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Short communication

Transient expression of heat- and acid-resistant foot-and-mouth disease virus P1-2A mutants in *Nicotiana benthamiana*

Varusha Pillay Veerapen[a], Albertha R. van Zyl[a], Edward P. Rybicki[a,b], Ann E. Meyers[a,*]

[a] Biopharming Research Unit, Department of Molecular and cell Biology, University of Cape Town, Private Bag X3, Rondebosch, 7701, South Africa
[b] Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Observatory, 7925, South Africa

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ABSTRACT

Recombinant foot-and-mouth disease virus-like particles (VLPs) can be expressed in a number of expression systems including plants. However, yields in plants have formerly been shown to be low, possibly due to their acid and/or heat lability, previously shown to affect VLP yields produced in other systems. This work describes the introduction of mutations into the FMDV structural protein-encoding gene (P1-2A) which have been previously shown to increase acid and thermostability. VLPs expressed in plants using the mutant constructs had negative rather than positive effects on yield and temperature and acid stability compared to the control.

1. Introduction

Foot-and-mouth disease virus (FMDV) is a single-stranded RNA virus belonging to the genus *Aphthovirus*, family *Picornaviridae* and is the etiological agent of foot-and-mouth disease(Puckette et al., 2017; Rueckert and Wimmer, 1984). The FMDV virion consists of 12 pentamers arranged in an icosahedral lattice and held together by electrostatic interactions and bonds (Acharya et al., 1989). Each pentamer consists of 5 subunits, each comprised of one copy of VP1, VP2, VP3 and VP4 capsid proteins (Vasquez et al., 1979). The RNA genome encodes the 3C-protease and the capsid protein precursor, P1-2A. The latter is proteolytically cleaved by 3C to yield capsid proteins VP0, VP1 and VP3, after which VP0 is cleaved into VP2 and VP4 upon virus maturation.

Recombinant co-expression of P1-2A with 3C in various expression hosts has been shown to result in the assembly of virus-like particles (VLPs) (Li et al., 2012; Lewis et al., 1991; Xiao et al., 2016; Cao et al., 2009; Roosien et al., 1990; Abrams et al., 1995; Mignaqui et al., 2013; Dus Santos et al., 2005; Dus Santos and Wigdorovitz, 2005; Porta et al., 2013a). In some cases, these VLPs are immunogenic in animals (Li et al., 2012; Cao et al., 2009; Mignaqui et al., 2013; Dus Santos et al., 2005; Porta et al., 2013a) making them ideal vaccine candidates. We have shown that the transient expression of FMDV P1-2A alone results in the expression of the VPs 0, 1 and 3 and subsequently in the assembly of VLPs using *Nicotiana benthamiana* as an expression platform (Veerapen et al., 2018). Moreover, these were shown to be immunogenic in mice.

In the field, when FMD virions are subjected to culture environments below neutral pH 6.5, they dissociate into pentameric subunits in order to release the RNA into an infected cell (Curry et al., 1995). This acid-sensitive characteristic seems to be determined by the presence of specific amino acids in the capsid proteins positioned at the inter-pentameric interfaces of the virion (Acharya et al., 1989; Yuan et al., 2017; Caridi et al., 2015). In an acidic environment, these residues become protonated leading to the disassembly of the capsid via electrostatic repulsions between capsid subunits (Acharya et al., 1989; Curry et al., 1995). Martin-Acebes et al (Martin-Acebes et al., 2010) have shown that a single amino acid substitution in this region (in VP3) can increase acid lability of FMD virions. Others have also shown that different single amino acid mutations (in VP1, VP2 and VP4) can influence acid lability of particles (Vazquez-Calvo et al., 2014; Liang et al., 2014). Further work by Liang et al (Liang et al., 2014) demonstrated how a mutation in the VP1 capsid protein from asparagine to aspartic acid (N17D) conferred acid resistance to the virion in the presence of ammonium chloride (NH_4Cl) which acts as a proton sink within endosomes. Liang et al (Liang et al., 2014) further showed how the double mutant VP1 N17D/VP4 S73N could also be a potential acid resistant mutant.

In addition to acid sensitivity, FMD virions are also thermolabile (Mateo et al., 2008). This has negative implications not only on the extent of immunogenicity but also for long term storage purposes when they are used for vaccines (de Los Santos et al., 2018; Rincón et al.,

* Corresponding author.
E-mail addresses: VRPVAR001@myuct.ac.za (V.P. Veerapen), alta.vanzyl@uct.ac.za (A.R. van Zyl), ed.rybicki@uct.ac.za (E.P. Rybicki), ann.meyers@uct.ac.za (A.E. Meyers).

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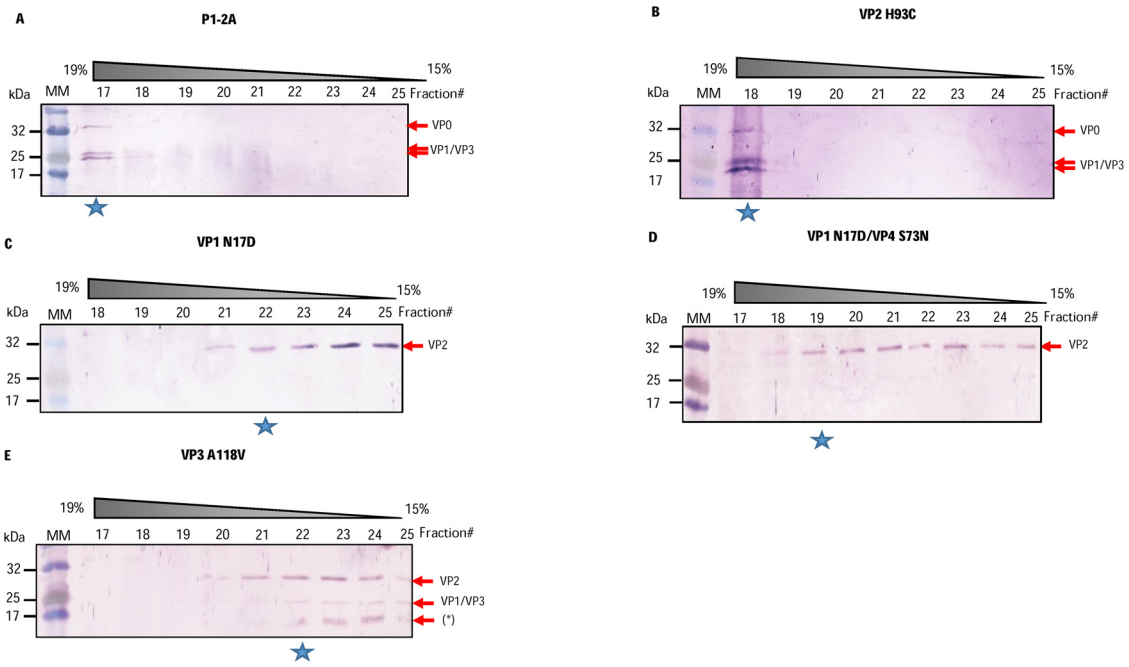

Fig. 1. Western blot analysis of the upper fractions of Optiprep™ gradient semi-purified leaf extracts. A) P1-2A, B) VP2 H93C, C) VP1 N17D, D) VP1 N17D/VP4 S73N and E) VP3 A118V. The blue star indicates fractions used for thermo and pH stability assays; (*) degraded protein; MM - Colour Prestained Protein Standard (New England Biolabs). (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.).

2014). Mateo et al (Mateo et al., 2008) were able to make at least 2 FMDVs more thermostable by engineering amino acid side chains near the capsid intersubunit interfaces. In addition, Porta et al (Porta et al., 2013b) showed that a mutation H93C on VP2 results in the production of more stable recombinant FMDV empty capsids. More recently, Scott et al. (Scott et al., 2017) screened various mutant FMD viruses for thermostability and showed how a mutation in VP2 (SAT 2 VP2 S93Y) conferred both acid and heat stability to the virus.

In the context of using VLPs as vaccine candidates, thermolability and acid sensitivity are undesirable, as they have negative connotations for vaccine stability if subjected to extreme temperatures or lower pH values during vaccine preparation and transit as well as VLP yield.

This report describes our efforts to improve the yield of plant produced FMD VLPs by introducing previously documented amino acid substitutions in the capsid sequences shown to increase both pH and thermostability.

Four different mutations in various capsid gene sequences were made and their influence on acid and thermostability as well as VLP yield analysed. Site-directed mutagenesis was carried out by PCR on a previously synthesised FMDV Type A *P1-2A* gene (Veerapen et al., 2018) to introduce the following amino acid substitutions shown to increase acid stability: VP1 N17D (Caridi et al., 2015; Liang et al., 2014) and VP4 S73N (Liang et al., 2014); acid and heat stability VP2 H93C (Porta et al., 2013b; Kotecha et al., 2015); and, the mutant VP3 A118V, which increases acid sensitivity, thereby making potential particles more unstable (Caridi et al., 2015; Martin-Acebes et al., 2010).

PCR products were cloned into the pEAQ-*HT* plant expression vector (Sainsbury et al., 2009) and transformed into *E. coli* to generate pEAQ-*HT*-VP1 N17D, pEAQ-*HTV*- VP1 N17D/VP4 S73N, pEAQ-*HT*-VP2 H93C and pEAQ-*HT*-VP3 A118V, which were subsequently transformed into *Agrobacterium tumefaciens* AGL-I.

These constructs were initially tested to verify that they expressed the capsid proteins, as well as to determine the optimal infiltration density and harvest day post infiltration. The construct pEAQ-*HT*-P1-2A, previously demonstrated to result in VLP assembly in plants, was included as a comparative positive control (Veerapen et al., 2018). Recombinant *A. tumefaciens* cultures were grown up as described in Veerapen et al (Veerapen et al., 2018) and syringe-infiltrated into *N. benthamiana* leaves at culture OD_{600} values of 0.25, 0.5 and 1.0. The plants were incubated at 22 °C for 3 to 7 days (16 h light:8 h dark) before harvesting. Leaf clippings were sampled at 3, 5 and 7 days post infiltration (dpi). Crude leaf extracts ground up in 1 × PBS, were separated on 15% SDS polyacrylamide gels and protein blotted onto nitrocellulose membranes which were probed with anti-P1-2A A1 rabbit serum generated in the Biopharming Research Unit (BRU) laboratory (diluted 1:100 in blocking buffer). The membranes were probed with alkaline phosphatase conjugated anti-rabbit antibody (Sigma-Aldrich) diluted 1: 5000. Proteins were detected with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) phosphate substrate (BCIP/NBT 1-component, KPL) and levels of expression quantitatively assessed by the presence/darkness of the bands representing VPs 0, 1, 2 and 3.

Blots showed that the highest levels of VP0, VP2 and VP3 using the VP1 N17D/VP4 S73N and VP3 A118V constructs were expressed using an infiltration OD_{600} of 1.0. The highest levels of the capsid proteins using VP1 N17D and VP2 H93C were expressed using an infiltration OD_{600} of 0.25 and 0.5, respectively. All the constructs expressed the highest level of capsid proteins after 5 dpi (data not shown).

In our previous work, it was found that VLPs could only be detected in purified, concentrated fractions of crude leaf material. Having established on the small scale described above, that in all cases FMDV capsid proteins were expressed, infiltration was scaled up for purification of the recombinant proteins to determine whether VLPs were assembled. Cultures were grown up and infiltration was performed using the optimal optical densities for large-scale vacuum infiltration (Veerapen et al., 2018); leaves were harvested at 5 dpi. Sixty grams of biomass expressing each construct was homogenized in 2 × NTE buffer pH 8.0 (0.1 M NaCl, 0.05 M Tris, 0.004 M EDTA-disodium dihydrate). The extract was filtered through Miracloth™(Merck) and centrifuged at 9600 × *g* for 10 min. Proteins in the supernatant were pelleted through a 30% Optiprep™ (Sigma- Aldrich) cushion by centrifugation at 175 000 × *g* for 3 h, at 12 °C in a SW 32 Ti rotor (Beckman). To purify VLPs, resuspended pellets were matured at room temperature for 30 min before being centrifuged through a continuous Optiprep™ gradient