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Short communication

Novel expression of immunogenic foot-and-mouth disease virus-like particles in *Nicotiana benthamiana*

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals and is endemic in Africa, parts of South America and southern Asia. The causative agent, FMD virus (FMDV) is a member of the genus *Aphthovirus*, family *Picornaviridae*. Vaccines currently used against FMDV are chemically inactivated virus strains which are produced under high-level biocontainment facilities, thus raising their cost. The development of recombinant FMDV vaccines has focused predominantly on FMDV virus-like particle (VLP) subunit vaccines for which promising results have been achieved. These VLPs are attractive candidates because they avoid the use of live virus in production facilities, but conserve the complete repertoire of conformational epitopes of the virus. Recombinant FMDV VLPs are formed by the expression and assembly of the three structural proteins VP0, VP1 and VP3. This can be attained by co-expression of the three individual structural capsid proteins or by co-expression of the viral capsid precursor P1-2A together with the viral protease 3C. The latter proteolytically cleaves P1-2A into the respective structural proteins. These VLPS are produced in mammalian or insect cell culture systems, which are expensive and can be easily contaminated. Plants, such as *Nicotiana benthamiana*, potentially provide a more cost-effective and very highly scalable platform for recombinant protein and VLP production. In this study, P1-2A was transiently expressed in *N. benthamiana* alone, without the 3C protease. Surprisingly, there was efficient processing of the P1-2A polyprotein into its component structural proteins, and subsequent assembly into VLPs. The yield was ~0.030 μg per gram of fresh leaf material. Partially purified VLPs were preliminarily tested for immunogenicity in mice and shown to stimulate the production of FMDV-specific antibodies. This study, has important implications for simplifying the production and expression of potential vaccine candidates against FMDV in plants, in the absence of 3C expression.

Foot-and-mouth disease (FMD) is a viral disease of domestic livestock that generates significant economic losses in affected countries (Guo et al., 2013; Huang et al., 1999). In the past, culling was implemented to prevent the spread of the disease, when an outbreak occurred. However, now other measures are preferentially exploited to control outbreaks. These include isolating potentially affected livestock and the use of prophylactic vaccination against Foot-and-mouth disease virus (FMDV).

FMD vaccines currently available on the market consist of purified, inactivated whole virus preparations (Guo et al., 2013; Robinson et al., 2016). However, these vaccines encounter some drawbacks: most of these are related to the manufacturing process including the high costs of biocontainment, the risk of failure of complete inactivation and subsequent viral escape, as well as the limitations that some countries such as the US have where production of vaccines using live virus is prohibited. These factors have led to the exploration of the development of recombinant vaccines. One of the proposed strategies is the production of virus-like particles (VLPs) as they lack the infectious viral genetic material which negates the risk of infection (Kushnir et al., 2012). It has been shown that co-expression of the FMDV ORF *P1-2A* encoding the three capsid proteins (VP0, VP1 and VP3), together with the non-structural *3C* protease which cleaves P1-2A into the component capsid proteins, results in appropriate cleavage and the assembly of the capsid proteins into VLPs. The latter have been produced in this way in

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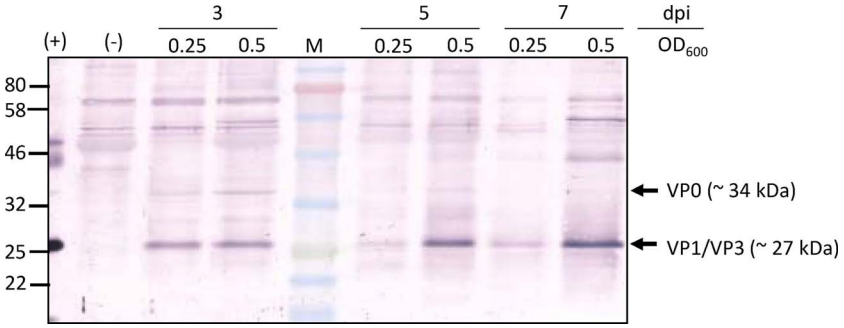

Fig. 1. Western blot analysis of *N. benthamiana* co-infiltrated with pEAQ-*HT*-3C and pEAQ-*HT*-P1-2A. Leaves were co-infiltrated with pEAQ-*HT*-P1-2A *A. tumefaciens* cultures having an OD_{600} = 0.5 and with pEAQ-*HT*-3C *A. tumefaciens* OD_{600} = 0.25 or 0.5. Western blots of crude leaf extracts harvested at 3, 5 and 7 days post infiltration and separated on 15% SDS-polyacrylamide gels were probed with a primary antibody (polyclonal anti-serum from guinea-pigs infected with FMDV, serotype A1 diluted 1:100) and an alkaline phosphatase-conjugated anti-guinea pig secondary antibody (Sigma-Aldrich) diluted 1: 10 000. (+): inactivated FMDV A positive control. (−): crude leaf extracts from leaves infiltrated with pEAQ-*HT* lacking any insert as a negative control. (MM): ColourPrestained Protein Standard (New England Biolabs).

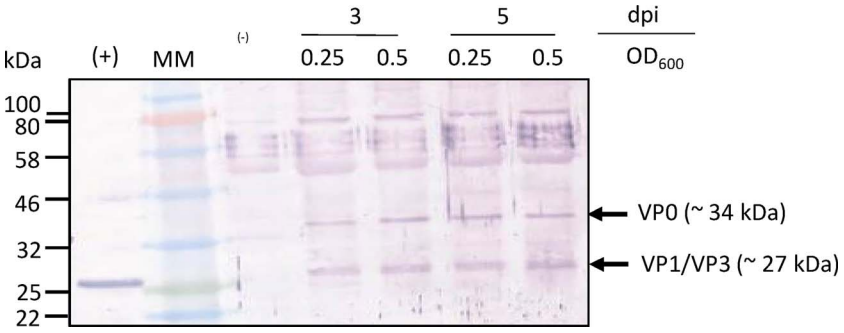

Fig. 2. Western blot analysis of *N. benthamiana* infiltrated with pEAQ-*HT*-P1-2A only. Leaves were infiltrated with pEAQ-*HT*-P1-2A *A. tumefaciens* cultures having an OD_{600} = 0.25 or 0.5. Western blots of crude leaf extracts harvested at 3 and 5 days post infiltration and separated on 15% SDS-polyacrylamide gels were probed with a primary antibody (polyclonal anti-serum from guinea-pigs infected with FMDV, serotype A1 diluted 1:100) and an alkaline phosphatase-conjugated anti-guinea pig secondary antibody (Sigma-Aldrich) diluted 1: 10 000. (+): inactivated FMDV A positive control. (−): crude leaf extracts from leaves infiltrated with pEAQ-*HT* lacking any insert as a negative control. (MM): ColourPrestained Protein Standard (New England Biolabs).

various host cell expression systems including silkworm larvae (Li et al., 2012), *E. coli* (Lewis et al., 1991; Xiao et al., 2016), insect cells (Cao et al., 2009; Porta et al., 2013b; Roosien et al., 1990), in mammalian cells via recombinant vaccinia virus (Abrams et al., 1995), in transfected mammalian cells (Mignaqui et al., 2013), and transgenic alfalfa plants and tomato fruits (Dus Santos et al., 2005; Dus Santos and Wigdorovitz, 2005). In most cases, the VLPs have been shown to be immunogenic when injected into animals (Mignaqui et al., 2013; Porta et al., 2013a).

This paper describes an investigation of the transient expression of FMDV VLPs in tobacco (*Nicotiana benthamiana*). DNA constructs encoding FMDV type A/Arg/01 P1-2A and 3C were cloned into the plant expression vector pEAQ-*HT* (Sainsbury et al., 2009) to generate pEAQ-*HT*-P1-2A and pEAQ-*HT*-3C. These were transformed into *Agrobacterium tumefaciens* AGL-I which were then co-infiltrated by syringe into *N. benthamiana* leaves. Crude extracts from leaves co-infiltrated with pEAQ-*HT*-P1-2A (OD_{600} = 0.5) and pEAQ-*HT*-3C (OD_{600} = 0.25 or 0.5) and sampled at 3, 5 and 7 days post-infiltration (dpi) were screened for FMDV VP0, VP1 and VP3 capsid protein expression. This was confirmed by western blot (Fig. 1), which showed two bands (black arrows) representing FMDV P1-2A cleaved capsid proteins VP0 (~34 kDa), and VP1 and VP3 which are both the same size and are visualised as a more intense band (~27 kDa) (Mignaqui et al., 2013). Based on the intensity of protein bands, the highest accumulation of VP1 and VP3-expressed proteins was seen at 5 and 7 dpi using a pEAQ-*HT*-3C culture infiltration OD_{600} of 0.5. However, a higher level of VP0 was observed at 3 dpi than at 5 dpi and 7 dpi using an OD_{600} of 0.5. Necrotic lesions on the infiltrated leaf regions were observed by 5 dpi which could account for the lower expression levels of the VPs by 7 dpi. Expression of 3C was also confirmed using the same parameters. A faint band of 26 kDa in size was observed at 3, 5 and 7 dpi suggesting expression of 3C, albeit at very low levels, while infiltrated leaves showed chlorosis (data not shown). The necrotic effects seen in infiltrated leaves suggest unfavorable conditions for VLP formation in the host cells.

The FMDV 3C-protease is a highly conserved viral enzyme and adopts a chymotrypsin-like fold. Its active site consists of the catalytic triad Cys^{163}-His^{46}-Asp^{84}, in a similar conformation to the Ser-His-Asp triad conserved in almost all serine proteases. The picornavirus 3C is thus classified as a chymotrypsin-like cysteine protease and the cleavage sites contain glutamine followed by small residues such as glycine, serine and alanine (Birtley et al., 2005; Puckette et al., 2017; Zunszain et al., 2010). *N. benthamiana* is known to harbour such cysteine proteases which might be involved in many processes, such as proprotein processing, programmed cell-death and especially in protein turnover. While the tobacco genome encodes for at least sixty putative cysteine proteinases (CysPs), they are poorly characterized. However, it is possible that the FMDV capsid precursor could be cleaved by any of the cysteine proteases present in the plant cells, (Duwadi et al., 2015; Hao et al., 2006).

To test whether the FMDV polyprotein could be cleaved by host plant cell proteases, leaves were infiltrated with only recombinant pEAQ-*HT*-P1-2A *A. tumefaciens* at OD_{600} values of 0.25 and 0.5 and leaf discs harvested at 3, 5 and 7 dpi. Surprisingly, bands representing VP0, VP1 and VP3 were observed on the western blot (Fig. 2) at days 3 and 5 post infiltration. This is very interesting, and it is important to mention that in similar studies carried out using alternative expression systems such as mammalian (Mignaqui et al., 2013) and insect cells (Roosien et al., 1990), expressed P1-2A recombinant protein was routinely detected as an uncleaved polyprotein of 83 kDa. The highest accumulation of the capsid proteins as determined by the intensity of the bands was observed at 5 dpi when infiltrated with OD_{600} values of both 0.25 and 0.5.

To determine whether the transiently expressed capsid proteins assembled into VLPs, the process was scaled up by vacuum infiltrating ~80 *N. benthamiana* plants at an OD_{600} of 0.5. Approximately 100 g of leaves were harvested at 5 dpi and the crude extract was filtered and centrifuged at 9 600*g* for 10 min. Clarified supernatant was loaded onto a 30% sucrose cushion to pellet the capsid proteins, which were then resuspended in NTE buffer (pH 8.0), matured to promote further VLP assembly and subsequently centrifuged to remove any insoluble material. The supernatant was loaded onto a linear sucrose gradient of 5–20% and ultracentrifuged. Gradient fractions (1.5 ml) were collected from the bottom of the tube, and were initially analysed by dot-blotting to determine the fractions in which the FMDV capsid proteins were present (data not shown). Selected fractions were further analysed by western blot (Fig. 3) using anti-FMDV A1 serum. FMDV capsid proteins were detected in fractions 4–14 with the highest protein accumulation

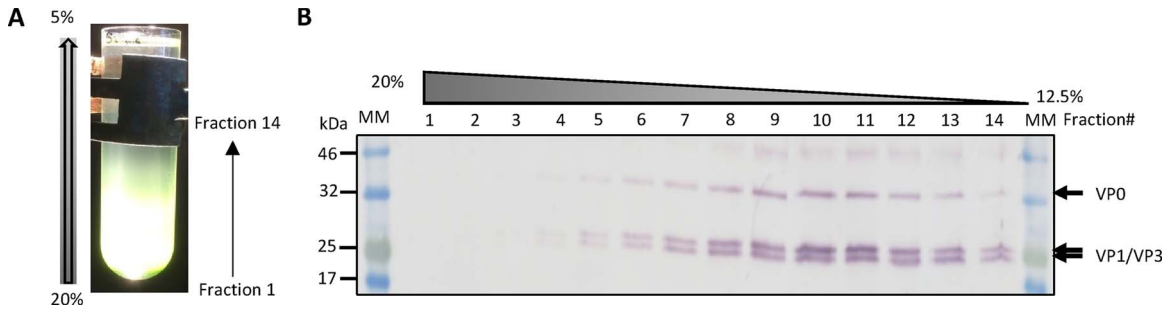

Fig. 3. Purified fractions from sucrose density gradient-purified leaf extract. Plants were vacuum infiltrated and harvested at 5 dpi. Protein was extracted by grinding up leaves in 2 x volume of NTE (0.1 M NaCl, 0.05 M Tris, 0.004 M EDTA-disodium dehydrate, pH 8.0), using a Waring-type blender. After centrifugation at 11 200 × *g* for 15 min, the extract was filtered through Miracloth™ (Merck) and the supernatant was loaded onto a 30% sucrose cushion. Centrifugation was carried out at 175 000 × *g* for 2 h 45 min in a SW 32 Ti rotor (Beckman). The pellet was resuspended in NTE buffer pH 8.0 with 3500 U Benzonase (Sigma-Aldrich), followed by maturation at room temperature for 20 min. The matured sample was then loaded onto a 5–20% continuous sucrose gradient (w/v in NTE) in a SW 32 Ti rotor (Beckman). The gradient was centrifuged at 175 000 × *g* for 2 h 30 min and the tube fractionated from the bottom (A). Fractions 1–14 were separated on a 15% SDS-polyacrylamide gel which was blotted onto nitrocellulose and probed with a primary antibody (polyclonal anti-serum from guinea-pigs infected with FMDV, serotype A01) diluted 1:100 and an alkaline phosphatase-conjugated anti-guinea pig secondary antibody (Sigma-Aldrich) diluted 1:10 000 (B). (MM): Colour Prestained Protein Standard (New England Biolabs).

found in fractions 9–11, as determined by the intensity of the bands.

TEM analysis of fractions 10 and 11 showed the presence of VLPs of approximately 25–30 nm (Fig. 4A), with dark halos seen around those which were decorated with anti-FMDV A1 serum (Fig. 4B). These VLPs are similar in size and shape to the related poliovirus-like particles recently expressed in plants by Marsian et al. (2017). The number of VLPs counted was on average 3 VLPs per field of view for 5 fields of view. No VLPs were viewed in the negative control samples (Fig. 4C). Quantitation of P1-2A in fractions 9–11 by ELISA ranged from 0.170 µg/ml to 0.197 µg/ml P1-2A, which represents a yield of ~0.030 µg VLPs per gram of fresh leaf material. Although this yield is relatively low when compared to the production of VLPs using mammalian cells and insect cells (Mignaqui et al., 2013; Porta et al., 2013b) and would need to be higher to produce vaccine doses of sufficient concentration, there are several methods that could be implemented for improving expression such as using a *P1-2A* gene which has been codon optimised for expression in N. *benthamiana* or co-infiltrating with a silencing suppressor construct.'

The plant-produced VLPs were preliminarily tested in mice for immunogenicity. Crude extracts were prepared from 100 g infiltrated *N. benthamiana* leaves as described above and then pelleted through a 30% Optiprep™ (Merck) cushion by ultracentrifugation at 175 000 x *g* at 12 °C in a SW 32 Ti rotor (Beckman). The pellet containing the VLPs was resuspended in NTE buffer (pH8.0), matured as described above and centrifuged for 15 min to remove any insoluble material. The supernatant was diluted at 1:50 in NTE buffer (pH 8.0) and TEM of a sample showed the presence of VLPs (Fig. 4D). The concentration of P1-2A in the supernatant was quantitated by I-ELISA using in-house produced *E. coli*-produced P1-2A as a standard. Maxisorp® plates (Nunc) were coated with 2-fold doubling dilutions (made in 10 mM Tris, pH 8.5) of *E. coli*-produced P1-2A or the plant-produced VLP preparation. Plates were probed with rabbit anti-P1-2A antiserum generated from rabbits immunized with *E. coli*-produced P1-2A (diluted 1:100 in TBS blocking buffer [1 x Tris-buffered saline [TBS] [0.0083 M Tris, 0.15 M NaCl], 5% Non-Fat Dry Milk [NFDM], pH 7.5]) and subsequently with 1:5000 diluted alkaline-phosphatase conjugated anti-rabbit secondary antibody (Sigma-Aldrich). SIGMA*FAST*™ p-NPP (Sigma Aldrich) was added to each well and plates incubated for 30 min after which the absorbance was read at A_{405} using a microplate spectrophotometer PowerWave XS™ (Biotek).

Mouse immunisations were carried out as approved by the Animal Research Ethics Committee at UCT (AEC 016_026). Three groups of 5

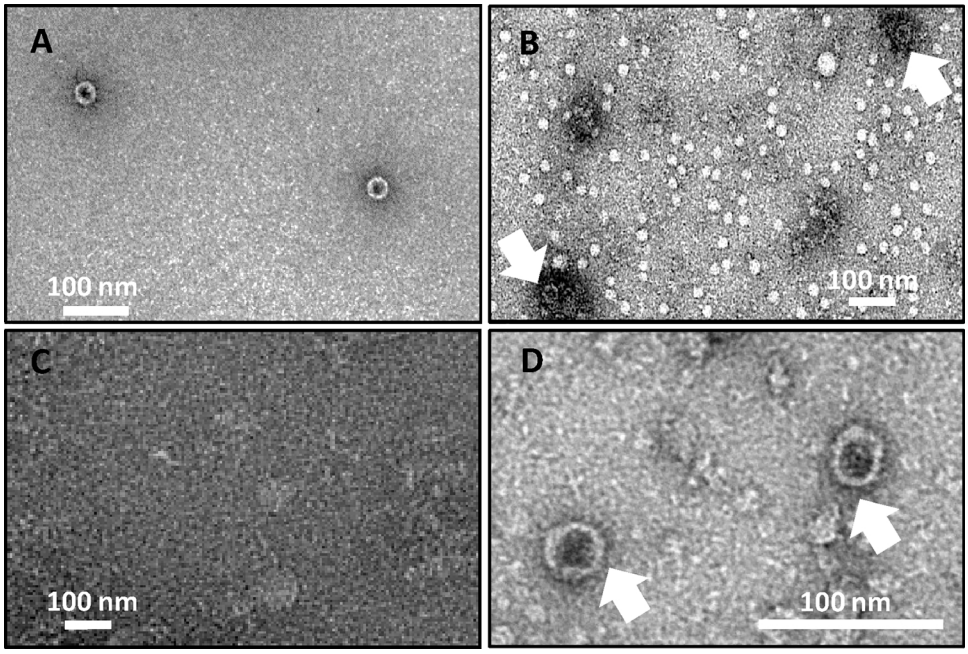

Fig. 4. TEM of P1-2A purified fractions. (A) Samples from sucrose-purified fractions 10 and 11 fixed to carbon-coated copper grids (mesh size 200) and negatively stained with 2% uranyl acetate (magnification: 64 000 x). (B) Samples from sucrose-purified fraction 10 were fixed to carbon-coated copper grids, decorated with polyclonal anti-serum from guinea-pigs infected with FMDV serotype A1 and subsequently alkaline phosphatase-conjugated anti-guinea pig secondary antibody (Sigma-Aldrich). Grids were washed and then stained with 2% uranyl acetate. Decorated VLPs are indicated by the white arrows. (C) Negative control consisting of a fixed sample prepared in the same way as in (B) from leaves infiltrated with cultures harbouring pEAQ-*HT* lacking any insert. (D) Sample from Optiprep™-prepared antigen used for mouse immunisations, fixed to grids and negatively-stained with 2% uranyl acetate (magnification: 53 000 x). VLPs are indicated by white arrows.

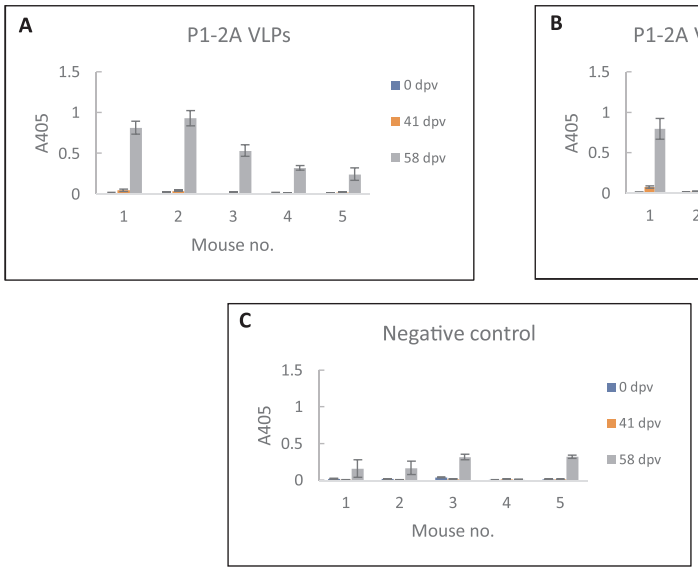

Fig. 5. Immunogenicity response of mice to P1-2A measured by I-ELISA. Sera were sampled at 0, 41 and 58 days post vaccination and diluted 1:50. (A) Mice 1–5 administered with P1-2A VLPs. (B) Mice 1–5 administered with P1-2A VLPs and Montanide ISA 50 V 2 (Seppic) adjuvant (1:1). (C) Mice 1–5 administered with purified plant extract from leaves infiltrated with pEAQ-*HT* lacking insert as a negative control. Error bars: ± standard deviation.

female Balb/C mice (8–10 weeks old) were immunized subcutaneously with P1-2A (Group 1), P1-2A formulated with Montanide ISA 50 V 2 (Seppic) adjuvant (1:1) (Group 2), or plant extract prepared similarly to the P1-2A immunogens as a negative control (Group 3). The immunogens were adjusted with NTE buffer (pH 8.0) to contain 5 µg P1-2A protein in a total volume of 100 µl and administered on day 0 with boosts at days 13, 27 and 44 post initial vaccination. Blood was collected on days 41 and 58.

Immunogenicity to P1-2A was determined by I-ELISA as described above with mouse sera diluted 1:50 in TBS blocking buffer. Serum from mice vaccinated with P1-2A only showed marginal increases in absorbance at 41 dpv which increased in varying significance for each mouse at 58 dpv (Fig. 5A). Serum from those vaccinated with P1-2A formulated with adjuvant, showed small increases in absorbances at 41 dpv but significantly higher increases after 58 dpv. Mouse serum from animals immunized with negative control showed minimal absorbance levels at 41 dpv, but there was a slight increase in absorbance levels for all but one mouse. In summary, mice immunized with adjuvanted P1-2A showed a four-fold increase in absorbance readings compared to this control group and those vaccinated with P1-2A only showed a two-fold increase. A one-way- ANOVA test performed on the data collected on the final bleeds, showed that the means of the A_{405} values of Group 1 (P1-2A VLPs) and Group 2 (P1-2A VLPs and adjuvant) differed significantly from Group 3 (negative control) ($p < 0.05$). The Tukey HSD test supported the difference of results between Groups 1 and 3 as well as Groups 2 and 3 ($p < 0.01$). These results indicate that P1-2A VLPs and adjuvanted P1-2A VLPs elicited a significant humoral immune response.

In conclusion, it has been shown that FMDV VPs can be produced transiently in *N. benthamiana* leaves in the absence of the FMDCV 3C-protease, and assemble into VLPs. To our knowledge, this is the first study which shows that the FMDV P1-2A capsid precursor can be proteolytically cleaved into capsid proteins VP0, VP1 and VP3 in the absence of the FMDV 3C-protease, unlike in similar studies performed previously in mammalian (Mignaqui et al., 2013) and insect (Roosien et al., 1990) cells where P1-2A was detected as an uncleaved polyprotein. This constitutes an advantage for the use of plants as an expression platform, as preliminary studies demonstrated how the protease can be toxic when present in large amounts. Despite the low numbers of VLPs in the antigen preparation, preliminary immunogenicity experiments showed that the partially purified preparations of P1-2A VLPs were significantly immunogenic in mice. Although the number of FMDV VLPs purified is low, further optimization to increase their assembly such as the inclusion of a longer maturation step or using site-directed mutagenesis of the P1-2A gene to replace amino acids which encourage better stabilization of the VLPs under higher temperatures or more acidic environments could be carried out (Mateo et al., 2008; Porta et al., 2013a). In addition, increasing expression levels of P1-2A by the use of codon-optimised sequences or the co-expression with silencing suppressors could be implemented. These results suggest that plant-produced FMDV VLPs have potential use as a vaccine candidate, production of which is simplified by exclusion of the expression of FMDV 3C-protease which can be toxic to host expression cells.

Conflicts of interest

There are no conflicts of interest.

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