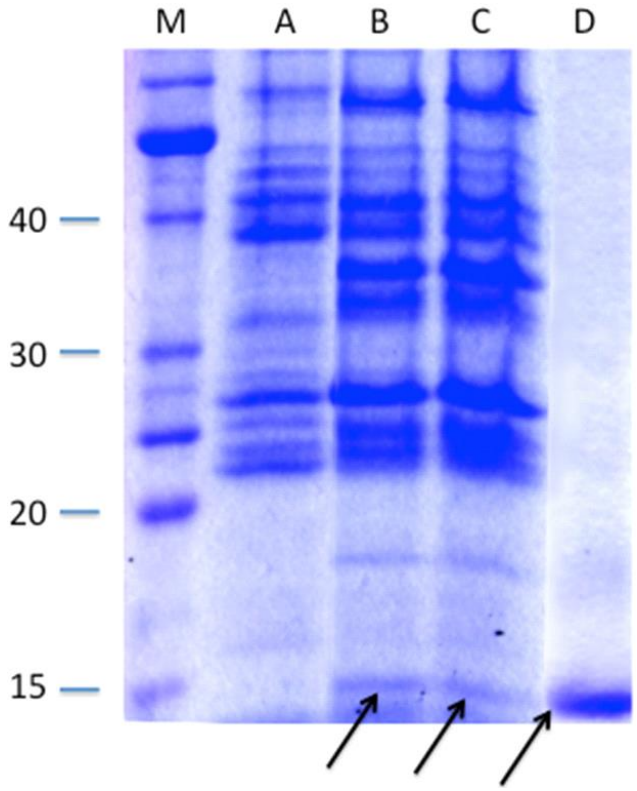

Figure 2. Analysis of total soluble proteins extracted from infiltrated and non-infiltrated plant by SDS-PAGE. Thirty micrograms of total protein were loaded per lane. Lanes are as follows: M, Molecular weight markers; A, non-infiltrated plants; B, plants infiltrated with M-GP5; C, plants infiltrated with T-GP5; D, purified protein from M-GP5. Arrows indicate the protein corresponding to the nanoparticles. Numbers on the left indicate the molecular weight markers in kDa.

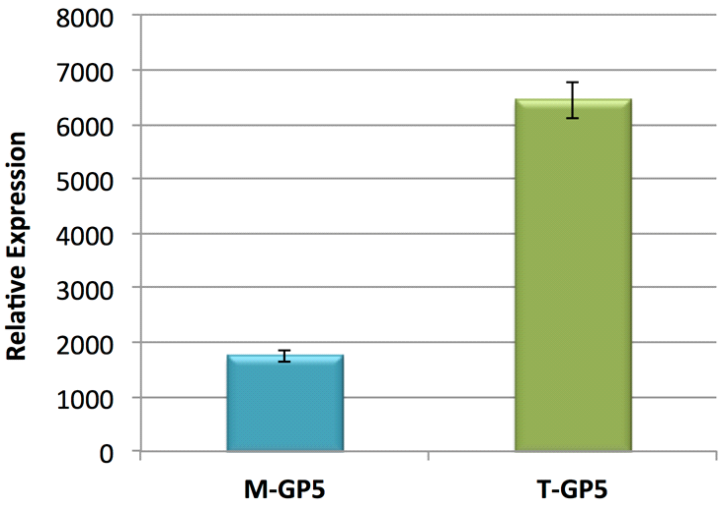

Figure 3. Analysis of the relative expression of M-GP5 and T-GP5 peptide nanoparticles by qRT-PCR. The relative expression of each gene (expressed as fold over the reference genes) was evaluated employing specific primers as described in the text. The expression of peptide nanoparticles was normalized against the expression of the endogenous, reference genes PP2 and L23.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Babapoor S, Neef T, Mittelholzer C, Girshick T, Garmendia A, Shang H, Khan MI, Burkhard P (2011). A Novel Vaccine Using Nanoparticle Platform to Present Immunogenic M2e against Avian Influenza Infection. Influenza Res. Treat. vol. 2011, Article ID 126794, 12 pages, 2011. doi:10.1155/2011/126794.

Bachmann MF, Jennings GT (2010). Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat. Rev. Immunol. 10:787-796.

Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM (1993). The influence of antigen organization on B cell responsiveness. Science 262:1448-1451.

Bae K, Choi J, Jang Y, Ahn S, Hur B (2009). Innovative vaccine production technologies: the evolution and value of vaccine production technologies. Arch. Pharm. Res. 32:465-480.

Botner A, Strandbygaard B, Sorensen KJ, Have P, Madsen KG, Madsen ES, Alexandersen S (1997). Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. Vet. Rec. 141:497-499.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Cano JP, Dee SA, Murtaugh MP, Pijoan C (2007). Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. Vaccine 25:4382-4391.

Chackerian B, Lenz P, Lowy DR, Schiller JT (2002). Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. J. Immunol. 169:6120-6126.

Chan HT, Chia MY, Pang VF, Jeng CR, Do YY, Huang PL (2013). Oral immunogenicity of porcine reproductive and respiratory syndrome virus antigen expressed in transgenic banana. Plant Biotechnol. J. 11:315-324.

Chen Q, Lai H (2013). Plant-derived virus-like particles as vaccines. Hum. Vaccine Immunother. 9:26-49.

Chen X, Liu J (2011). Generation and immunogenicity of transgenic potato expressing the GP5 protein of porcine reproductive and respiratory syndrome virus. J. Virol. Methods 173:153-158.

Chia MY, Hsiao SH, Chan HT, Do YY, Huang PL, Chang HW, Tsai YC, Lin CM, Pang VF, Jeng CR (2011). Evaluation of the immunogenicity of a transgenic tobacco plant expressing the recombinant fusion protein of GP5 of porcine reproductive and respiratory syndrome virus and B subunit of Escherichia coli heat-labile enterotoxin in pigs. Vet. Immunol. Immunopathol. 140:215-225.

Coconi-Linares N, Ortega-Davila E, Lopez-Gonzalez M, Garcia-Machorro J, Garcia-Cordero J, Steinman RM, Cedillo-Barron L, Gomez-Lim MA (2013). Targeting of envelope domain III protein of DENV type 2 to DEC-205 receptor elicits neutralizing antibodies in mice. Vaccine 31:2366-2371.

Cruz JL, Zuniga S, Becares M, Sola I, Ceriani JE, Juanola S, Plana J, Enjuanes L (2010). Vectored vaccines to protect against PRRSV. Virus Res. 154:150-160.

Diaz I, Darwich L, Pappaterra G, Pujols J, Mateu E (2006). Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. Virology 351:249-259.

Dokland T (2010). The structural biology of PRRSV. Virus Res. 154:86-97.

Fang Y, Treffers EE, Li Y, Tas A, Sun Z, van der Meer Y, de Ru AH, van Veelen PA, Atkins JF, Snijder EJ, Firth AE (2012) Efficient -2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. Proc. Natl. Acad. Sci. USA 109:E2920-2928.

Han M, Yoo D (2014). Engineering the PRRS virus genome: Updates and perspectives. Vet. Microbiol. 174:279-295.

Hanada K, Suzuki Y, Nakane T, Hirose O, Gojobori T (2005). The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol. Biol. Evol. 22:1024-1031.

Holtkamp D, Kliebenstein JB, Neumann EJ, Zimmerman JJ, Rotto HF, Yoder TK, Wang C, Yeske PE, Mowrer CL, Haley CA (2013). Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J. Swine Health Prod. 21:72-84.

Hu J, Zhang C (2014). Porcine reproductive and respiratory syndrome virus vaccines: current status and strategies to a universal vaccine. Transbound. Emerg. Dis. 61:109-120.

Hu Y, Zandi R, Anavitarte A, Knobler CM, Gelbart WM (2008). Packaging of a polymer by a viral capsid: the interplay between polymer length and capsid size. Biophys . J. 94(4):1428-1436.

Huang C, Zhang Q, Feng WH (2015) Regulation and evasion of antiviral immune responses by porcine reproductive and respiratory syndrome virus. Virus Res. 202:101-111.

Jegerlehner A, Tissot A, Lechner F, Sebbel P, Erdmann I, Kundig T, Bachi T, Storni T, Jennings G, Pumpens P, Renner WA, Bachmann MF (2002). A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. Vaccine 20:3104-3112.

Jiang Y, Xiao S, Fang L, Yu X, Song Y, Niu C, Chen H (2006). DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. Vaccine 24:2869-2879.

Kaba SA, Brando C, Guo Q, Mittelholzer C, Raman S, Tropel D, Aebi U, Burkhard P, Lanar DE (2009). A nonadjuvanted polypeptide nanoparticle vaccine confers long-lasting protection against rodent malaria. J. Immunol. 183:7268-7277.

Kappes MA, Faaberg KS (2015) PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. Virology 479-480:475-486.

Kappes MA, Miller CL, Faaberg KS (2013) Highly divergent strains of porcine reproductive and respiratory syndrome virus incorporate multiple isoforms of nonstructural protein 2 into virions. J. Virol. 87: 13456-13465.

Kappes MA, Miller CL, Faaberg KS (2015) Porcine reproductive and respiratory syndrome virus nonstructural protein 2 (nsp2) topology and selective isoform integration in artificial membranes. Virology 481:51-62.

Kimman TG, Cornelissen LA, Moormann RJ, Rebel JM, Stockhofe-Zurwieden N (2009). Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27:3704-3718.

Kumar BV, Raja TK, Wani MR, Sheikh SA, Lone MA, Gowher N, Azooz MM, Muhammad Y, Maryam S, Parvaiz A (2013). Transgenic plants as green factories for vaccine production. Afr. J. Biotechnol. 12:6147-6158.

Li B, Xiao S, Wang Y, Xu S, Jiang Y, Chen H, Fang L (2009a). Immunogenicity of the highly pathogenic porcine reproductive and respiratory syndrome virus GP5 protein encoded by a synthetic ORF5 gene. Vaccine 27:1957-1963.

Li J, Jiang P, Li Y, Wang X, Cao J, Wang X, Zeshan B (2009b). HSP70 fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus enhanced the immune responses and protective efficacy against virulent PRRSV challenge in pigs. Vaccine 27:825-832.

Liu D, Shi L, Han C, Yu J, Li D, Zhang Y (2012). Validation of reference genes for gene expression studies in virus-infected *Nicotiana*

benthamiana using quantitative real-time PCR. PLoS One 7:e46451.
Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.
Lunney JK, Benfield DA, Rowland RR (2010). Porcine reproductive and respiratory syndrome virus: an update on an emerging and re-emerging viral disease of swine. Virus Res. 154:1-6.
Mardassi H, Massie B, Dea S (1996). Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology 221: 98-112.
Murtaugh MP, Stadejek T, Abrahante JE, Lam TT, Leung FC (2010). The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. Virus Res. 154:18-30.
Nam HM, Chae KS, Song YJ, Lee NH, Lee JB, Park SY, Song CS, Seo KH, Kang SM, Kim MC, Choi IS (2013). Immune responses in mice vaccinated with virus-like particles composed of the GP5 and M proteins of porcine reproductive and respiratory syndrome virus. Arch. Virol. 158:1275-1285.
Neumann EJ, Kliebenstein JB, Johnson CD, Mabry JW, Bush EJ, Seitzinger AH, Green AL, Zimmerman JJ (2005). Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J. Am. Vet. Med. Assoc. 227:385-392.
Ostrowski M, Galeota JA, Jar AM, Platt KB, Osorio FA, Lopez OJ (2002). Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76:4241-4250.
Pimentel TA, Yan Z, Jeffers SA, Holmes KV, Hodges RS, Burkhard P (2009). Peptide nanoparticles as novel immunogens: design and analysis of a prototypic severe acute respiratory syndrome vaccine. Chem. Biol. Drug Des. 73:53-61.
Plagemann PG, Rowland RR, Faaberg KS (2002). The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch. Virol. 147:2327-2347.
Qiu HJ, Tian ZJ, Tong GZ, Zhou YJ, Ni JQ, Luo YZ, Cai XH (2005). Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. Vet. Immunol. Immunopathol. 106:309-319.
Raman S, Machaidze G, Lustig A, Aebi U, Burkhard P (2006). Structure-based design of peptides that self-assemble into regular polyhedral nanoparticles. Nanomedicine 2:95-102.
Rao AL, Duggal R, Lahser F, Hall TC (1994). Analysis of RNA replication in plant viruses. In: Methods in Molecular Virology Techniques. Academic Press. 4:216-236.
Rascón-Castelo E, Burgara-Estrella A, Mateu E, Hernández J (2015). Immunological features of the non-structural proteins of porcine reproductive and respiratory syndrome virus. Viruses 7:873-886.
Rebeaud F, Bachmann M (2012). Virus-Like Particles as Efficient Delivery Platform to Induce a Potent Immune Response. In: Baschieri S (Ed.). Innovation in Vaccinology: From Design, Through to Delivery and Testing. Springer. pp. 87-122.
Rowland RR, Morrison RB (2012). Challenges and opportunities for the control and elimination of porcine reproductive and respiratory syndrome virus. Transbound. Emerg. Dis. 59(1):55-59.
Salguero FJ, Frossard JP, Rebel JM, Stadejek T, Morgan SB, Graham SP, Steinbach F (2015) Host-pathogen interactions during porcine reproductive and respiratory syndrome virus 1 infection of piglets. Virus Res. 202:135-143.
Schmidt GW, Delaney SK (2010). Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. Mol. Genet. Genomics 283: 233-241.
Snijder EJ, Kikkert M, Fang Y (2013). Arterivirus molecular biology and pathogenesis. J. Gen. Virol. 94:2141-2163.
Veit M, Matczuk AK, Sinhadri BC, Krause E, Thaa B (2014) Membrane proteins of arterivirus particles: structure, topology, processing and function. Virus Res. 194:16-36.
Vimolmangkang S, Gasic K, Soria-Guerra R, Rosales-Mendoza S, Moreno-Fierros L, Korban SS (2012). Expression of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus in soybean seed yields an immunogenic antigenic protein. Planta 235: 513-522.
Virgen-Ortiz JJ, Ibarra-Junquera V, Escalante-Minakata P, Osuna-Castro JA, Ornelas-Paz J, Mancilla-Margalli NA, Castaneda-Aguilar RL (2013). Improving sodium dodecyl sulfate polyacrylamide gel electrophoresis detection of low-abundance protein samples by rapid freeze centrifugation. Anal Biochem. 443(2):249-251.
Wissink EH, van Wijk HA, Kroese MV, Weiland E, Meulenberg JJ, Rottier PJ, van Rijn PA (2003). The major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome virus contains a neutralization epitope in its N-terminal ectodomain. J. Gen. Virol. 84:1535-1543.
Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134.
Zhang H, Kono H, Kubota S (2014). An Integrated Epidemiological and Economic Analysis of Vaccination against Highly Pathogenic Porcine Reproductive and Respiratory Syndrome (PRRS) in Thua Thien Hue Province, Vietnam. Asian Australas. J. Anim. Sci. 27:1499-1512.
Zheng Q, Chen D, Li P, Bi Z, Cao R, Zhou B, Chen P (2007). Co-expressing GP5 and M proteins under different promoters in recombinant modified vaccinia virus ankara (rMVA)-based vaccine vector enhanced the humoral and cellular immune responses of porcine reproductive and respiratory syndrome virus (PRRSV). Virus Genes 35:585-595.