



# Transient Bluetongue virus serotype 8 capsid protein expression in *Nicotiana benthamiana*



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## ABSTRACT

Bluetongue virus (BTV) causes severe disease in domestic and wild ruminants, and has recently caused several outbreaks in Europe. Current vaccines include live-attenuated and inactivated viruses; while these are effective, there is risk of reversion to virulence by mutation or reassortment with wild type viruses. Subunit or virus-like particle (VLP) vaccines are safer options: VLP vaccines produced in insect cells by expression of the four BTV capsid proteins are protective against challenge; however, this is a costly production method. We investigated production of BTV VLPs in plants via *Agrobacterium*-mediated transient expression, an inexpensive production system very well suited to developing country use. Leaves infiltrated with recombinant pEAQ-*HT* vectors separately encoding the four BTV-8 capsid proteins produced more proteins than recombinant pTRA vectors. Plant expression using the pEAQ-*HT* vector resulted in both BTV-8 core-like particles (CLPs) and VLPs; differentially controlling the concentration of infiltrated bacteria significantly influenced yield of the VLPs. *In situ* localisation of assembled particles was investigated by using transmission electron microscopy (TEM) and it was shown that a mixed population of core-like particles (CLPs, consisting of VP3 and VP7) and VLPs were present as paracrystalline arrays in the cytoplasm of plant cells co-expressing all four capsid proteins.

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## 1. Introduction

Bluetongue virus (BTV) is the causative agent of Bluetongue (BT) disease, an insect-borne, infectious but non-contagious disease of both domesticated and wild ruminants. BTV is a double-stranded RNA (dsRNA) virus that is the type species of the genus *Orbivirus* in the family Reoviridae [29]. BTV was first discovered and reported on in South Africa as “malarial catarrhal fever” in 1902 [16] and has since spread worldwide. At present, 26 serotypes of the virus have been described [19]. The virulence and mortality rate of the different virus strains vary considerably, depending on the species of animal being infected [9,14,20,41].

There has been a gradual movement of Bluetongue disease into previously unaffected areas in northern Europe and an emergence of several different BTV serotypes in the United States and Northern Australia [3]. Bluetongue disease is now considered endemic in northern Europe, probably as a result of climate change leading to migration of viruliferous vectors from Africa, with the most recent outbreak of BTV serotype 8 (BTV-8) occurring in Northern Europe in August 2006 [38,40]. The virus was shown to

have originated in sub-Saharan Africa, although its route of entry into the Netherlands is unclear. The disease is also common in South Africa with occurrences correlating with high rainfall areas.

Virus-like particles (VLPs), which resemble the mature virus particle in size and shape but lack any infectious viral genomic material, are considered good immunogens as they are stable, elicit a strong immune response and present the viral antigens in a conformation that is more appropriate than subunit protein vaccines [10,18,25].

BTV VLPs consisting only of the 4 immunogenic structural proteins (virus capsid proteins) of BTV and containing no BTV RNA have been produced in a variety of expression systems. BTV has complex, multi-layered virions. The virus capsid proteins VP2 and VP5 form an outer shell that is laid onto the foundation provided by the assembly of VP3 and VP7, which together constitute the inner shell [7]. When VP3 and VP7 are expressed in insect cells using a baculovirus expression system, they form core-like particles (CLPs) on their own. In the same expression system, VP2 and VP5 form VLPs when co-expressed with VP3 and VP7 [7,28]. VLPs produced in this manner have been shown to protect sheep against live virus challenge [27]. However, despite their efficacy, the cost of producing VLPs in insect cells is traditionally fairly high, not very scalable and there is the risk of contamination [22]. Thus,

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alternative methods of production of such vaccines would be desirable.

The transient expression of heterologous proteins in plants has attracted much interest in recent years as a method for recombinant protein production. There are many advantages of using plants over other eukaryotic expression hosts including their high yield of biomass, ease and affordability of scale-up and no risk of contamination by human or animal pathogens [31,39]. A recent study compared the production of four recombinant pharmaceutical proteins using different expression systems. The study found that when assuming all downstream processing costs are equal for different expression platforms, the estimated production costs of recombinant proteins in plants are lower than for other technologies [22]. Transient expression systems are flexible and allow for rapid expression of high concentrations of recombinant protein in a matter of days [6]. There are many examples to date of recombinant vaccine protein production in plants, including the production of VLPs [2,35].

Thuenemann et al. [37] have demonstrated the successful production of BTV-8 VLPs in *Nicotiana benthamiana* using the Cowpea mosaic virus-based HyperTrans (CPMV-*HT*) and associated pEAQ transient expression system [32]. This involved the infiltration of plants with 4 individual constructs encoding VP2, VP3, VP5 and VP7. However, although particulate structures were obtained, there was an over-representation of VP3 protein expression, leading to the formation of considerable numbers of subcore-like particles. To overcome this, the authors down-regulated VP3 production by controlling expression of the VP3 gene with the wild-type CPMV 5' UTR. Plants were infiltrated with dual-expressing constructs encoding VP3 and VP7 together, and VP2 and VP5 together, which resulted in a shift of particle production biased to that of VLPs.

There are twenty-five different serotypes of BTV-25 circulating in South Africa alone. The current vaccine used in South Africa consists of 3 cocktails of 5 different live-attenuated BTV serotypes [5]. For effective recombinant vaccines to accommodate such requirements, the method for producing VLPs representing different serotypes will have to be flexible. It may be easier to fine-tune the production of plant-produced VLPs for vaccine cocktails by making constructs encoding each VP representative of each serotype and simply varying the ratios of infiltrated constructs in order to direct serotype-specific VLP assembly.

In order to increase expression levels and to facilitate easier purification of heterologous proteins from plants, expression vectors that target all the co-expressed proteins to different organelles within the plant cells can be used [21] to increase VLP production.

In this study, co-expression of the four BTV-8 VPs in *N. benthamiana* was compared by using the binary *Agrobacterium tumefaciens* pTRA suite of vectors pTRAc-*HT*, pTRAc-AH and pTRAc-rbcs1-cTP, which target recombinant protein to the cytosol, apoplast and chloroplast respectively, in order to determine whether the expression yields could be improved by targeting the recombinant proteins to different subcellular compartments. In addition to the pTRA vectors, the VPs were also co-expressed using pEAQ-*HT* which has the P19 silencing suppressor sequence of TBSV incorporated into the T-DNA, enabling expression of the gene of interest and the silencing suppressor from a single plasmid [32] to compare which system best co-expressed the four VPs. In addition, the effects of varying the infiltration ratios of the four recombinant *Agrobacterium* cultures containing VP recombinant constructs were analyzed as well as variation of the density of infiltrated recombinant *Agrobacterium* cells.

## 2. Material and methods

### 2.1. Constructs

The VP2, VP3, VP5 and VP7 gene sequences (GenBank accession numbers: AM498052, AM498053, AM498056 and AM498057, respectively) of BTV-8 were codon optimized for expression in *N. benthamiana* and synthesized by GeneArt (Germany). The gene sequences used in this study were the same as the genes used by Thuenemann et al. [37].

The genes were cloned into 3 different pTRA binary vectors (kindly provided by Dr Rainer Fischer, Fraunhofer Institute, Aachen, Germany): pTRAc-*HT*, pTRAc-AH and pTRAc-rbcs1-cTP. These are designed to target recombinant proteins to the cytosol, apoplast and chloroplast, respectively [21]. The 5' and 3' termini of all 4 BTV-8 genes were modified by PCR to add restriction sites to facilitate cloning into the pTRA vector suite. Similarly, all 4 genes were modified and cloned into the pEAQ-*HT* vector obtained from George Lomonosoff, John Innes Centre, UK [32]. The constructs generated are shown in Table 1.

**Table 1**  
Summary of plant expression vectors tested and constructs made.

Vector	Restriction sites (5'/3')	Insert	Construct	Subcellular target
pTRAc- <i>HT</i>	<i>NcoI/XhoI</i>	VP2	pTRAc- <i>HT</i> VP2co	Cyttoplasm
		VP3	pTRAc- <i>HT</i> VP3co	
		VP5	pTRAc- <i>HT</i> VP5co	
		VP7	pTRAc- <i>HT</i> VP7co	
pTRAc-AH	<i>NcoI/XhoI</i>	VP2	pTRAc-AH VP2co	Apoplast
		VP3	pTRAc-AH VP3co	
		VP5	pTRAc-AH VP5co	
		VP7	pTRAc-AH VP7co	
pTRAc-rbcs1-cTP	<i>MluI/XhoI</i>	VP2	pTRAc-rbcs1-cTPVP2co	Chloroplast
		VP3	pTRAc-rbcs1-cTPVP3co	
		VP5	pTRAc-rbcs1-cTPVP5co	
		VP7	pTRAc-rbcs1-cTPVP7co	
pEAQ- <i>HT</i>	<i>AgeI/XhoI</i>	VP2	pEAQ- <i>HT</i> VP2co	Cytoplasm
		VP3	pEAQ- <i>HT</i> VP3co	
		VP5	pEAQ- <i>HT</i> VP5co	
		VP7	pEAQ- <i>HT</i> VP7co	

## 2.2. *A. tumefaciens*–mediated transient expression

Fifty to 100 ng of the pTRA and pEAQ-HT recombinant plasmids was electroporated into *A. tumefaciens* GV3101::pMP90RK and *A. tumefaciens* LBA4404, respectively as described by Maclean et al. [21]. The pTRA and pEAQ-HT plasmid were transformed into different *Agrobacterium* strains to allow for appropriate antibiotic selection of recombinant clones [13]. Recombinant pTRA clones were selected on Luria Bertani (LB) media plates at 27 °C containing 50 µg/mL carbenicillin, 30 µg/mL kanamycin and 50 µg/mL rifampicin. The pEAQ-HT clones were selected under the same conditions except that carbenicillin was omitted from the LB plates. Starter cultures of recombinant pTRA and pEAQ-HT *Agrobacterium*, including *A. tumefaciens* LBA4404 (pBIN-NSs) containing the NSs silencing-suppressor gene of tomato spotted wilt virus (TSWV) [26], were supplemented with the relevant antibiotics and grown in LB broth as previously described by Maclean et al. [21]. Furthermore, to prevent clumping of recombinant *Agrobacterium* LBA4404 cells, 2 mM magnesium sulfate (MgSO<sub>4</sub>) was added to the medium. The starter cultures were used to inoculate induction medium [21] with the addition of the appropriate antibiotics, including 20 µM acetosyringone, and grown overnight at 27 °C with agitation. The cells were harvested the following morning at 1000 × g for 5 min and resuspended in infiltration medium [21]. The cell suspensions were incubated at 22 °C for 2 h with agitation to allow for expression of the *vir* genes prior to infiltration. After incubation the cultures were diluted to the required optical density in infiltration medium.

For each one of the expression vectors used, the relevant recombinant *Agrobacterium* constructs carrying the capsid genes were co-infiltrated into 4–6-week-old *N. benthamiana* leaves with a blunt-ended syringe. Recombinant *Agrobacterium* pTRA strains were co-infiltrated with pBIN-NSs. The pEAQ-HT recombinant strains did not require co-infiltration with the silencing suppressor as it has the P19 silencing suppressor integrated on the T-DNA of the plasmid [32]. As a negative control the plants were infiltrated with infiltration medium. The plants were grown at 22 °C under 16 h/8 h light/dark cycles. Three leaf discs were harvested from leaves 3, 4 and 5 from the top of the plant (1 leaf disc each, clipped with the lid of a microcentrifuge tube) for each vector co-expressing recombinant protein to form VLPs at 1, 3, 5 and 7 days post infiltration (dpi).

## 2.3. Protein extraction and western blot analysis

The leaf discs were ground up in liquid nitrogen and resuspended in 210 µL bicine buffer (50 mM bicine (pH 8.4), 20 mM sodium chloride (NaCl), 0.1% *N*-lauroyl-sarcosine (NLS), 1 mM DL-dithiothreitol (DTT) and 1 × Complete Mini, EDTA-free protease inhibitor cocktail (Roche)) by vortexing. Extracts were clarified by centrifugation at 15,000 × g on a bench top centrifuge.

For western blot analysis, the crude plant extracts were incubated at 90 °C for 10 min in loading buffer [33]. The proteins were separated on 8% SDS polyacrylamide gels where equal amounts of total protein (120 µg as determined by spectrometry) were loaded in each lane. After electrophoresis the proteins were transferred onto nitrocellulose membranes using a Trans-blot<sup>®</sup> SD semi-dry transfer cell (Bio-Rad). Membranes were probed with a 1:2000 dilution of BTV-8 sheep serum [37] and subsequently with a 1:10,000 dilution of anti-goat/sheep 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).

## 2.4. Density gradient purification of VLPs

Plants co-infiltrated with the 4 pEAQ-HT constructs were harvested at 9 dpi and immediately cut up into fine pieces and homogenized in three volumes of ice cold bicine buffer (50 mM bicine (pH 8.4), 20 mM sodium chloride (NaCl), and 1 × Complete Mini, EDTA-free protease inhibitor cocktail (Roche)) lacking NLS and DTT. The homogenate was clarified by centrifugation at 1000 × g for 10 min after which the supernatant was filtered through four layers of Miracloth<sup>™</sup> (Merck). The crude plant sap was overlaid onto 5 mL of a 40% iodixanol (Optiprep<sup>™</sup>, Sigma–Aldrich) cushion prepared in 50 mM Tris–HCl, pH 8.4 and 20 mM NaCl after which it was centrifuged for 2 h at 79,000 × g in a SW 32 Ti rotor (Beckman). The 40% iodixanol cushion was collected after centrifugation from the bottom of the tube and overlaid onto 5 mL of a 20–60% step gradient (1 mL of each gradient in 10% incrementing steps) and centrifuged as above. Fractions of 0.5 mL were collected from the bottom of the tubes and analyzed by western blotting (as described above) and TEM.

## 2.5. Transmission electron microscopy (TEM)

Copper grids (mesh size 200) were floated for 2 min on a 1:200 dilution of BTV-8 sheep serum and washed twice with sterile water. Thereafter the grids were floated on a 1:10 dilution of crude plant extract for 5 min and washed three times with sterile water. The samples were negatively stained for 1 min with 2% uranyl acetate. Fractionated samples from the density gradients were treated similarly except they were not captured onto the grids with anti-BTV-8 sheep serum. All grids were viewed using a Technai G2 TEM.

## 2.6. In situ TEM of BTV-8 capsid proteins co-expressed in *N. benthamiana*

*Agrobacterium* carrying the BTV-8 pEAQ-HT VP2co, pEAQ-HT VP3co, pEAQ-HT VP5co and pEAQ-HT VP7co constructs were cultured and combined (as described above) in a ratio of 1:1:2:1 (VP2:VP3:VP5:VP7) and syringe-infiltrated into the abaxial surfaces of six-week-old *N. benthamiana* plants.

At 9 dpi 1 mm × 3 mm infiltrated leaf fragments were fixed overnight in 2.5% glutaraldehyde (25% glutaraldehyde diluted in 0.1 M phosphate buffer [20 mM sodium dihydrogen(ortho)phosphate (NaH<sub>2</sub>PO<sub>4</sub>) and 80 mM disodium hydrogen(ortho)phosphate (Na<sub>2</sub>HPO<sub>4</sub>), pH 7.4]) at 4 °C. The following day the leaf fragments were washed 3 times, 5 min for each wash, in 0.1 M phosphate buffer (pH 7.4). The leaf fragments were fixed for 1 h in one part 2% osmium tetroxide and one part 0.2 M phosphate buffer (40 mM NaH<sub>2</sub>PO<sub>4</sub> and 160 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4), followed by washing twice for 5 min with 0.1 M phosphate buffer and two washes of 5 min each with sterile water.

After washing, the leaf fragments were sequentially dehydrated by incubating for 5 min each in 30%, 50%, 70%, 80%, 90% and 95% ethanol. The fragments were incubated for 10 min in 100% ethanol; this step was repeated twice. Finally, the leaf fragments were further dehydrated by 10 min incubation in 100% acetone, repeated twice. The leaf fragments were mixed O/N in 1:1 acetone: Spurr's (Agar Scientific) resin. The following day half of the 1:1 acetone: Spurr's resin mixture was removed and replaced with 100% Spurr's resin to yield a 1:3 acetone: Spurr's resin mixture. The samples were mixed for 4 h at room temperature, after which the acetone/resin mixture was removed and replaced with 100% Spurr's resin. The leaf fragments were incubated in 100% Spurr's resin for three days at 4 °C. The 100% Spurr's resin was replaced with fresh resin and incubated for 4 h at room temperature after which the resin was replaced again and incubated overnight at room temperature. The

following day the specimens were embedded in 100% Spurr's resin and baked for 24 h at 60 °C.

The embedded leaf specimens were cut into ultrathin sections with a diamond knife and collected onto copper grids (mesh size 200). The copper grids were stained with 2% uranyl acetate for 10 min after which they were rinsed with water. Thereafter, the grids were blotted dry and transferred to lead citrate for 10 min after which the grids were washed with water and blotted dry. Grids were viewed using a Technai G2 TEM.

### 3. Results and discussion

#### 3.1. Expression of BTV-8 capsid proteins in *N. benthamiana*

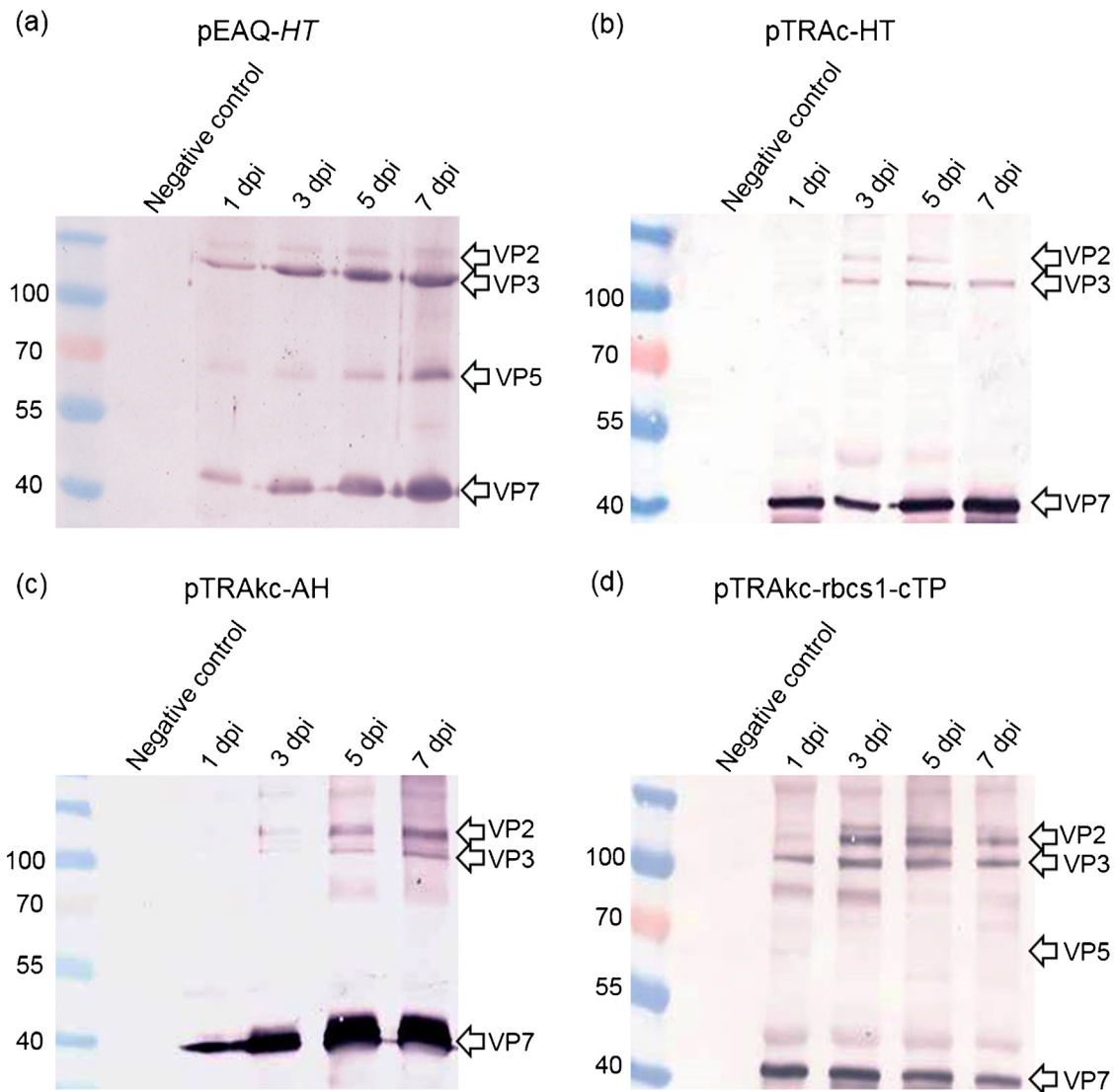
Each set of 4 recombinant constructs (listed in Table 1) was initially co-infiltrated into *N. benthamiana* leaves using a syringe with a relative vector concentration ratio of 1:1:1:1 (VP2:VP3:VP5:VP7), at a final OD<sub>600</sub> value of 0.25 for each construct. Preliminary screening of pEAQ-*HT* infiltrated leaf tissue after 1, 3, 5 and 7 days post infiltration (dpi) using western blot analysis showed that

although VP2, 3 and 7 were present in crude extracts, VP5 did not co-express (results not shown).

It has been shown that an increase in the density of *Agrobacterium* cells infiltrated into lettuce played an important role in increasing transgene expression and expressed protein levels [43]. To determine whether an increase in cell density would influence BTV-8 VP expression using the pTRA and pEAQ-*HT* sets of constructs, infiltration of *N. benthamiana* using different OD<sub>600</sub> values of the recombinant *Agrobacterium* constructs was tested.

The concentration of the recombinant *Agrobacterium* constructs encoding VP5 was subsequently doubled to an OD<sub>600</sub> of 0.5 (for both pTRA and pEAQ-*HT* constructs) and infiltration carried out at a concentration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7). Co-infiltration with the pEAQ-*HT* constructs resulted in expression of all 4 capsid proteins at 5 and 7 dpi. Only expression of VP3 and VP7 was detected during the time-trial using the pTRAc-*HT* and pTRAc-rbcs1-cTP vectors, whereas co-infiltration with the pTRAc-AH constructs resulted in expression of VP5 and VP7 (data not shown).

The cell concentrations used for co-infiltration was further increased to OD<sub>600</sub>=0.5 for constructs expressing VP2, VP3 and



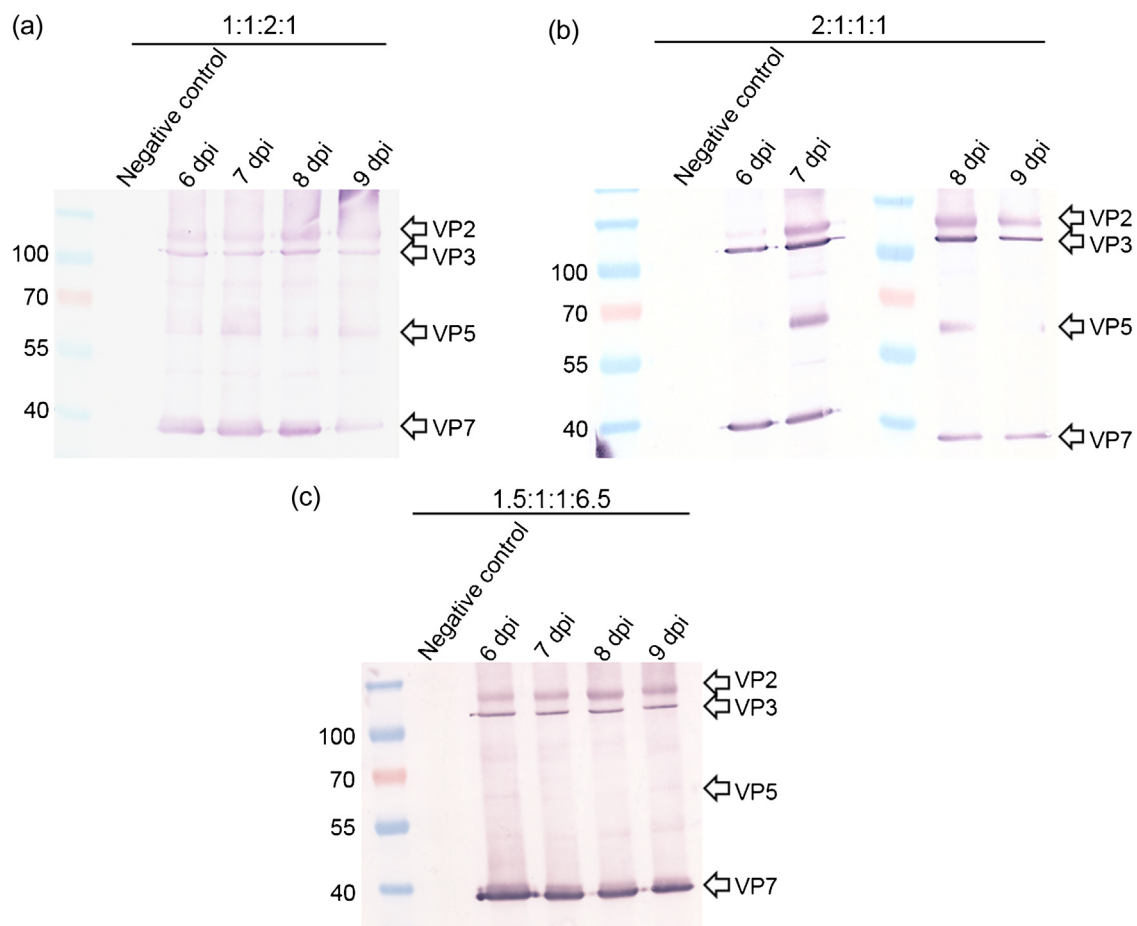
**Fig. 1.** Western blot analysis of crude *N. benthamiana* extracts to detect BTV-8 capsid protein co-expression. BTV-8 capsid protein co-expression (VP2, VP3, VP5 and VP7) over 7 days using 3 different vectors: (a) pEAQ-*HT*, (b) pTRAc-*HT*, (c) pTRAc-AH and (d) pTRAc-rbcs1-cTP. Each set of recombinant constructs was co-infiltrated at OD<sub>600</sub> of 0.5 for each individual construct (with an OD<sub>600</sub> = 1.0 for VP5), using an infiltration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7). Equal amounts (120 µg) of total protein were loaded in each lane. In all cases PageRuler™ Prestained Protein Ladder (Thermo Scientific) was used as a size marker and the negative control was crude leaf extract of a plant infiltrated with infiltration medium.

VP7 while the concentration of VP5-carrying constructs was further increased to  $OD_{600} = 1.0$ . Successful co-expression of all 4 recombinant proteins using the pEAQ-HT plant expression vector was detected at 5 and 7 dpi (Fig. 1a). Expected band sizes of 111 kDa, 102 kDa, 59 kDa and 38 kDa corresponding to VP2, 3, 5 and 7 respectively, were visualized with the most protein detected at 7 dpi.

Using the pTRAc-HT plant expression vector, accumulation of VP2, VP3 and VP7 was detected at 3 and 5 dpi (Fig. 1b); after 7 dpi VP2 was not detected. Co-expression with the pTRAc-AH constructs, resulted in expression of VP7 from 1 to 7 dpi, with VP2 and VP3 visible from 3 dpi onwards (Fig. 1c). The pTRAc-rbcs1-cTP set of constructs resulted in the expression of VP2, VP3 and VP7 from 1 to 7 dpi (Fig. 1d). Noticeably, expression of VP5 was not detected in any crude extracts sampled from 1 to 7 dpi for the pTRA plant expression vectors (Fig. 1b–d). In addition, there was a difference between the different ratios of expressed VP proteins (particularly VP3 and VP7) using the two different systems.

The lack of detection of VP5 expression using the pTRA suite of vectors could be due to the fact that this protein is generally expressed at low levels due to its inherent nature, and that the *HyperTrans* (CPMV-HT) transcription system expresses proteins at significantly higher levels than the p35S promoters of the pTRA vectors; thus in the case of VP5, this protein is expressed by the pEAQ-HT vector at a level which it is detectable by western blot and not when using the pTRA vectors.

For comparison of the pTRA suite of vectors themselves, it is important to recognize that the resultant VP products using the pTRAc-HT constructs had a  $6\times$  his tag on the C-terminus and it is possible that this may have indirectly influenced VP5 longevity in the plant host cell: it has been shown that VP5 interacts strongly with VP7 and assembly of VP5 precedes that of VP2 in the making of VLPs [24]. It is possible that in the use of the pTRAc-HT constructs, CLPs (consisting of VP3 and VP7) may not have assembled correctly due to the presence of the  $6\times$  his tags and that although VP5 was expressed, it was degraded by plant host proteases due to the absence of CLP scaffolds for it to interact with and therefore was not detectable on the western blots. This may be substantiated by our observation in a different experiment for which results are not shown, where although pEAQ-HT constructs encoding C-terminal his-tagged VP3 and VP7 yielded proteins which were detectable by western blot, no CLP assembly was determined by transmission electron microscopy (TEM). In the case of apoplast or chloroplast-targeted BTV VPs (directed by pTRAc-AH and pTRAc-rbcs1-cTP expression vectors, respectively), the assembly of the CLPs or VLPs will likely take place in these particular organelles, after expression of the individual VPs which have N terminal signal sequences directing them either to the chloroplast or apoplast. In these cases, similarly to cytoplasm-targeted protein, VP5 may be degraded due to the lack of CLP scaffolding available. In addition, the cytoplasm of both animal and plant cells has a pH of 7.0–7.5, whereas the apoplast and chloroplast have pH values of 5.8 and 8.0, respectively [12,36].



**Fig. 2.** Western blot analysis of crude *N. benthamiana* extracts. BTV-8 capsid protein co-expression (VP2, VP3, VP5 and VP7) using pEAQ-HT constructs infiltrated at VP2:VP3:VP5:VP7 ratios of (a) 1:1:2:1, (b) 2:1:1:1 and (c) 1.5:1:1:6.5. A starting cell concentration of  $OD_{600} = 0.5$  for each recombinant construct was maintained and the concentration was adjusted according to the ratios used. PageRuler™ Prestained Protein Ladder (Thermo Scientific) was used as a size marker and the negative control was crude leaf extract of a plant infiltrated with infiltration medium.

These pH values could impair the correct assembly of CLPs and VLPs in the appropriate organelles, thus further abrogating the stabilisation of VP5 via particle formation and thus leading to its degradation.

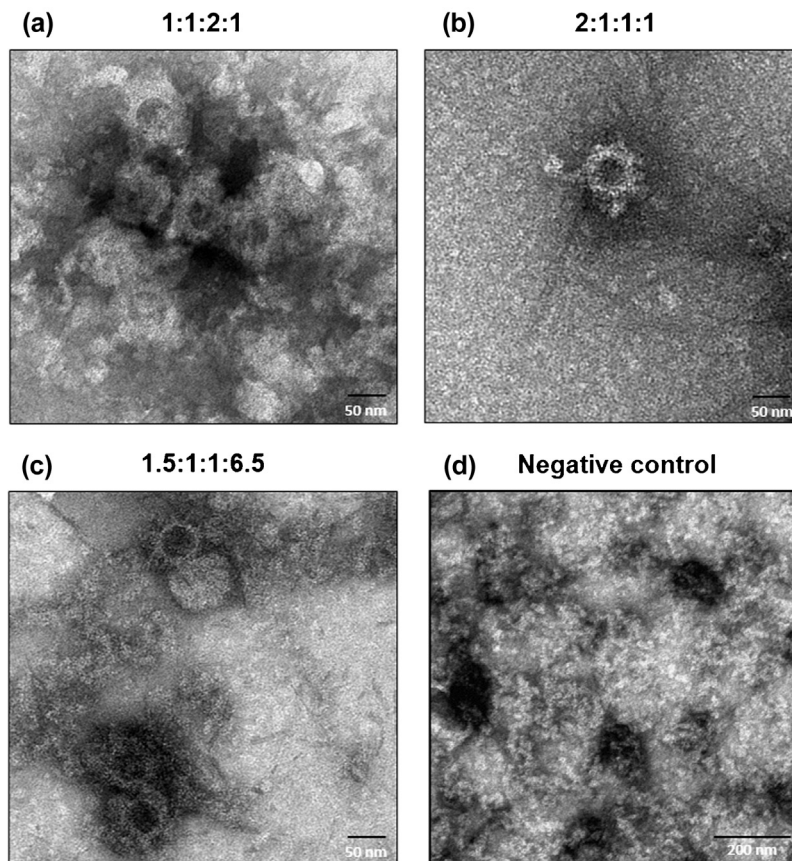
There may be several reasons for the observation of the difference in VP3:VP7 ratios between the pEAQ-*HT* and pTRA suite of vectors. One explanation could be the fundamental difference in gene size: the VP3 protein is much larger than VP7 and therefore if both proteins are expressed at the same rate, there will be more VP7 than VP3. Possibly the difference could be explained by a variable effect of the HT regulatory sequences (in the pEAQ-*HT* vector) on genes of different lengths (VP3 and VP7 are approximately 2.7 kb and 1 kb, respectively). It is not likely the difference is attributed to the 6× his tag, as although it is known that the presence of terminally fused 6× his tags can influence protein expression, in this case only the pTRAc-*HT* constructs (and not pTRAKc-AH nor pTRAKc-rbcs1-cTP constructs) result in recombinant proteins with 6× his tags.

There is a small chance that the difference in expression levels observed between the pTRA vector suite and the pEAQ-*HT* vector could be attributed to the presence of the P19 silencing suppressor which is integrated into the T-DNA of the pEAQ-*HT* vector. This ensures that both the gene of interest and the silencing suppressor are transferred to the same plant host cells, thereby ensuring concomitant suppression of post transcriptional gene silencing activity and expression of recombinant protein [32]. In the case of the pTRA vectors, *Agrobacterium* cells hosting the silencing suppressor plasmid (pBIN-NSs) – P19 was not available in the laboratory in a separate plasmid at the time – were co-infiltrated

with the plant expression vectors containing the genes of interest. Co-infiltration does not necessarily guarantee that both the silencing suppressor plasmid and the recombinant plasmid with the gene of interest are taken up and expressed by the same plant cells. The use of two different silencing suppressors could also have influenced expression levels as they influence the suppression process differently. P19 binds siRNA and miRNA non-specifically, resulting in a reduced amount of free siRNA duplexes and the silencing response is reduced by P19 interfering with siRNA loading of the RNA-induced silencing complex [15]. On the other hand, NSs interferes with siRNA synthesis by preventing long dsRNA from being processed into siRNA by dicer-like proteins [34].

Further increasing the cell concentrations above an OD<sub>600</sub> of 1.0 resulted in necrosis of the infiltrated *N. benthamiana* leaves and did not enhance co-expression of the capsid proteins. It is possible that the higher concentration of *Agrobacterium* cells may have been detrimental to normal plant regulatory functions [43], causing the plant cells to die off which results in abrogation of heterologous protein expression.

Further work could include calculations of the molar ratio of all four proteins in order to help find a better infiltration ratio. However, since the pEAQ-*HT* set of constructs showed the highest co-expression levels of all 4 VPs by qualitative comparison, further optimization experiments were carried out using only this set of constructs. The harvest time post infiltration was extended to 9 dpi in order to determine whether protein levels perhaps increased beyond 7 dpi. The most intense bands for all 4 proteins were observed at 7 and 8 dpi, with recombinant protein levels showing a slight decrease after 8 dpi (Fig. 2a).



**Fig. 3.** TEM analysis of crude extracts. Transmission electron micrographs of leaves infiltrated with the three different ratios tested of *A. tumefaciens* harbouring the recombinant pEAQ-*HT* plasmids at 6 dpi—(a) 1:1:2:1, (ii) 2:1:1:1, (b) 1.5:1:1:6.5. (c) A negative control crude extract of a plant infiltrated with infiltration medium only was also viewed. The crude extracts were captured onto copper grids with anti-BTV sheep serum and viewed using a Technai G2 transmission electron microscope (FEI). Scale bars: (a–c) 50 nm and (d) 200 nm.

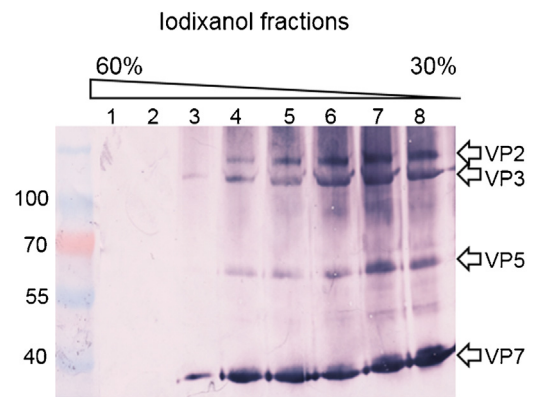
The effects of using different infiltration ratios of the four recombinant pEAQ-*HT Agrobacterium* constructs were also tested in order to find an optimal co-expression approach for the 4 VPs. In addition to the ratio of 1:1:2:1 used above, the ratios of 2:1:1:1 and 1.5:1:1:6.5 (VP2:VP3:VP5:VP7) were tested. The 2:1:1:1 ratio was chosen as VP2 is the largest protein and the ratio of 1.5:1:1:6.5 correlated to the ratio of the number of copies of each of the VPs on the BTV virion as determined by cryo-electron microscopy [11,27,29]. Co-infiltration of the recombinant pEAQ-*HT* constructs in a ratio of 2:1:1:1 resulted in the presence of VP2 (111 kDa), VP3 (102 kDa) and VP7 (38 kDa) on the western blot at 6 dpi with no VP5 visible (Fig. 2b). At 7 and 8 dpi, all 4 VPs constituting the VLPs were observed, with VP5 becoming less visible after 9 dpi. In comparison, infiltration at a cell density ratio of 1.5:1:1:6.5 (Fig. 2c) resulted in co-expression of all 4 capsid proteins at 6 dpi, with VP5 at barely detectable levels. From 7 to 9 dpi only VP2, VP3 and VP7 could be detected on the western blot. In summary, from these qualitative western blots, the use of the infiltration ratio of 2:1:1:1 and harvesting of leaves at 7 and 8 dpi showed that all 4 VP proteins were expressed at similar levels.

### 3.2. Electron microscopy of crude leaf extracts

TEM was carried out on crude leaf extracts of the 6–9 dpi pEAQ-*HT* samples that were infiltrated using the relative vector concentration ratios of 1:1:2:1, 2:1:1:1 and 1.5:1:1:6.5 to assess whether co-infiltration resulted in the assembly of intact core-like particles (CLPs) or virus-like particles (VLPs). It was expected that they could be distinguished from each other by a difference in size, with VLPs having diameters ranging in size from 72 to 80 nm and CLPs ranging in diameter from 60 to 69 nm. The formation of both CLPs and VLPs (Fig. 3) were confirmed in samples harvested at all 3 time points (6, 7 and 8 dpi) tested. Although CLPs were seen in crude extracts from *N. benthamiana* infiltrated with all 3 ratios tested (Fig. 3), there were no VLPs visible in samples from *N. benthamiana* leaves which were co-infiltrated using the ratios of both 2:1:1:1 and 1.5:1:1:6.5 (VP2:VP3:VP5:VP7, Fig. 3b and c). The western blot (Fig. 2) and EM results (Fig. 3) indicated the importance of VP5 in the assembly of VLPs; it appeared that VP5 was not as readily expressed as the other VPs. Therefore, increasing the infiltration volume of the recombinant *Agrobacterium* carrying VP5, increased the amount of VP5 being successfully co-expressed with the other VPs.

### 3.3. Purification of putative BTV-8 VLPs

Since VLPs were observed in crude leaf extracts from samples infiltrated with pEAQ-*HT* constructs in the ratio of 1:1:2:1, the number of plants infiltrated with this particular construct was scaled up to produce larger amounts of VLPs for purification using density gradient centrifugation. In addition, several small modifications were made to the method of purification to promote VLP stability: plant tissue was not ground up in liquid nitrogen but homogenized in buffer, and the extraction buffer containing bicine (similar to that used by Theuenemann et al. [37] did not include DTT or NLS; DTT is a redox reagent that reduces disulphide bridges of proteins and prevents intra-molecular disulphide bonds from forming between cysteine residues, while NLS is a detergent that denatures proteins by reducing solvent-inaccessible disulfide bonds. The infiltrated plant tissue was harvested at 9 dpi, as this was found to be the day at which the most intact VLPs could be purified by ultracentrifugation, as determined by TEM analysis. Furthermore, the best ratio for all 4 VPs were also detected at 9 dpi with western blot analysis (Fig. 2a). The purified fractions were separated on an 8% SDS polyacrylamide gel.



**Fig. 4.** Western blot analysis of density gradient fractions. Fractions 1–8 from 30 to 60% iodixanol gradient centrifugation of pEAQ-*HT* VLPs probed with anti-BTV-8 antiserum. PageRuler™ Prestained Protein Ladder (Thermo Scientific) was used as a size marker.

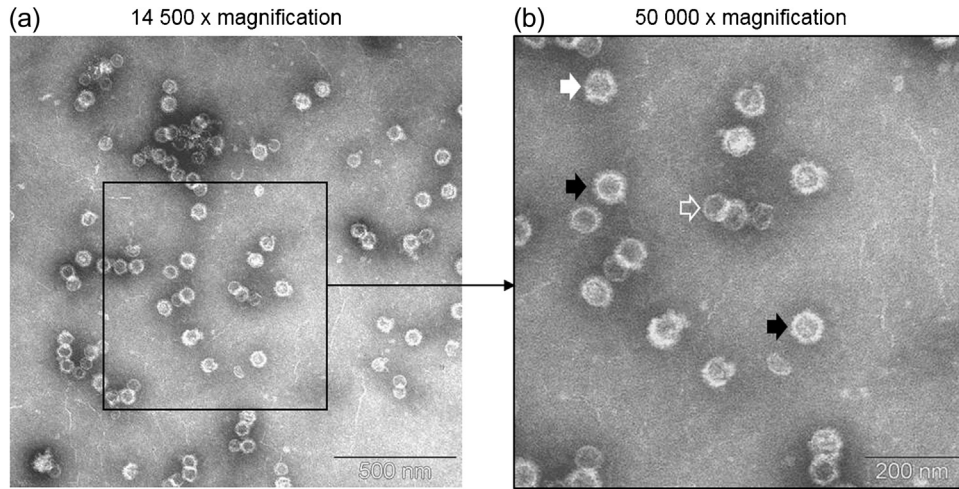
Western blot analysis of the first 8 fractions collected from the iodixanol gradient after centrifugation showed the presence of all four bands constituting the BTV-8 VLPs (VP2, 3, 5 and 7) in fractions 4 (approximately 40–50% iodixanol) to 8 (20–30% iodixanol, Fig. 4). These samples were also resolved on a Coomassie-stained SDS-polyacrylamide gel (not shown): only VP3, VP5 and VP7 were observed on the gel.

### 3.4. Electron microscopy of density gradient-purified VLP fractions

TEM of fraction 4 from the density gradient showed a mixed population of subcore-like particles, CLPs and VLPs based on diameter measurements (Fig. 5a and b). Subcore-like particles had a diameter of 52–54 nm and were hexagonal, angular or round, depending on their orientation on the grid. CLPs measured 60–69 nm in diameter and VLPs measured 72–80 nm in diameter. Ten fields of view, at a magnification of 14,500× showed approximately 80 particles in each view, of which approximately 10% were VLPs (Fig. 5a, one field of view shown). Fig. 5b shows more detail of the particles, at 50,000× magnification, making it easier to distinguish CLPs (single shelled particles—white arrow) from VLPs (double shelled particles—black arrows). Although all 4 VP proteins were detected in fractions 5–8 by western blot (Fig. 4), only CLPs were observed by TEM analysis of these samples (results not shown). Since VLPs are comprised of all 4 VPs, they are more dense and therefore will be found at the lower end of the gradient which may explain their absence in the higher fractions 5–8. These results indicate that although VP2 and VP5 are co-purified in the gradient with CLPs, the VLPs may not be stable during purification where VP2 and VP5 dissociate from the scaffolding surface provided by the CLPs. Thus, even though the proteins are detected on western blots they are not incorporated into fully formed particles. These results indicated that purification of VLPs needs to be further optimized to separate VLPs from CLPs (Fig. 5a and b). This could be achieved by changing the iodixanol gradient range or varying the gradient slope to separate the two particle types further.

### 3.5. In situ TEM of BTV-8 capsids

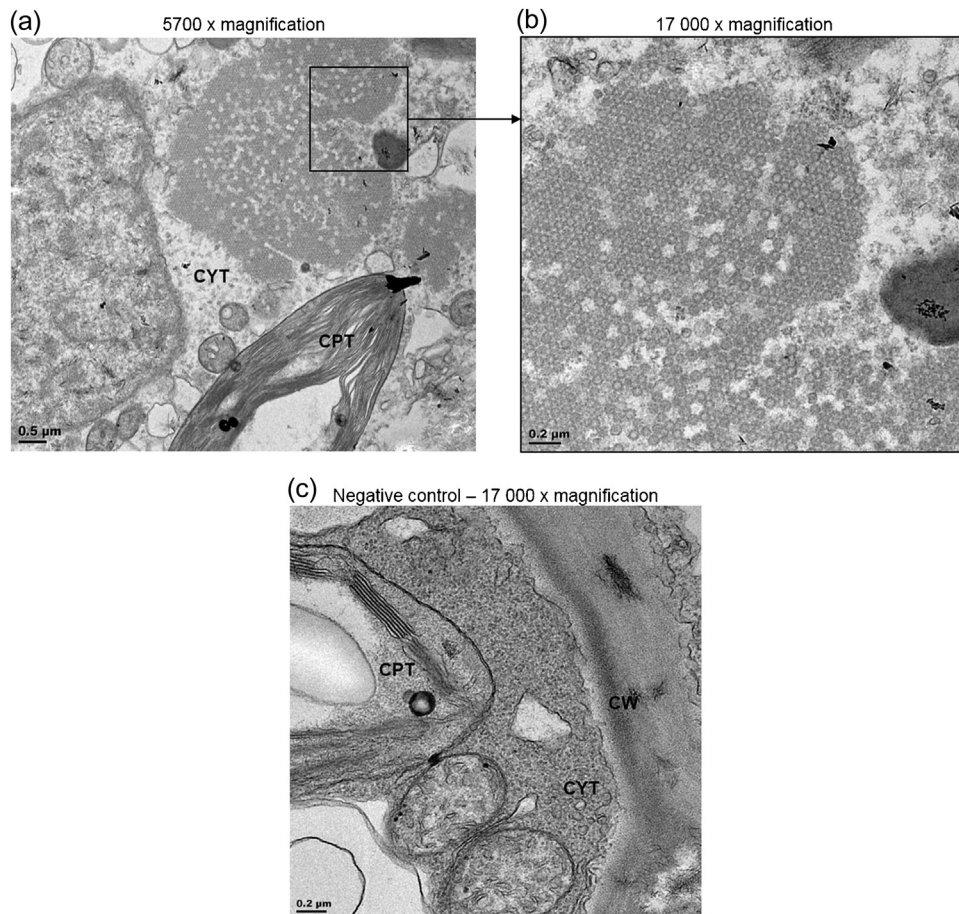
Leaves infiltrated with recombinant pEAQ-*HT A. tumefaciens* constructs using a relative vector concentration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7) were harvested at 9 dpi, embedded in Spurr's resin and subsequently viewed using TEM. It was confirmed with western blot analysis that all four capsid proteins were successfully expressed at 9 dpi (results not shown).



**Fig. 5.** TEM analysis of fraction 4 taken from 30 to 60% iodixanol gradient. (a) 14,500 $\times$  magnification of BTV8 VLPs and CLPs ranging in size from 70 to 88 nm in diameter. (b) A 50,000 $\times$  magnification of the same view. Black arrows indicate VLPs measuring 80 nm in size, the white arrow shows a CLP of 65 nm and the empty white arrow indicates a subcore-like particle (measuring 53 nm). Scale bars: (a) 500 nm and (b) 200 nm. The images were obtained using a Technai G2 transmission electron microscope.

Paracrystalline arrays consisting of spherical particles were observed in the cytoplasm of the plant cells where particles were accumulated (Fig. 6). The particles ranged from 61 to 83 nm in diameter, indicating the existence of a mixed population of particles consisting of both CLPs and VLPs. Fig. 5a and b shows the same field of view at 5700 $\times$  and 17,000 $\times$  magnification,

respectively. CLPs and VLPs did not accumulate in every plant cell (results not shown), but when a cell did contain particles, there were more than 2000 particles per field of view (Fig. 6a). No similar structures were observed in the negative control samples infiltrated with infiltration media (Fig. 6c). French and Roy [8] showed that insect cell-produced CLPs also aggregated as



**Fig. 6.** TEM analysis of leaf sections that were infiltrated with recombinant pEAQ-HT plasmids using an infiltration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7). (a) and (b) represent one field of view at 5700 $\times$  and 17,000 $\times$  magnification, respectively. (c) Negative control leaf infiltrated with infiltration medium at 17,000 $\times$  magnification. Scale bars: (a): 0.5  $\mu$ m, (b) and (c): 0.2  $\mu$ m. CW: cell wall, CPT: chloroplast, CYT: cytoplasm.



paracrystalline arrays within the cytoplasm. BTV virions assemble within the cytoplasm of infected host cells, whereafter they are released from the cells in a process mediated by the non-structural BTV protein, NS3 [1,23]. The *in situ* results from this study together with the work done by French and Roy [8] suggest that recombinantly-produced CLPs and VLPs assemble within the cytoplasm, similar to native BTV virions. The absence of NS3 from recombinantly-produced particles possibly results in their retention within the plant cell cytoplasm.

#### 4. Conclusions

In recent years, plants have become an increasingly popular vehicle for expressing recombinant proteins [30,31]. They have many advantages, including the ease of scale up, lower cost of production compared to that in mammalian or insect cells, their ability to carry out post translational modifications such as glycosylation, as well as providing the opportunity to target protein expression to different subcellular locations and thereby increase stability and accumulation. They have an added advantage for the production of veterinary vaccines in particular, since regulatory bodies still allow animals to be dosed with crude plant extracts or preparations with limited processing for medicinal purposes, whereas regulations regarding dosing of medicines to humans are far more stringent [17]. Since the downstream cost of making plant-produced proteins ranges from 65 to 95% of the total cost of manufacture [42], the requirement for less stringent purity of products contributes even further in favour of making veterinary vaccines in plants.

The expression of subunit vaccines in plants for several different animal viral diseases, including avian influenza, Foot-and-mouth disease and Newcastle disease has previously been reported [17]. Virus-like particles are particularly appealing for veterinary vaccines as they stimulate strong humoral and cellular immune responses and have potential for use as vectors to present multimeric epitopes against different strains of the same viruses [4]. While the successful plant production of BTV-8 VLPs has been reported elsewhere [37], this study – performed simultaneously – was aimed at comparing the ability of several different plant expression vectors to transiently co-express the 4 capsid proteins of BTV-8 (VP2, VP3, VP5 and VP7) in *N. benthamiana* leaves, and to form intact VLPs which could potentially be used as a vaccine. This is the first time that recombinant BTV VLPs have been shown to aggregate in paracrystalline arrays in the cytoplasm of plant cells when transiently expressed.

The approach used here to produce BTV VLPs in plants contrasts to that of Thuenemann et al. [37], in that it is more flexible in terms of being able to vary individual *Agrobacterium* construct densities for “tuning” protein expression. This approach may be necessary for making vaccines for BTV serotypes other than BTV-8, as there is no guarantee that the individual VPs of these will be expressed in the same ratios as has been shown for BTV-8.

#### Conflict of interest

The authors of this manuscript have no conflicts of interest.

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#### References

- [1] M. Boyce, J. Wehrfritz, R. Noad, P. Roy, Purified recombinant bluetongue virus VP1 exhibits RNA replicase activity, *J. Virol.* 78 (2004) 3994–4002.
- [2] Q. Chen, H. Lai, Plant-derived virus-like particles as vaccines, *Hum. Vaccin. Immunother.* 9 (2013) 26–49.
- [3] P. Coetzee, M. Stokstad, E. Venter, M. Myrmel, M. Van Vuuren, Bluetongue: a historical and epidemiological perspective with the emphasis on South Africa, *Virol. J.* 9 (2012) 198.
- [4] E. Crisci, J. Bárcena, M. Montoya, Virus-like particles: the new frontier of vaccines for animal viral infections, *Vet. Immunol. Immunopathol.* 148 (2012) 211–225.
- [5] B. Dungu, C. Potgieter, B. Von Teichman, T. Smit, Vaccination in the control of bluetongue in endemic regions: the South African experience, *Dev. Biol.* 119 (2004) 463–472.
- [6] R. Fischer, C. Vaquero-Martin, M. Sack, J. Drossard, N. Emans, U. Commandeur, Towards molecular farming in the future: transient protein expression in plants, *Biotechnol. Appl. Biochem.* 30 (1999) 113–116.
- [7] T.J. French, J.J. Marshall, P. Roy, Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins, *J. Virol.* 64 (1990) 5695–5700.
- [8] T.J. French, P. Roy, Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV, *J. Virol.* 64 (1990) 1530–1536.
- [9] B.M. Gorman, The bluetongue viruses, *Curr. Top. Microbiol. Immunol.* 162 (1990) 1–19.
- [10] E.V. Grgacic, D.A. Anderson, Virus-like particles: passport to immune recognition, *Methods* 40 (2006) 60–65.
- [11] S.S. Hassan, P. Roy, Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry, *J. Virol.* 73 (1999) 9832–9842.
- [12] W.H. Heldt, K. Werdan, M. Milovancev, G. Geller, Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space, *Biochim. Biophys. Acta* 314 (1973) 224–241.
- [13] R. Hellens, P. Mullineaux, H. Klee, Technical focus: a guide to *Agrobacterium* binary Ti vectors, *Trends Plant Sci.* 5 (2000) 446–451.
- [14] M.A. Hofmann, S. Renzullo, M. Mader, V. Chaignat, G. Worwa, B. Thuer, Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland, *Emerg. Infect. Dis.* 14 (2008) 1855–1861.
- [15] Y.C. Hsieh, R.T. Omarov, H.B. Scholthof, Diverse and newly recognized effects associated with short interfering RNA binding site modifications on the tomato bushy stunt virus p19 silencing suppressor, *J. Virol.* 83 (2009) 2188–2200.
- [16] D. Hutcheon, Malarial catarrhal fever of sheep, *Vet. Rec.* 14 (1902) 629–633.
- [17] H.Y. Ling, A. Pelosi, A.M. Walmsley, Current status of plant-made vaccines for veterinary purposes, *Expert Rev. Vaccines* 9 (2010) 971–982.
- [18] F. Liu, S. Ge, L. Li, X. Wu, Z. Liu, Z. Wang, Virus-like particles: potential veterinary vaccine immunogens, *Res. Vet. Sci.* 93 (2012) 553–559.
- [19] N.S. Maan, S. Maan, M.N. Belaganahalli, E.N. Ostlund, D.J. Johnson, K. Nomikou, P.P.C. Mertens, Identification and differentiation of the twenty six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2, *PLoS One* 7 (2012) e32601.
- [20] N.J. MacLachlan, Bluetongue: pathogenesis and duration of viraemia, *Vet. Ital.* 40 (2004) 462–467.
- [21] J. Maclean, M. Koekemoer, A.J. Olivier, D. Stewart, I.I. Hitzeroth, T. Rademacher, R. Fischer, A.L. Williamson, E.P. Rybicki, Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization, *J. Gen. Virol.* 88 (2007) 1460–1469.
- [22] M. Merlin, E. Gecchele, S. Capaldi, M. Pezzotti, L. Avesani, Comparative evaluation of recombinant protein production in different biofactories: the green perspective, *Biomed. Res. Int.* (2014) .
- [23] P.P. Mertens, J. Diprose, S. Maan, K.P. Singh, H. Attoui, A.R. Samuel, Bluetongue virus replication, molecular and structural biology, *Vet. Ital.* 40 (2004) 426–437.
- [24] E.L. Nason, R. Rothagel, S.K. Mukherjee, A.K. Kar, M. Forzan, B.V. Prasad, P. Roy, Interactions between the inner and outer capsids of bluetongue virus, *J. Virol.* 78 (2004) 8059–8067.
- [25] R. Noad, P. Roy, Virus-like particles as immunogens, *Trends Microbiol.* 11 (2003) 438–444.
- [26] M. Prins, O. Resende Rde, C. Anker, A. van Schepen, P. de Haan, R. Goldbach, Engineered RNA-mediated resistance to tomato spotted wilt virus is sequence specific, *Mol. Plant Microbe Interact.* 9 (1996) 416–418.
- [27] P. Roy, Bluetongue virus proteins, *J. Gen. Virol.* 73 (Pt. 12) (1992) 3051–3064.
- [28] P. Roy, Genetically engineered particulate virus-like structures and their use as vaccine delivery systems, *Intervirology* 39 (1996) 62–71.
- [29] P. Roy, Genetically engineered structure-based vaccine for bluetongue disease, *Vet. Ital.* 40 (2004) 594–600.
- [30] E.P. Rybicki, Plant-produced vaccines: promise and reality, *Drug Discov. Today* 14 (2009) 16–24.

- [31] E.P. Rybicki, Plant-made vaccines for humans and animals, *Plant Biotechnol. J.* 8 (2010) 620–637.
- [32] F. Sainsbury, E.C. Thuenemann, G.P. Lomonosoff, pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants, *Plant Biotechnol. J.* 7 (2009) 682–693.
- [33] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1989.
- [34] E. Schnettler, H. Hemmes, R. Huisman, R. Goldbach, M. Prins, R. Kormelink, Diverging affinity of tospovirus RNA silencing suppressor proteins, NSs, for various RNA duplex molecules, *J. Virol.* 84 (2010) 11542–11554.
- [35] N. Scotti, E.P. Rybicki, Virus-like particles produced in plants as potential vaccines, *Expert Rev. Vaccines* 12 (2013) 211–224.
- [36] R.M. Spanswick, A.G. Miller, Measurement of the cytoplasmic pH in *Nitella translucens*: comparison of values obtained by microelectrode and weak acid methods, *Plant Physiol.* 59 (1977) 664–666.
- [37] E.C. Thuenemann, A.E. Meyers, J. Verwey, E.P. Rybicki, G.P. Lomonosoff, A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles, *Plant Biotechnol. J.* 6 (2013) 12076.
- [38] J.F. Toussaint, F. Vandenbussche, J. Mast, L. De Meester, N. Goris, W. Van Dessel, E. Vanopdenbosche, P. Kerkhofs, K. De Clercq, S. Zientara, C. Sailleau, G. Czaplicki, G. Depoorter, J.M. Dochy, Bluetongue in Northern Europe, *Vet. Rec.* 159 (September (10)) (2006) 327.
- [39] T. Tzfira, V. Citovsky, Agrobacterium-mediated genetic transformation of plants: biology and biotechnology, *Curr. Opin. Biotechnol.* 17 (2006) 147–154.
- [40] L. van Wuijckhuise, D. Dercksen, J. Muskens, J. de Bruijn, M. Scheepers, R. Vrouenraets, Bluetongue voor het eerst in Nederland: beschrijving van de klinische verschijnselen en differentiaaldiagnose: bekende ziektebeelden maar toch net iets anders en op te veel bedrijven, *Tijdschr. Diergeneesk.* 131 (2006) 649–654.
- [41] P. Vellema, Bluetongue in sheep: question marks on bluetongue virus serotype 8 in Europe, *Small Rumin. Res.* 76 (2008) 141–148.
- [42] L.R. Wilken, Z.L. Nikolov, Recovery and purification of plant-made recombinant proteins, *Biotechnol. Adv.* 30 (2012) 419–433.
- [43] T. Wroblewski, A. Tomczak, R. Michelmore, Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*, *Plant Biotechnol. J.* 3 (2005) 259–273.