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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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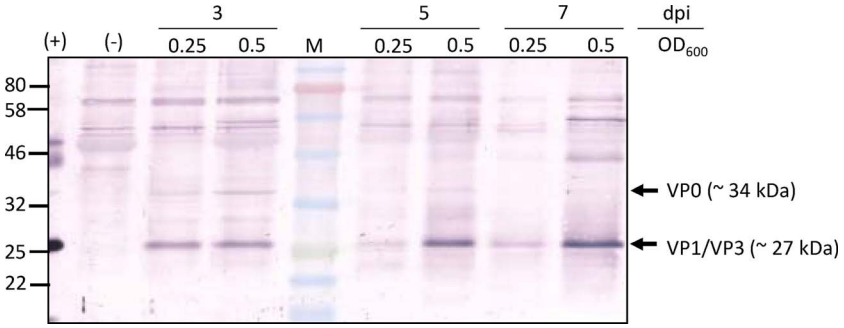

Fig. 1. Western blot analysis of *N. benthamiana* co-infiltrated with pEAQ-*HT*-3C and pEAQ-*HT*-P1-2A. Leaves were co-infiltrated with pEAQ-*HT*-P1-2A *A. tumefaciens* cultures having an OD_{600} = 0.5 and with pEAQ-*HT*-3C *A. tumefaciens* OD_{600} = 0.25 or 0.5. Western blots of crude leaf extracts 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).

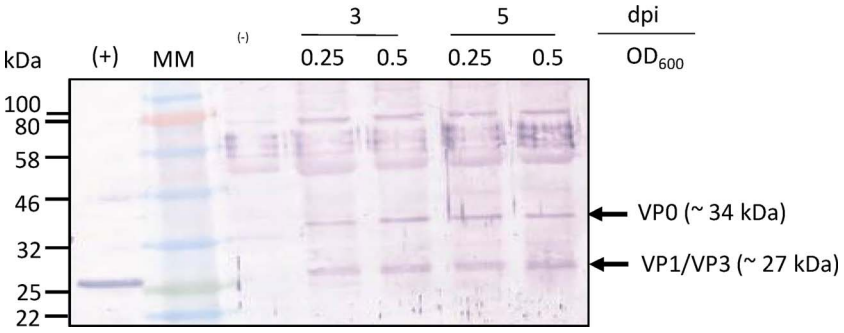

Fig. 2. Western blot analysis of *N. benthamiana* infiltrated with pEAQ-*HT*-P1-2A only. Leaves were infiltrated with pEAQ-*HT*-P1-2A *A. tumefaciens* cultures having an OD_{600} = 0.25 or 0.5. Western blots of crude leaf extracts harvested at 3 and 5 days post infiltration and separated on 15% SDS-polyacrylamide 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