



Short communication

Minimally processed crude leaf extracts of *Nicotiana benthamiana* containing recombinant foot and mouth disease virus-like particles are immunogenic in mice

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ARTICLE INFO

Article history:

Received 7 March 2018

Received in revised form 27 August 2018

Accepted 20 September 2018

Keywords:

Foot-and-mouth disease virus

Virus-like particles

Transient expression

Nicotiana benthamiana

ABSTRACT

Foot-and-mouth disease (FMD) remains one of the most feared viral diseases affecting cloven-hoofed animals, and results in severe economic losses. Currently available vaccines are based on inactivated FMD virus (FMDV). The use of recombinant FMDV-like particles (VLPs) as subunit vaccines has gained importance because of their immunogenic properties and safety. We evaluated the production of FMD VLPs, via *Agrobacterium*-mediated transient expression, and the immunogenicity of these structures in mice. Leaves were infiltrated with pEAQ-HT and pRIC 3.0 vectors encoding the capsid precursor P1-2A and the protease 3C. The recombinant protein yield was 3–4 mg/kg of fresh leaf tissue. Both groups of mice immunized with purified VLPs and mice immunized with the crude leaf extract elicited a specific humoral response with similar antibody titers. Thus, minimally processed plant material containing transiently expressed FMD VLPs could be a scalable and cost-effective technology for the production of a recombinant subunit vaccine against FMDV.

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1. Introduction

Foot and mouth disease virus (FMDV) is the etiological agent of foot and mouth disease (FMD), one of the most contagious diseases in mammals, affecting cloven-hoofed animals like cattle, swine, goats and sheep. The clinical signs of FMD include high fever and the formation of vesicular lesions on the feet, in and around the oral cavity, and on the mammary glands of females. Consequently, affected animals have severe lameness and reduced food intake and milk production, which lead to significant economic losses resulting from reduced productivity, losses due to culling of infected animals or even of the whole herd in areas of disease outbreaks, and the associated restrictions to international trade [1]. Due to the highly contagious nature and economic impact of FMD on livestock, strict disease control is required. One of the

current control measures used to prevent the spread of FMD includes regular mass vaccination of animals in endemic regions. Currently, the FMD vaccine is produced by growing FMDV in BHK-21 cell cultures under biosecure conditions and inactivating it with binary ethylenimine [2,3]. This vaccine has proved to be effective in controlling FMD outbreaks across the world, but there are still a number of concerns and limitations regarding its use and production, particularly in FMD-free countries. These concerns and limitations include the potential escape of infectious virus to the environment, the high costs of the high-containment facilities required for manufacturing, the problem of discriminating between vaccinated and field-virus infected animals, the need for adequate cold chain of formulated vaccines, and difficulties of certain serotypes and subtypes to grow efficiently in cell cultures [4,5]. In order to address these problems, new technologies have been investigated. Among these, the most promising subunit vaccine candidates are based on recombinant FMDV-like particles [6–8]. Virus-like particles (VLPs) are highly effective as immunogens since they display the complete antigenic epitopes of the virus in the correct conformation and in a highly repetitive manner [9]. Moreover, since these structures do not contain the infectious genomic material, they have unique advantages in terms of safety [8,10].

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The production of recombinant FMD VLPs requires the simultaneous expression of the capsid precursor P1-2A and the protease 3C, the latter of which cleaves the precursor to produce the structural proteins VP0, VP3, and VP1. These proteins subsequently self-assemble to produce the viral capsid [11]. FMD VLPs have been produced in different expression systems with different levels of success (reviewed in [4,12]). Plants are an economic and safe platform for vaccine development due to ease in scalability and low risk of contamination with endotoxins or mammalian pathogens, as well as reduced regulatory issues [13]. Traditionally, production of recombinant proteins in plants has relied on the generation of stable transgenic lines, but this has the limitation that it usually takes a long time to produce a stable transformed transgenic plant. This problem can be solved by transient expression technologies, which enable the production of recombinant proteins at a timescale that is comparable to other prokaryotic and eukaryotic expression systems, and can be scaled up to commercially relevant production levels.

Until 2018, only two approaches to produce plant-based FMD VLPs (both using transgenic plants) had been reported. Although these approaches were not able to conclusively demonstrate that the recombinant capsid proteins assembled into VLPs, immunization with foliar extracts from transgenic alfalfa [14] and tomato plants [15] proved to evoke a strong antibody response and protection in mice and guinea pigs, respectively. During the last few years, there has been an increasing trend towards the use of transient expression of recombinant proteins in plants, mostly because this approach is fast and flexible, allowing the rapid expression of high concentrations of heterologous proteins in a matter of days [13]. This could be particularly interesting in the face of an outbreak with a new FMDV serotype, when it is necessary to rapidly produce the new vaccine antigen. In line with this, our group has recently reported for the first time the production and assembly of FMD VLPs in *Nicotiana benthamiana* by transient expression in the absence of the viral 3C protease [16]. Although the yield of the partially purified VLPs was low, preliminary experiments in mice showed that these structures could stimulate the production of FMDV-specific antibodies.

It is widely accepted that, in addition to protein expression levels, downstream processing is a bottleneck, not only for plant molecular farming but for other different production systems [17], contributing up to 80% of the total production costs [18,19]. Therefore, the implementation of minimally-processed plant tissue as an immunogen, which allows reducing downstream costs, could be a great advantage both in general and particularly for veterinary applications.

Based on all the above arguments, the main purpose of the present work was to evaluate an agroinfiltration-mediated transient expression system [20] for the production of recombinant FMD VLPs by using two different expression vectors, and to compare the use of these structures as immunogens in mice, either purified or in the crude extracts.

2. Materials and methods

2.1. Constructs and virus

The sequences encoding for the capsid precursor P1-2A and the protease 3C from FMDV A/Arg/01 were codon-optimized for expression in *N. benthamiana* and synthesized as a single ORF by GenScript (Piscataway, NJ, USA). The complete cassette P12A3C (2973 bp) was subcloned into the replicating vector pRIC 3.0 [21] or the non-replicating vector pEAQ-HT [22], using *AflIII/XhoI* or *AgeIII/XhoI* restriction enzymes sites, respectively. The recombinant vectors were named pRIC-P12A3C and pEAQ-P12A3C.

FMDV serotype A/Arg/01, propagated in BHK-21 cells, inactivated with binary ethyleneimine and purified by sucrose gradient

(iFMDV) was used as a positive control for protein characterization assays and to formulate the experimental vaccine (positive control).

2.2. Transformation of *Agrobacterium tumefaciens*

A. tumefaciens GV3101:pMP90RK and *A. tumefaciens* LBA4404 were electroporated with 400 ng of the recombinant pRIC-P12A3C and pEAQ-P12A3C vectors, respectively in a 0.1 cm gap electroporation cuvette using a Bio-Rad Gene Pulser set at 1.8 kV, 25 μ F and 200 Ω . The cells were incubated in Luria Bertani (LB) broth for 2 h at 27 °C and were then plated on LB agar plates containing the appropriate antibiotics. Recombinant pEAQ-HT clones were selected on LB agar plates containing 30 μ g/ml kanamycin and 50 μ g/ml rifampicin. The pRIC 3.0 clones were selected under the same conditions except that plates also contained 50 μ g/ml carbenicillin. The transformation was verified by colony PCR. To that purpose, a sterile toothpick was used to pick up a few cells from each colony and transfer them to a PCR tube, where cells were resuspended in the PCR reaction mixture. This mixture contained Pfu DNA polymerase (1.5 U; Thermo Scientific), dNTPs (0.2 mM), MgSO₄ (2 mM), Pfu DNA polymerase buffer and specific primers for either pEAQ-HT or pRIC 3.0 vectors. The cycling conditions were: 94 °C, 5 min; followed by 35 cycles of 95 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min 30 s. After PCR was complete, 10 μ l of each reaction was subjected to electrophoresis on a 1% agarose gel, to verify that the desired genetic construct was present.

2.3. Transient protein expression in *N. benthamiana* leaves

Starter cultures of recombinant pRIC-P12A3C and pEAQ-P12A3C *Agrobacterium* were used to inoculate induction media (LB broth with 10 mM MES, 2 mM MgCl₂ and 20 μ M acetosyringone) with the addition of the appropriate antibiotics, and grown overnight in an orbital shaker at 27 °C, 120 rpm. The cells were pelleted by centrifugation at 1000 g for 10 min and resuspended in infiltration medium (3% sucrose, 200 μ M acetosyringone, 10 mM MES and 10 mM MgCl₂, pH 5.6). The cultures were diluted to an OD₆₀₀ of 0.5 and 1.0 in infiltration medium and kept at room temperature for 1–2 h to allow induction of *vir* genes. Small-scale infiltration was performed by direct injection of leaves with the different vectors, to select optimal conditions for recombinant protein expression. For this purpose, leaves from 6 to 8-week-old *N. benthamiana* plants were infiltrated by injecting the bacterial suspension into the abaxial air spaces from the underside of the leaf, using a blunt-ended syringe. Six leaves (three plants, two leaves per plant) were infiltrated with each bacterial culture (pEAQ-P12A3C and pRIC-P12A3C) at both OD₆₀₀ values (0.5 and 1.0) and harvested at 3 and 4 days post infiltration (dpi). As negative controls, plants were infiltrated with *Agrobacterium* cultures containing the empty pEAQ or pRIC vector (mock-infiltrated leaves) at an OD₆₀₀ of 1.0. The plants were grown at 22 °C under conditions of 16 h light, 8 h dark at light intensity of 60–80 μ E/m²/s.

Vacuum infiltration of *N. benthamiana* plants was performed as described by Maclean, et al. [23] with the conditions previously set up in the small scale infiltration assay. To that end, 20 plants were infiltrated by submerging each plant into 1 L of each *Agrobacterium* cultures at OD₆₀₀ values of 0.5 for pEAQ-P12A3C, and 1.0 for pRIC-P12A3C and empty vectors. The plants were grown for 4 days as described above.

2.4. Protein extraction and western blot

For expression optimization studies, six 8-mm leaf disks (clipped with the lid of a microcentrifuge tube, fresh weight

approximately 20 mg) were harvested from the agroinfiltrated leaves, pooled, ground up in liquid nitrogen and resuspended in 150 μ l of extraction buffer (PBS 1X supplemented with Roche EDTA-free complete protease inhibitor cocktail). Extracts were clarified by centrifugation at 15,000g on a bench top centrifuge. For western blot analysis, the crude plant extracts were incubated at 90°C for 10 min in loading buffer [24]. The proteins were separated on 10% SDS polyacrylamide gels where equal amounts of total protein (150 μ g as determined by Bradford assay) were loaded in each lane. After electrophoresis the proteins were transferred onto nitrocellulose membranes using a Trans-blot®SD semi-dry transfer cell (Bio-Rad). Membranes were probed with a 1:100 dilution of anti-FMDV A/Arg/01 guinea pig serum and subsequently with a 1:30,000 dilution of anti-guinea pig alkaline phosphatase-conjugated secondary antibody (Sigma–Aldrich). Detection was performed with 5-bromo-4-chloro-3-indoxyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) phosphatase substrate (BCIP/NBT 1-component, KPL).

Vacuum infiltrated plant leaves were harvested at 4 dpi and 20–40 g of leaf tissue was used for protein extraction. Leaves were ground into a fine powder in liquid nitrogen using a mortar and pestle, and three volumes (w/v) of extraction buffer were added. Extracts were clarified by centrifugation at 15,000g and filtered through two layers of Miracloth™ (Merck). The expression levels of FMDV proteins in these filtered crude extracts were quantified by enzyme-linked immunosorbent assay (ELISA).

2.5. Sucrose gradient

The filtered crude extracts from the mock-infiltrated leaves or from the leaves infiltrated with pEAQ-P12A3C or pRIC-P12A3C were loaded onto a double sucrose cushion (40–20%) in NTE buffer (0.1 M NaCl, 0.05 M Tris, 0.004 M EDTA-disodium dehydrate, pH 8.0), and centrifuged at 150,000g for 1.5 h in a SW 32 Ti rotor (Beckman). The interface layer was collected, solubilized in NTE buffer, overlaid onto a 15–45% sucrose gradient and centrifuged at 44,000g for 16 h at 4°C. Fractions (1 ml) were collected from the bottom of the tubes and analyzed by ELISA. As a positive control, iFMDV was run in the same conditions. Fractions corresponding to empty capsids (75S) were pooled, quantified by ELISA, and used for transmission electron microscopy (TEM) analysis and mouse immunizations. The same fractions obtained with the crude extract of mock-infiltrated leaves were also pooled and used as negative control (mock-purified). Fractions corresponding to iFMDV virions (146S) were pooled, quantified by ELISA and used as control for TEM analysis.

2.6. ELISA

The analysis of sucrose gradient fractions, and the quantification of recombinant proteins in crude leaf extracts and in the pooled fractions purified by sucrose gradient, was carried out by ELISA as previously described [25]. Briefly, microtiter plates were coated with anti-FMDV rabbit serum (1/3000) in carbonate-bicarbonate buffer, pH 9.6, at 4°C overnight. After washing with PBS 0.1% Tween-20 and blocking with 1% ovalbumin in PBS 0.1% Tween-20, samples were added and incubated on plates at 37°C for an hour. Known amounts of iFMDV were two-fold serially diluted and added to the wells for standard curve generation. Plates were then incubated with anti-FMDV guinea pig serum, followed by horseradish peroxidase-conjugated anti-guinea pig goat serum (KPL). O-phenylenediamine-H₂O₂ was used as substrate. The reaction was stopped with sulfuric acid 12%. Absorbance was recorded at 492 nm (A₄₉₂) in a microplate reader (Thermo Scientific MultiskanFC).

2.7. Transmission electron microscopy (TEM)

Pooled fractions obtained from the sucrose gradient were fixed to carbon-coated copper grids (mesh size 200), washed and negatively stained with 2% uranyl acetate for 1 min. Grids were viewed using a Technai G2 transmission electron microscope.

2.8. Immunization of mice and analysis of humoral immunity

The experiment was performed in accordance with protocols approved by the Institutional Committee for Care and Use of Experimental Animals, CICUAE (approval reference CICUAE INTA-CICVyA 60/2015). Eight-week-old BALB/c male mice (obtained from INTA-Castelar, Buenos Aires, Argentina) were randomly divided into six groups, with seven mice in each group. Groups A and B received 500 ng of purified VLPs produced by pEAQ-P12A3C and pRIC-P12A3C, respectively; as a negative control, group C received the same volume of mock-purified sample; group D received 120 μ l of crude leaf extract containing 150 ng of FMDV proteins produced by pRIC-P12A3C, and group E received the same volume of crude leaf extract of mock-infiltrated leaves. Mice in group F were immunized with 500 ng of iFMDV as a positive control. All the vaccines were formulated with Montanide ISA 50 (Seppic) as adjuvant in an adjuvant:antigen proportion of 60:40. Mice were intraperitoneally inoculated with 0.3 ml of each formulation and boosted 21 and 35 days post-inoculation (dpi). Sera were collected at 14, 28 and 42 dpi for humoral immunity analysis. Anti-FMDV antibodies from immunized mice serum were measured by ELISA. Immunolon II plates were coated with anti-FMDV rabbit serum in carbonate-bicarbonate buffer, pH 9.6, at 4°C overnight. After washing and blocking, iFMDV was added. Serial dilutions of mice serum samples were incubated on plates for half an hour. Anti-FMDV antibodies were detected using horseradish peroxidase-conjugated anti-mouse goat serum. O-phenylenediamine-H₂O₂ was used as substrate. The reaction was stopped with sulfuric acid 12%. The cut-off was established as the mean A₄₉₂ of the sera at 0 dpi plus three standard deviations (SD). Antibody titers are expressed as the log₁₀ of the reciprocal of the highest serum dilution which gives A₄₉₂ readings above the cut-off value.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad InStat Software (version 3.06). Kruskal–Wallis test and Dunn's multiple comparisons test were performed to assess statistically significant differences. P-values were considered to be significant if less than 0.05.

3. Results

The capsid precursor (P1–2A) and the protease 3C were codon optimized and cloned together in frame in two different vectors for expression in *N. benthamiana*, producing pEAQ-P12A3C and pRIC-P12A3C. These recombinant vectors were transformed in *A. tumefaciens* and further inoculated in *N. benthamiana* leaves. To determine the best conditions for recombinant protein expression, a small-scale agroinfiltration was initially performed.

Analysis of total protein extracts by western blot showed that the FMDV capsid precursor was successfully synthesized and processed by the protease 3C into the expected structural proteins VP0 (37 kDa), VP3 (23 kDa) and VP1 (23 kDa) using both the pEAQ-HT and pRIC 3.0 vectors, under all the conditions assayed (Fig. 1). No specific band was detected in the crude extract from mock-infiltrated leaves. When using the pEAQ-P12A3C vector, higher expression levels were detected at 4 dpi with an OD₆₀₀ = 0.5. Similar results were observed with pRIC-P12A3C with most

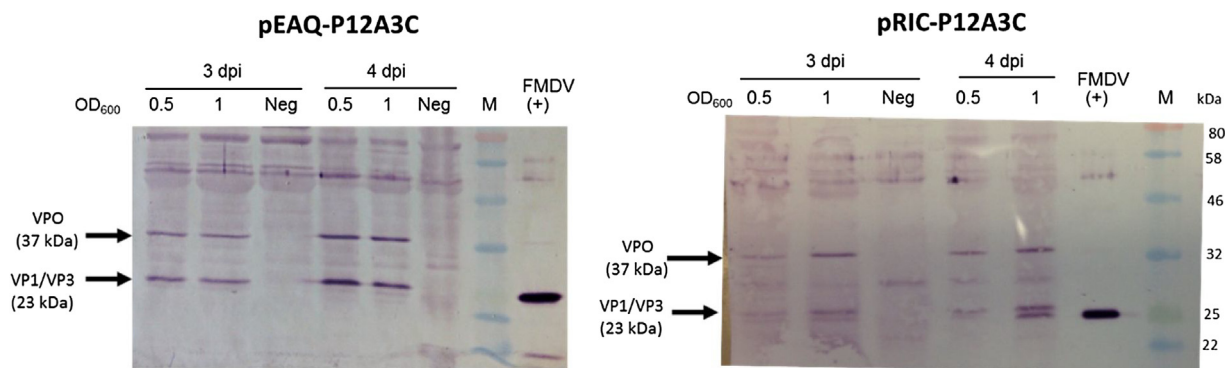


Fig. 1. Western blot analysis of crude *N. benthamiana* extracts. Leaves were infiltrated with pEAQ-P12A3C or pRIC-P12A3C *A. tumefaciens* cultures (approx. 1 ml per leaf) with an OD_{600} = 1.0 or 0.5, and mock-infiltrated (Neg), and harvested on days 3 and 4. The expected migration positions of the viral proteins VP0, VP3 and VP1 are indicated by arrows. Purified iFMDV was used as a positive control (+). M: Color Prestained Protein Standard (New England Biolabs).

protein detected at 4 dpi using an OD_{600} = 1.0. High molecular weight non-specific bands were also observed in lanes loaded with crude leaf extracts. This could be due to an excess of plant material loaded into the gel which binds non-specifically to the polyclonal guinea pig serum used as primary antibody. Increasing the time of harvest (5–7 days) resulted in necrosis of leaves infiltrated with the recombinant vectors, but not of mock-infiltrated leaves (data not shown), probably because of the toxicity of the 3C protease, which has proved to have adverse effects on protein expression [26–30].

With the optimal optical density of *Agrobacterium* cultures and time of harvest, the process was scaled up by vacuum infiltration. The expression levels of FMDV proteins in the crude extracts were quantified by ELISA and proved to be very similar for both recombinant vectors, reaching 3–4 mg/kg of fresh leaf tissue.

To determine whether the expressed proteins assembled into VLPs, the crude extract from the mock-infiltrated leaves or from the leaves infiltrated with pEAQ-P12A3C or pRIC-P12A3C was further analyzed by density gradient centrifugation. Crude extracts from leaves infiltrated with both recombinant vectors showed defined peaks of specific protein that sedimented at the same rate as the iFMDV empty capsids (75S) (Fig. 2). From these profiles it seems that one third of the recombinant proteins in the crude extracts are assembled into VLPs (~1–1.3 mg/kg of fresh leaf tissue). This was also confirmed by ELISA, analyzing the pooled fractions 15–17. Antigenic peaks in the fractions corresponding to pentamers (12S) were also identified (Fig. 2). No specific signal was observed in fractions obtained from the crude extracts of mock-infiltrated leaves.

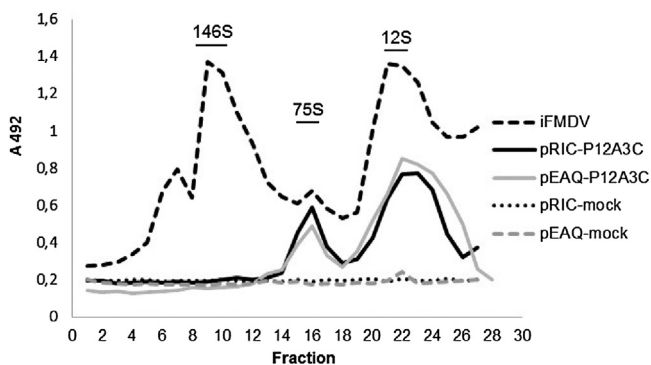


Fig. 2. Assembly of recombinant structural proteins into VLPs. Crude extracts of leaves infiltrated with pEAQ-P12A3C or pRIC-P12A3C (or mock-infiltrated) were loaded onto a double sucrose cushion (40–20%) and the interface layer was overlaid onto a 15–45% sucrose gradient. Fractions were collected and analyzed by ELISA. The known positions of the iFMDV A/Arg/01 virions (146S), empty capsids (75S), and pentamers (12S) are indicated.

In order to confirm that the peaks observed in the sucrose gradient were in fact FMDV VLPs, fractions 15–17 were pooled and analyzed by TEM. Electron microscopy of the pooled purified fractions obtained from the crude extracts of leaves infiltrated with pEAQ-P12A3C or pRIC-P12A3C, revealed the presence of spherical particles of approximately 30 nm in diameter (Fig. 3A–H), very similar in shape and size to natural FMDV virions (Fig. 3K and L), suggesting the presence of recombinant VLPs. Conversely to complete virions, VLPs looked with a dark center since they are empty capsids. As expected, no similar structures were found in fractions 15–17 from the mock-purified samples (Fig. 3I and J).

To assess the immunogenicity of the recombinant VLPs, mice were immunized either with recombinant VLPs purified by sucrose gradient or with the crude extract without further processing. The antigenic mass inoculated in mice was 500 ng because we have previously demonstrated that this is enough to develop an efficient humoral immune response in all the vaccinated animals [25]. In group D, the antigenic mass was limited by the inoculation volume because the concentration of specific protein in the crude extract without purification was not as high as in the purified samples.

FMDV-specific antibodies were detected from day 14 and antibody titers increased after each booster immunization in groups receiving recombinant VLPs as well as in the positive control group (Fig. 4). The VLPs produced by the different vectors induced similar humoral responses in mice. Moreover, animals immunized with the crude extract showed almost the same antibody titers as the ones receiving purified VLPs, although the antigenic mass was three times lower (Fig. 4). The titers of all these groups were significantly higher than those observed in negative controls.

4. Discussion

Many efforts have been made to develop new FMD vaccines that meet the requirements that would be expected for an ideal vaccine, such as safety during production, induction of a rapid and long-lasting immune response, and cost-effectiveness. Molecular farming for the production of recombinant proteins has emerged as a promising approach with significant advantages in terms of cost and safety over other eukaryotic expression systems [13,31–33].

Most of the attempts to develop plant-based anti-FMDV vaccines have concentrated on expressing the capsid protein VP1 either in transgenic plants or transiently, since this protein contains the immunodominant epitope from the virus (reviewed in [34]). However, vaccines based on individual viral proteins rarely present epitopes in their native conformation, making them less effective than whole-virus preparations. Therefore, to improve the

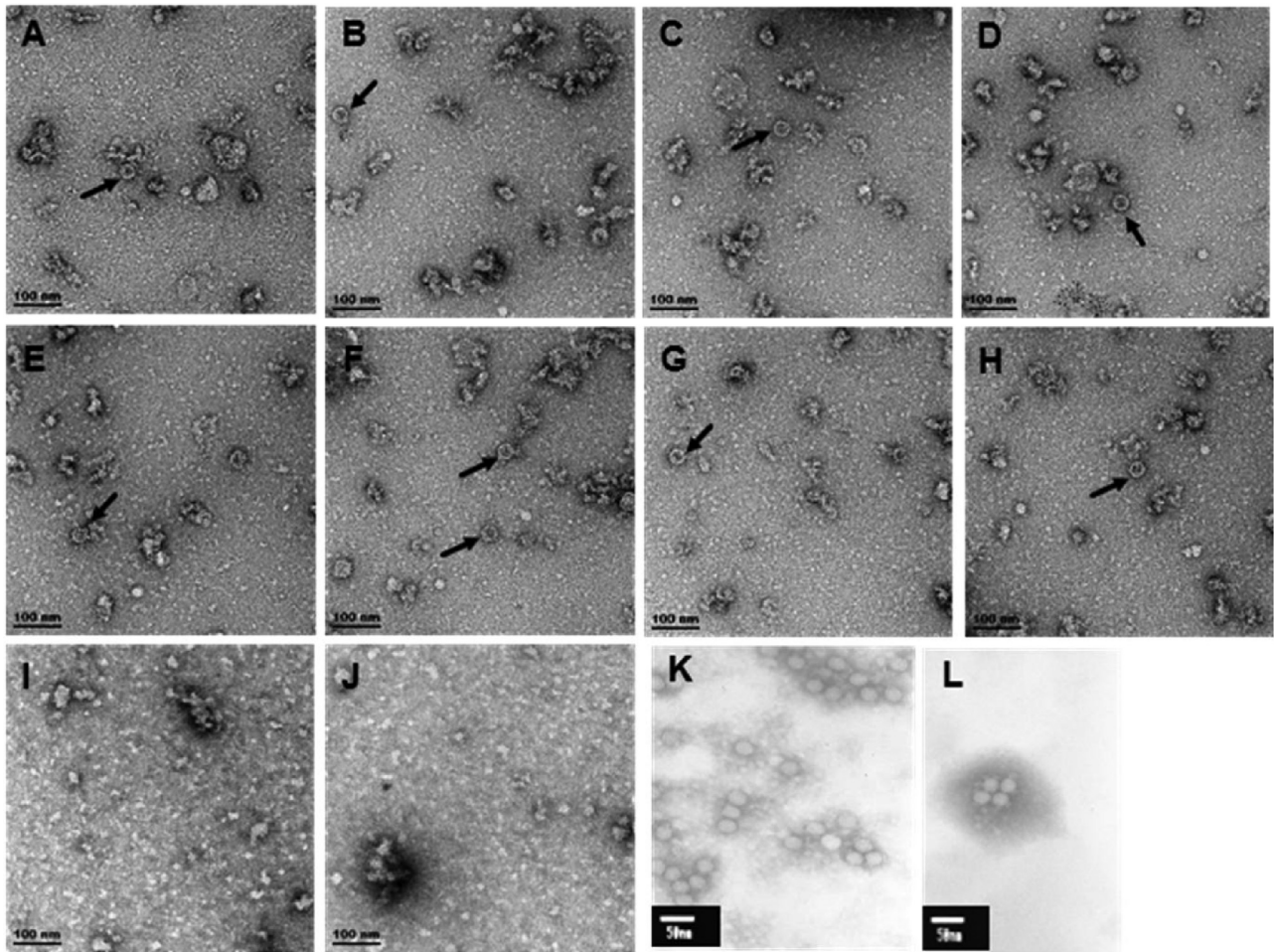


Fig. 3. TEM analysis of sucrose-purified peak fractions (fr. 15–17) obtained with the crude extracts of leaves infiltrated with pRIC-P12A3C (A–H), or with the crude extracts of mock-infiltrated leaves (I–J). The samples were fixed to carbon-coated copper grids and negatively-stained with 2% uranyl acetate (65,000 \times magnification). Black arrows indicate spherical structures measuring approximately 30 nm in size. Scale bar, 100 nm. Sucrose-purified iFMDV virions (146S) in different concentrations [(1000 μ g/ml of total FMDV proteins (K), and 20 μ g/ml (L)] are included for comparison (80,000 \times magnification). Scale bar, 50 nm.

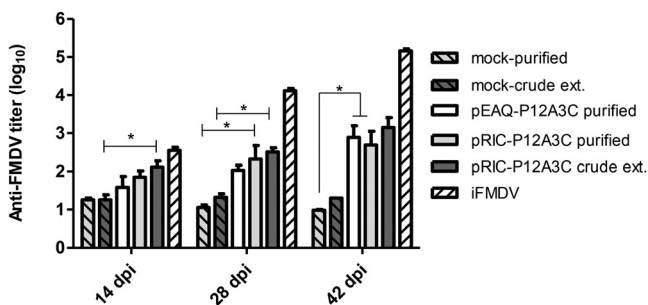


Fig. 4. Humoral response in mice immunized with recombinant proteins and purified inactivated viral antigen (iFMDV). The specific antibody titers were measured by ELISA. The cut-off was established as the mean OD_{492} of the sera at 0 dpi plus three standard deviations (SD). Antibody titers are expressed as the \log_{10} of the reciprocal of the highest serum dilution which gives OD_{492} readings above the cut-off value. Bars represent the mean ($n = 7$) of the antibody titer values \pm S.E.M., at 14, 28 and 42 days post-infection (dpi) for each group. * $p < 0.05$.

immunogenicity of subunit vaccines, alternative strategies have been explored. These strategies include the production of FMDV empty capsids or VLPs, which, based on their particulate nature and repetitive protein motif structure, are considered the most promising antigen for FMD vaccine development [8,29,35,36].

Our group has recently described the use of an agroinfiltration-mediated transient expression system for the production of recombinant FMD VLPs. This was achieved by infiltrating tobacco leaves with *A. tumefaciens* cultures containing only the capsid precursor (pEAQ-P1-2A), demonstrating that this precursor could be proteolytically cleaved by host plant cell proteases [16].

In the present study, the complete cassette containing the capsid precursor and the protease 3C (P12A3C) from FMDV A/Arg/01 was used to produce recombinant FMD VLPs in *N. benthamiana* by transient expression. This strategy was analyzed using two different vectors (pEAQ-HT and pRIC 3.0), which proved to achieve very similar yields (3–4 mg/kg of fresh leaf material). This expression level was significantly higher than the one reached in the previous study without the protease 3C [16]. The use of expression vectors encoding the complete cassette P12A3C ensures the availability of the 3C protease to cleave the capsid precursor as soon as it is translated, making the complete processing of this polyprotein more efficient. Therefore, although it has been well documented that the viral protease has toxic effects, the use of the complete cassette, the harvesting of leaves at earlier times, and the use of codon-optimized sequences for expression in *N. benthamiana* could account for the higher expression levels observed in this study.

Sedimentation of the crude extracts in a sucrose gradient revealed that the recombinant capsid proteins self-assembled into

empty capsids (75S). The pentameric subunits (12S) that were also observed in these samples could indicate subunits particles that are below the critical threshold concentration required for their assembly to occur [37], or could be produced by dissociation of previously assembled VLPs. The high peak corresponding to 12S particles was also present in the iFMDV used as positive control. This is usually observed in virus preparations and corresponds to breakdown products of 146S virions after virus inactivation procedures or unassembled components generated during virus replication *in vitro*.

The recombinant FMD VLPs were consistently observed by electron microscopy in very similar amounts in the different fields of view analyzed. Although these structures were partially purified by sucrose cushion and sucrose gradient, it seems that some plant material is still present in the final product, suggesting that the purification protocol could be further optimized. FMDV virions (146S) used as positive control could be observed in large quantities only when the sample was concentrated (1000 µg/ml) (Fig. 3K). However, when the concentration of FMDV proteins was lower (20 µg/ml) only a few virions could be found (Fig. 3L). The concentration of FMDV proteins in the pooled fractions obtained from the crude extracts of infiltrated leaves was 3–4 µg/ml for pEAQ-P12A3C and pRIC-P12A3C, respectively. This could be the reason why only 1–2 VLPs could be observed per field when these samples were analyzed by TEM.

The recombinant VLPs produced by transient expression with both vectors (pEAQ-P12A3C and pRIC-P12A3C) proved to be immunogenic in BALB/c mice, a well-studied murine model [38–40]. It is important to highlight that, in the present study, the crude leaf extract without purification elicited a humoral immune response similar to the purified VLPs, even with one third of the antigenic mass. This could be due to the structure of the antigenic particle in those types of samples. During the process of self-assembly of the viral capsid, one copy of each protein spontaneously forms the protomer (5S), subsequently five protomers form the pentamer (12S), and finally twelve pentamers assemble into the empty capsid (75S). It is likely that purified empty capsids or VLPs were not highly stable and would subsequently become dissociated into more stable -but less immunogenic- pentameric capsid subunits. Conversely, in the crude leaf extracts the presence of plant cell components could have interacted with the pentameric subunits (12S) favoring their self-assembly into VLPs, which are known to be more immunogenic. This adjuvant-like effect of *N. benthamiana* foliar extracts have been already reported in mice [41].

The problem of capsid instability could be overcome by incorporating different mutations in VP2, designed to strengthen interpentamer interactions. The introduction of a disulphide bond replacing a histidine in position 93 for a cysteine has been shown to enable the production of thermostable and pH-stable recombinant FMD VLPs for an A serotype [35]. Other mutations designed to increase noncovalent interactions have also been described for other serotypes [42]. It is worth noting that other researchers have used a significantly larger dose (5–10 µg) of a recombinant protein vaccine to immunize mice, obtaining a specific humoral immune response similar to the one described in this work [43,44].

The downstream processing is the most expensive part of the production of plant-produced proteins, and is estimated to account for at least 80% of the total manufacturing costs [18]. Therefore, the use of partially or minimally processed plant material could be of great interest for veterinary applications, especially as the regulatory burden is lower for veterinary health than for human use [31].

In conclusion, here we demonstrated that recombinant FMD VLPs can be successfully produced by transient expression in tobacco plants, using either replicative or non-replicative systems,

and that these structures are immunogenic in mice. The use of minimally-processed plant tissue as an immunogen, which significantly reduces downstream costs, could be a great advantage both in general and particularly for veterinary applications.

Declarations of interest

None.

Acknowledgments

We thank Dr. George Lomonosoff for providing the pEAQ- HT vector (John Innes Centre, Norwich, UK). We are truly grateful to Maureen Nwaxigombe and Varusha Veerapen for their technical assistance with sucrose gradient assays, and to Mohamed Jaffer from the EM unit at UCT for help with the microscopy. This work was supported by a bilateral cooperation program grant (SA/13/12) between Argentina and South Africa, implemented and administered by the Ministry of Science, Technology and Productive Innovation (MINCYT) of Argentina and the National Research Foundation (NRF) of South Africa.

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