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A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles

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Summary

Plant expression systems based on nonreplicating virus-based vectors can be used for the simultaneous expression of multiple genes within the same cell. They therefore have great potential for the production of heteromultimeric protein complexes. This work describes the efficient plant-based production and assembly of Bluetongue virus-like particles (VLPs), requiring the simultaneous expression of four distinct proteins in varying amounts. Such particles have the potential to serve as a safe and effective vaccine against Bluetongue virus (BTV), which causes high mortality rates in ruminants and thus has a severe effect on the livestock trade. Here, VLPs produced and assembled in *Nicotiana benthamiana* using the cowpea mosaic virus–based *HyperTrans* (CPMV-*HT*) and associated pEAQ plant transient expression vector system were shown to elicit a strong antibody response in sheep. Furthermore, they provided protective immunity against a challenge with a South African BTV-8 field isolate. The results show that transient expression can be used to produce immunologically relevant complex heteromultimeric structures in plants in a matter of days. The results have implications beyond the realm of veterinary vaccines and could be applied to the production of VLPs for human use or the coexpression of multiple enzymes for the manipulation of metabolic pathways.

Introduction

The efficient production of complex heteromultimeric protein complexes, in which the various components are present in differing stoichiometries, represents a major challenge to plant expression methods. It requires the production of all the components of the complex within the same cell at appropriate levels. This has proved very difficult to achieve, especially with transient expression approaches using replicating virus vectors. Nonreplicating systems, such as the cowpea mosaic virus–based HyperTrans (CPMV-*HT*) system (Sainsbury and Lomonossoff, 2008; Sainsbury *et al.*, 2009), offer considerable potential benefits for the production of heteromultimeric complexes: it is possible to control the level of expression of the individual components, and multiple constructs can be expressed in the same cell without the problem of virus exclusion found with replicating systems. We therefore examined whether the CPMV-*HT* system can be used to efficiently produce virus-like particles (VLPs) of Bluetongue virus (BTV).

Bluetongue is a severe disease of ruminants, notably sheep and cattle, causing facial swelling, lameness and infertility amongst other symptoms and leading to mortality in some cases. The causal agent, the multicomponent dsRNA Bluetongue virus, is spread by an insect vector, *Culicoides sp.*, and occurs in its vector's habitat in temperate climates throughout much of the world (Carpenter *et al.*, 2009). BTV is the type member of genus *Orbivirus* in the family *Reoviridae*, with 26 known serotypes (Maan *et al.*, 2012). When bluetongue first broke out in the United Kingdom in autumn of 2007, the disease was already rapidly spreading throughout continental Europe, causing high mortality rates in sheep and having a detrimental effect on the livestock trade through trade restrictions and loss of stock. The only effective weapon against the disease is control of the spread of BTV through rigorous vaccination programmes. Currently available commercial vaccines are based on both inactivated virus and live, attenuated strains and protect against a single serotype or multiple serotypes when provided as a cocktail (Savini *et al.*, 2008). However, the possibility of recombination between the live vaccine strain(s) and wild-type virus in infected animals, leading to the emergence of new infectious strains (Batten *et al.*, 2008), has motivated efforts to develop safer vaccines.

One approach in the development of an inherently safe vaccine has been the production of Bluetongue virus-like particles (VLPs) (French *et al.*, 1990; Roy *et al.*, 1992). BTV has a nonenveloped icosahedral structure, with four main structural proteins (VP3, VP7, VP5 and VP2) arranged in concentric shells around the segmented double-stranded RNA genome and minor structural and nonstructural proteins involved in virus replication. French *et al.* have shown that these four structural proteins, expressed in insect cells using a baculovirus expression system, assemble into virus-like particles devoid of nucleic acid (French *et al.*, 1990). The most internal of the structural proteins, VP3, is a 100-kDa protein, 120 copies of which form 60 dimers, which assemble into a $T = 1$ particle of 55 nm diameter (Grimes *et al.*, 1998) (schematic in

Figure 1c). During virus assembly, trimers of VP7 form an icosahedral shell of $T = 13$ symmetry on the scaffold provided by VP3, resulting in the production of core particles consisting of 120 copies of VP3 and 780 copies of VP7 (Prasad *et al.*, 1992). Using a baculovirus expression system in insect cells, core-like particles (CLPs) of VP3 and VP7 have been produced (French and Roy, 1990). These are stable structures, although lacking 60 trimers of VP7 at the fivefold axes (Hewat *et al.*, 1994), but do not induce a protective immune response in sheep. For this, a third shell consisting of 180 copies of VP2 and 360 copies of VP5 is needed. The major immunogenicity determinant, VP2, is a 102-kDa protein forming triskelion-shaped trimers on the surface of BTV (Hassan and Roy, 1999). Between these structures, 120 trimers of the 59-kDa VP5, which are involved in virus entry into cells, are positioned (Forzan *et al.*, 2004). Inoculation of sheep with insect cell–expressed triple-shelled VLPs of BTV-10 containing all four structural proteins induces protective immunity against challenge with live virus of the same serotype. In addition, inoculation of sheep with similar structured BTV-1 VLPs produced in insect cells also induced neutralizing antibodies to BTV-1 and protected sheep challenged with live virus of the same serotype (Stewart *et al.*, 2012). These results show that BTV VLPs have the potential to be used as an inherently safe vaccine (Roy *et al.*, 1992). However, the high cost of production of such VLPs may be one barrier that has prevented wide-scale development of these vaccines. Over the past three decades, plants have become an increasingly popular alternative host for the heterologous expression of complex high-value proteins, providing cost-effective expression without the risk of contaminating animal pathogens. Transient expression systems in particular allow for very rapid expression, development and testing of new constructs in a matter of days (Rybicki, 2010).

Here, we report the plant-based high-level expression of assembled subcore-, core- and virus-like particles of BTV serotype 8 using the CPMV-*HT* and associated pEAQ vector system (Sainsbury and Lomonossoff, 2008; Sainsbury *et al.*, 2009). Purified preparations of the VLPs, consisting of all four structural proteins, elicited an immune response in sheep and provided protective immunity against challenge with a South African BTV-8 field isolate. This demonstrates that CPMV-*HT* technology provides an economically viable method for producing complex VLPs, such as those of BTV, with the desired biological properties. It represents a significant advance in the use of plant-based systems for the production of complex biopharmaceuticals. The methods employed could also be applied to other situations where the expression of multiple proteins is required, such as the reconstruction of metabolic pathways.

Results

BTV coat proteins transiently expressed in *Nicotiana benthamiana* leaves assemble into subcore-, core- and virus-like particles

To determine whether BTV-8 structural proteins could be expressed in plant leaves, constructs containing each of the four structural proteins VP3, VP7, VP5 and VP2 were created. The genes encoding these proteins were codon-optimized for *N. benthamiana* expression and cloned between the CPMV untranslated regions (UTRs) in the pEAQ-*HT* vector (Figure 1b). Transient expression was achieved by infiltration of *N. benthamiana* leaves with cultures of *Agrobacterium tumefaciens* carrying these constructs, either individually or in various combinations. All constructs produced

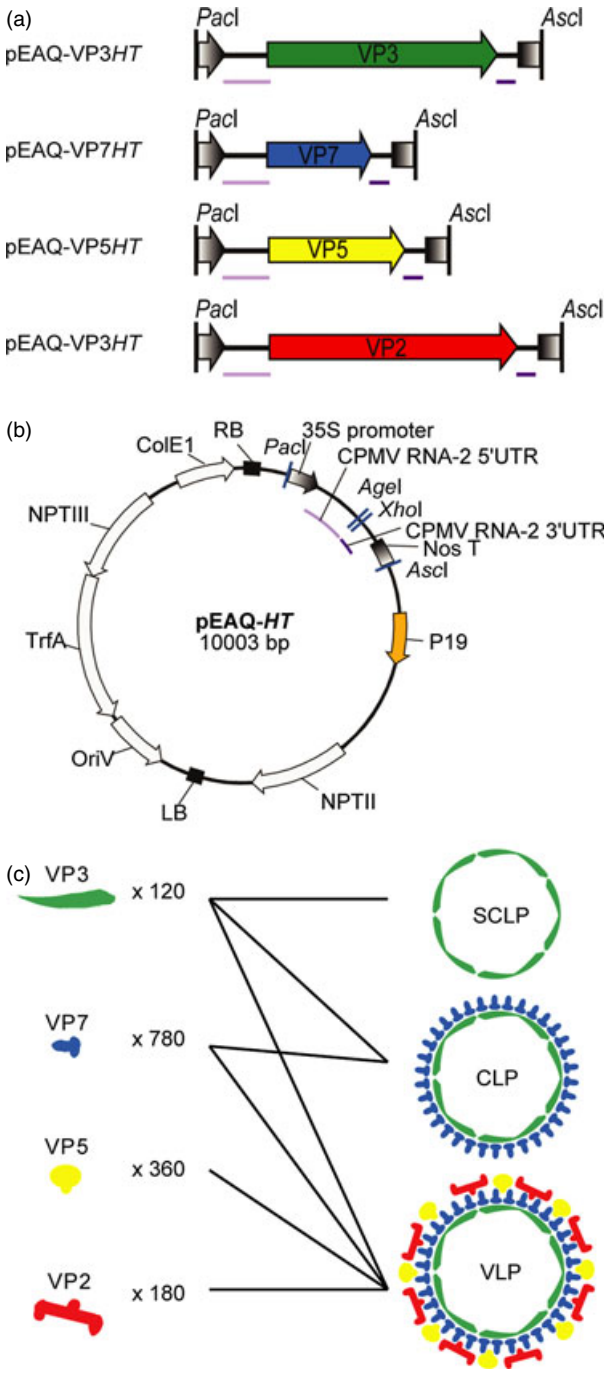

Figure 1 Schematic representations of constructs for plant-based expression of Bluetongue virus (BTV)-8 proteins and their assembly into particle structures. Maps of the cowpea mosaic virus–based *HyperTrans* (CPMV-*HT*) expression cassettes (a) and pEAQ-HT expression vector backbone (b) for expression of codon-optimized versions of BTV8 proteins VP3, VP7, VP5 and VP2. (c) Schematic of major BTV structural proteins, indicating how many copies of each structural protein come together to form subcore-, core- and virus-like particles (SCLP, CLP and VLP). pEAQ vectors contain an expression cassette for P19, a suppressor of gene silencing (Silhavy *et al.*, 2002), required for high-level heterologous expression in plants.

chlorotic symptoms in the infiltrated leaf tissue. Individual expression of VP3 and VP5 produced necrotic symptoms, whilst in combination with the other proteins, no necrosis was observed

(Figure S1). Total protein extracts from leaf tissue infiltrated with the individual BTV-8 constructs in each case contained a distinct band, not present in the negative control, corresponding to the size of the relevant structural protein; the identity of these protein bands was confirmed by mass spectrometry (Figure S2).

To investigate whether the BTV-8 structural proteins expressed in leaf tissue could assemble into appropriate particulate structures, *N. benthamiana* leaves were either infiltrated with the VP3 gene alone, coinfiltrated with the VP3 and VP7 genes or infiltrated with all four structural protein genes. Plant extracts were fractionated by centrifugation on sucrose or iodixanol step gradients, and the protein content of fractions was analysed by SDS-PAGE followed by Coomassie blue staining (Figure 2a, Figure S3). In all cases, the majority of the BTV-specific proteins were found in the 45%–55% sucrose fractions (30%–40% iodixanol fractions), which were largely devoid of contaminating plant proteins. This indicates that the BTV proteins had assembled into high-molecular-weight structures. Furthermore, the cosedimentation of the BTV proteins in extracts of leaves coinfiltrated with VP3 and VP7 or with VP3, VP7, VP2 and VP5 strongly suggested that the proteins had interacted to form supramolecular structures.

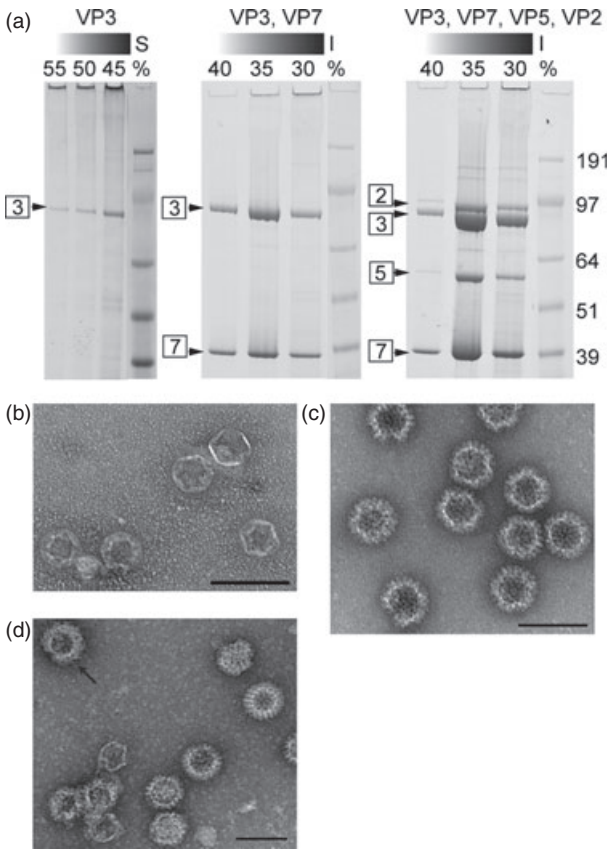

Figure 2 Assembly of BTV-8 structural proteins into particles. Leaves were infiltrated with pEAQ-*HT* constructs as indicated, harvested 8 dpi and extracted, and extracts subjected to sucrose (S) or iodixanol (I) density gradient centrifugation, as indicated. (a) Fractions were collected from the bottom and separated by denaturing SDS-PAGE followed by Coomassie blue staining. Marker sizes indicated on far right and location of viral proteins VP2, VP3, VP5 and VP7 (boxed) to the left of each gel. (b-d) Imaging of fractions from gradients of VP3 showing three different orientations of SCLPs (b), VP3 and VP7 showing CLPs (c), and VP3, VP7, VP5 and VP2 showing VLPs (arrow) and assembly intermediates (d). Scale bars, 100 nm.

The complexes formed by the BTV proteins were analysed by transmission electron microscopy of the gradient fractions in which they were found. Expression of VP3 alone resulted in the formation of particles closely resembling BTV subcore-like particles (SCLPs) obtained by stripping CLPs of the VP7 layer (Loudon and Roy, 1991). These VP3 SCLPs had an average diameter of 54.9 (±1.3) nm and three distinct appearances (hexagonal, angular and round), depending on their orientation on the grid (Figure 2b). Coexpression of VP3 and VP7 yielded larger particles of 69.6 (±0.9) nm in diameter. These particles had a striking appearance consistent with that of BTV core particles and insect cell–produced CLPs (Figure 2c). The distinct difference in appearance compared with the SCLPs is likely due to the layer of VP7 trimers arranged in a concentric shell on top of the VP3 layer. Coexpression of all four structural proteins of BTV-8 resulted in a mixture of particle types, some of which appeared to be CLPs, whilst others were larger (Figure 2d). The larger particles had a less-structured appearance than CLPs, with some appearing to be assembly intermediates between CLPs and VLPs. Although these larger particles indicate that VP5 and VP2 can associate with CLPs in plant cells, the stoichiometry of the structural proteins (Figure 2a) is indicative of partial conversion of SCLPS and CLPs into fully assembled VLPs. Based on densitometric analysis (Figure S4) and electron microscopy, we estimate that these assembly intermediates constitute at least 50% of the particles produced by coinfiltration with four pEAQ-*HT* constructs.

Modulation of VP3 expression levels increases recovery of virus-like particles

To increase the yield of fully assembled VLPs in leaves expressing the four BTV-8 structural proteins, their relative expression levels were modulated. Due to the generally higher abundance of the smaller proteins, protein staining of a preparation of fully assembled virus produces bands of similar intensity for all four of the main structural proteins in an SDS-PAGE gel (Martin and Zweerink, 1972). Coexpression of the four BTV-8 structural proteins from separate pEAQ-*HT* constructs resulted in preparations with an over-representation of VP3, resulting in the presence of substantial numbers of subcore-like particles or assembly intermediates (Figure 2a).

To address this issue, we made use of the fact that multiple expression cassettes, each containing a gene of interest flanked by CPMV UTRs as well as the promoter and terminator, can be cloned into the vector pEAQexpress (Sainsbury *et al.*, 2009) (Figure 3a). Thus, plasmids were created which were designed to express either the two core proteins (VP3 plus VP7) or the two outer shell proteins (VP5 plus VP2) from the same T-DNA. To control the level of CLP synthesis, two different versions of the VP3 + VP7 construct were produced. In addition to the codon-optimized genes under control of the *HyperTrans* UTRs for maximum expression (pEAQex-VP7*HT*-VP3*HT*), another (pEAQex-VP7*HT*-VP3wt) was made with codon-optimized genes but containing VP3 under control of the CPMV wild-type 5′ UTR, that is, not containing the *HyperTrans* mutation. This latter construct was designed to down-regulate the synthesis of CLPs and shift the equilibrium towards VLPs when coexpressed with VP2 and VP5. Combining two proteins on the same T-DNA also halved the minimum number of different *Agrobacterium* T-DNA

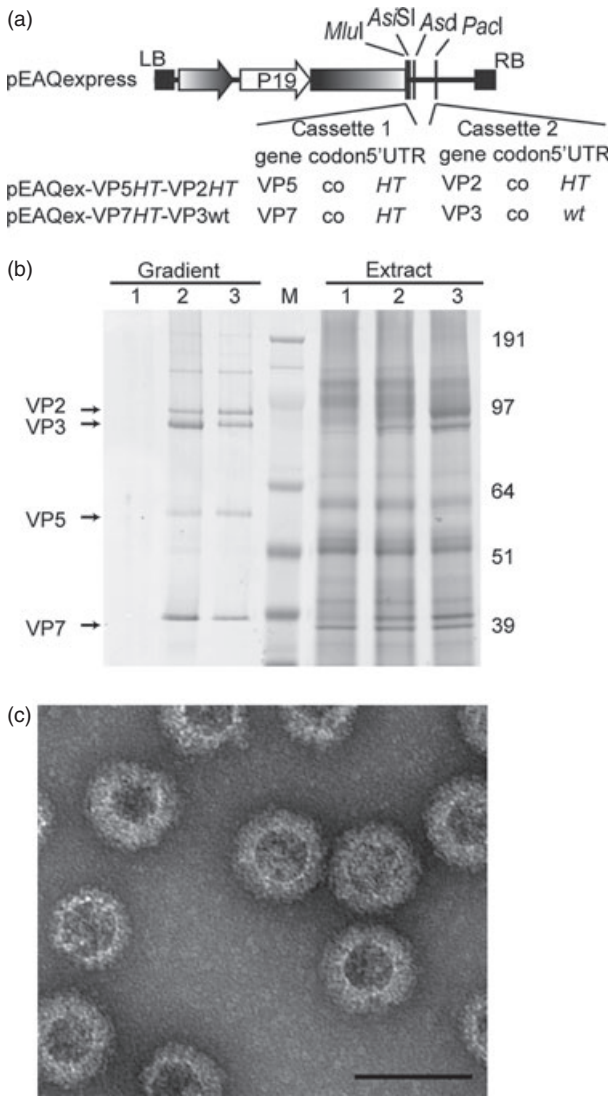

Figure 3 The stoichiometry of recovered VLPs can be influenced by changes in expression constructs. pEAQexpress vectors were made to incorporate two Bluetongue virus (BTV)-8 genes (VP5 and VP2, or VP7 and VP3) on the same construct to ensure coexpression. These constructs contained codon-optimized (co) genes and either *HyperTrans* (*HT*) or wild-type (wt) 5′UTRs. (a) Schematic representation of pEAQexpress vectors. (b) pEAQexpress vectors and pEAQ-*HT* vectors were coinfiltrated in combinations (1, 2 or 3) to codeliver all four BTV-8 genes. Leaves were harvested 8 dpi, and particles from clarified extracts purified on sucrose gradients. Clarified extracts (right) and peak gradient fractions (fraction 3; left) were run on denaturing SDS-PAGE and proteins stained with Coomassie blue. Infiltration combinations: (1) pEAQ-*HT* negative control; (2) pEAQ-VP7*HT* + pEAQ-VP3*HT* + pEAQ-VP5*HT* + pEAQ-VP2*HT*; (3) pEAQexpress-VP7*HT*-VP3wt + pEAQexpress-VP5*HT*-VP2*HT*; lane M: marker. Size markers are indicated on the right; location of BTV proteins is indicated on the left. (c) Purified particles arising from coinfiltration of pEAQex-VP5*HT*-VP2*HT* and pEAQex-VP7*HT*-VP3wt were imaged by TEM. Scale bar, 100 nm.

transfer events necessary to enable VLP production in any given cell from four to two. Restriction of the expression level of VP3 by the use of wild-type CPMV 5′ UTR produced VLP preparations with similar band intensities for all four BTV proteins on Coomassie blue-stained SDS-PAGE, indicative of high levels of fully assembled VLPs (Figure 3b). Further gel analysis by densitometry revealed that, whilst levels of the outer layer proteins (VP2 and VP5) were similar in the two preparations, there was a marked reduction in band intensity of the core proteins (VP3 and VP7) when VP3 expression was controlled by the wild-type 5′UTR (Figure S4). This indicates that down-regulation of VP3 expression does not have a negative impact on VLP production, whilst reducing the burden of CLPs and SCLPs in the preparation. Visualization of these particles by TEM showed a far higher degree of VLP integrity in these preparations compared with coinfiltration of four separate codon-optimized constructs (Figure 3c). The particles deemed to be assembled VLPs had a mean diameter of 86.8 (±3.0) nm. Thus, for the larger-scale production of VLPs in plants, we utilized coinfiltration of *N. benthamiana* leaves with pEAQex-VP7*HT*-VP3wt and pEAQex-VP5*HT*-VP2*HT*. To further maximize the ratio of VLP to CLP/SCLP in the preparations, the most pure and stoichiometrically correct fractions of the density gradients were subjected to a second round of gradient centrifugation. The final yield of VLPs produced in this way was approximately 70 mg VLPs per kg leaf wet weight, as determined by protein staining and absorbance measured at a wavelength of 280 nm of the most stoichiometrically correct fractions. Total particulate BTV-8 protein yield was estimated to be more than 200 mg per kg leaf wet weight. To produce a preparation of CLPs, *N. benthamiana* leaves were infiltrated with pEAQex-VP7*HT*-VP3wt and CLPs isolated by two rounds of gradient centrifugation.

Plant-produced BTV-like particles induce an immune response in sheep

To test the immunogenicity of plant-produced BTV-8 VLPs, two sheep were injected with 20 μg of adjuvanted VLPs and received boost inoculations at 21 and 42 days post-initial inoculation. Serum was collected 18 days after each inoculation and on day 56. Serum samples from both sheep tested positive for BTV antibodies after the first boost using a Bluetongue virus antibody test kit (cELISA, VMRD Inc., Pullman, WA). Serum from day 56 (final bleed) was used to probe a Western blot of the original plant-produced particles (Figure 4). Signals for all four structural proteins could be identified. Degradation products were also recognized, indicating some loss of protein integrity in samples stored at 4 °C for 10 months. Prebleed serum used to probe a corresponding Western blot did not show any positive reaction with the proteins (data not shown). These results show that plant-produced BTV-8 VLPs stimulate an immune response in sheep, producing antibodies mainly against the major immunogenicity determinant (VP2) and the most abundant structural protein (VP7).

Plant-produced BTV-8 VLPs confer protective immunity in sheep

To test the efficacy of plant-produced BTV-8 VLPs as a potential vaccine against BTV-8, four groups of five sheep were injected with either 50 μg VLP, 200 μg CLP, 5×10^4 $TCID_{50}$/mL live, attenuated, monovalent BTV-8 used in the multivalent commercial vaccine (Onderstepoort Biological Products, South Africa) or PBS, in the presence of an adjuvant. On day 28, animals received a booster injection of the same composition. On day 63, animals were challenged with 1 mL infected sheep blood containing live BTV-8, and the clinical reactions were monitored for 2 weeks postchallenge. The severity of clinical bluetongue after challenge with the virulent virus was expressed in a numerical form as the clinical reaction index (CRI) as described by Huismans *et al.*

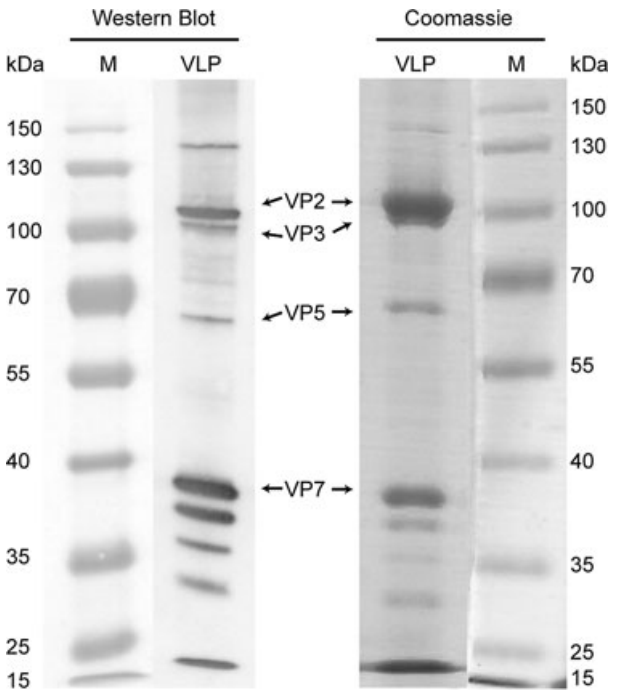

Figure 4 Immunogenicity of plant-made BTV-8 particles. Sheep were inoculated with 20 μg VLPs, boosted with 2 further doses after 21 and 42 days, and serum collected 56 days after the first inoculation. Bluetongue virus (BTV)-8 VLPs were run on SDS-PAGE and either stained by Coomassie blue or transferred by Western blotting. The Western blot was probed with the collected serum.

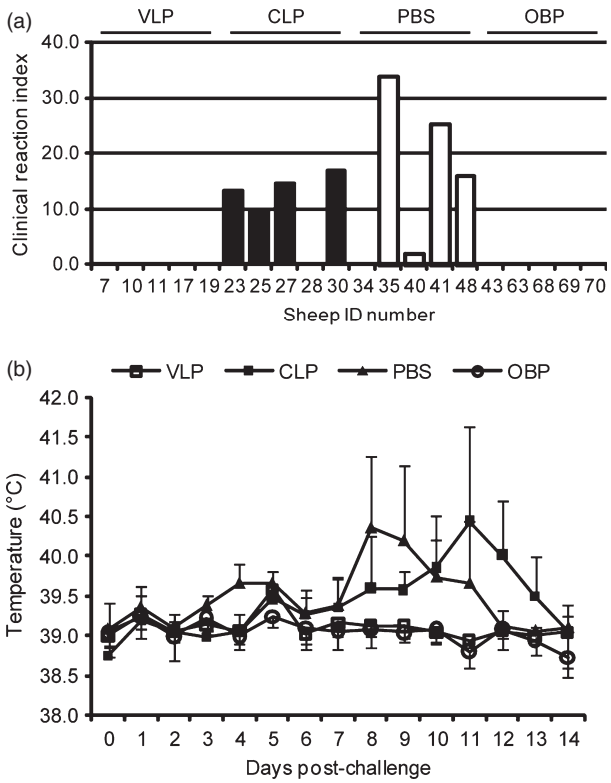

Figure 5 Protective efficacy of plant-made Bluetongue virus (BTV)-8 particles. A challenge experiment was performed by inoculating groups of 5 sheep with 50 μg plant-produced BTV-8 VLP, 200 μg plant-produced BTV-8 CLP, OBP monovalent, live, attenuated BTV-8 vaccine or PBS on day 0, boosting on day 28 and challenging with live BTV-8 on day 63. Animals were monitored for 14 days postchallenge and clinical reaction index determined (a). Mean recorded temperatures of each group of animals are presented with standard deviations indicated above (CLP and PBS) or below (VLP and OBP) each data point (b).

(1987a) (Figure 5a). The plant-produced BTV-8 VLP vaccine had the same protective efficacy as the live, attenuated, monovalent BTV-8 vaccine strain, with animals showing no clinical symptoms of bluetongue disease. Plant-produced CLPs were poorly protective, with a mean CRI of 10.8 compared with a mean CRI of 15.5 for the control group injected with PBS. Animal body temperature was recorded for 14 days postchallenge. None of the animals receiving VLP or live, attenuated vaccine developed an elevated temperature (>40.0 °C), in contrast with the CLP and PBS groups (Figure 5b). None of the animals died prematurely.

Serum neutralization tests were carried out weekly throughout the 91-day experiment (Table 1). Both the VLP group and the group vaccinated with the monovalent, live, attenuated BTV-8 vaccine strain showed high serum neutralization titres after day 28. However, whilst high levels of neutralizing antibodies were induced by the monovalent, attenuated BTV-8 vaccine as soon as 7 days after vaccination, plant-based VLPs only induced high antibody levels after booster injection, showing that a booster is necessary for protective efficacy of the plant-produced VLP vaccine. Neither the PBS control nor plant-produced CLPs induced a protective antibody response. However, plant-produced CLPs did appear to offer some protection against challenge, similar to insect cell–produced CLPs (Stewart *et al.*, 2012).

Discussion

The results presented here demonstrate that it is possible to coexpress four structural proteins within the same plant cell using the CPMV-*HT* expression system and that correct assembly into complex structures (VLPs) with the appropriate immunological properties readily occurs within days of infiltration. The most efficient way to produce BTV VLPs was to coexpress the core proteins (VP3 and VP7) on one plasmid and the outer proteins (VP2 and VP5) on another. As well as reducing the number of constructs that needed to be coinfiltrated, this approach also enabled the differential expression of the core and outer proteins. Down-regulation of VP3 synthesis through the use of the wild-type (wt) rather than the *HT* 5′UTR resulted in the production of fewer CLPs and shifted the equilibrium towards fully assembled VLPs. The ability to regulate translation levels of individual proteins within the same cell is a useful tool of the CPMV-*HT* expression system. In the case of BTV, it has been used to efficiently produce a protein complex where the individual components are present in varying stoichiometries; such an approach could also be applied to other complex viruses, such as human rotavirus. Furthermore, it could equally be used to reconstruct a metabolic pathway where the component enzymes are present at different levels. Thus, the results obtained with the production of BTV VLPs have implications outside the veterinary field.

The ability to express high levels of assembled BTV VLPs that can stimulate protective immunity in target animals (sheep) is both a very valuable proof of efficacy, as well as a further demonstration of the potential utility of plant-made pharmaceuticals. The timescale for production means that it is possible to respond quickly to outbreaks of emerging diseases. The present study made use of small-scale expression methodology (syringe

Table 1 Neutralizing antibody titres during challenge experiment

		Day													
		Vaccination				Boost					Challenge				
Group	Animal	0	7	14	21	28	35	42	49	56	63	70	77	84	91
VLP	7	0	0	8	8	16	256	256	256	256	512	128	512	512	4096
	10	0	0	0	4	16	256	256	256	256	64	64	128	128	64
	11	0	0	0	0	4	32	32	32	64	16	8	16	32	64
	17	0	0	0	0	4	256	256	256	64	128	64	64	128	4096
	19	0	0	0	2	4	256	256	256	64	16	8	16	64	128
CLP	23	0	0	0	0	2	0	0	0	8	0	0	256	1024	512
	25	0	0	0	0	0	0	0	0	0	0	0	2048	2048	4096
	27	0	0	0	0	0	0	0	0	0	0	0	32	512	2048
	28	0	0	4	0	8	0	0	0	0	0	0	256	1024	4096
	30	0	0	0	0	0	0	0	0	8	0	0	2048	2048	4096
PBS	34	0	0	0	0	0	0	0	0	0	0	0	32	0	512
	35	0	0	0	0	0	0	0	0	0	0	0	2048	1024	1024
	40	0	0	0	0	0	0	0	0	0	0	0	0	0	32
	41	0	0	0	0	0	0	0	0	0	0	0	0	0	512
	48	0	0	0	0	0	0	0	0	0	0	0	512	256	512
OBP vaccine strain	43	0	2	128	128	256	256	256	256	256	32	512	512	512	512
	63	0	2	256	256	256	256	256	256	256	128	256	1024	256	n/a
	68	0	64	256	256	256	256	256	256	256	128	64	512	512	512
	69	0	32	32	64	256	256	256	256	256	128	256	128	64	256
	70	0	16	64	34	256	256	256	256	256	64	128	64	256	256

Five sheep in each group were vaccinated with 50 μg plant-produced Bluetongue virus (BTV)-8 VLP, 200 μg plant-produced BTV-8 core-like particles (CLP), OBP monovalent, live, attenuated BTV-8 vaccine or PBS (control) on day 0, boosted with the same on day 28 and challenged with live BTV-8 on day 63. Serum samples were taken every 7 days, and neutralizing antibody titres were determined by serum neutralisation plaque reduction assay. Results are expressed as the reciprocal of the dilution factor causing a 50% reduction in cytopathic effect.

infiltration of individual leaves), but techniques for scaling up expression by the vacuum infiltration of whole plants have recently been developed for coexpression of multiple proteins and shown to give comparable yields to syringe infiltration (Vézina *et al.*, 2009). Using such techniques, in combination with scalable downstream processing techniques such as size exclusion and ion-exchange chromatography, it should be possible to produce sufficient quantities of BTV-8 VLPs in plants for this to be a viable route to the production of recombinant subunit vaccines to protect animals against Bluetongue disease. This should be a valuable addition to the veterinary armamentarium, as such vaccines made by more conventional expression systems are not cost-effective for animal use. Furthermore, the methods utilized in this study could be applied to production of a wide variety of VLPs relevant to both veterinary and human diseases.

Experimental procedures

Constructs

Constructs for plant-based expression were based on the Netherlands NET2006/04 strain of BTV-8 (Maan *et al.*, 2008). Gene sequences for VP2, VP3, VP5 and VP7 were codon-optimized for *Nicotiana* translation synthesized by GeneArt (Life Technologies, Grand Island, NY) with flanking AgeI and XhoI sites. These genes were inserted into pEAQ-*HT (*Sainsbury *et al.*, 2009*)* by restriction cloning, producing pEAQ-VP2*HT*, pEAQ-VP3*HT*, pEAQ-VP5*HT* and pEAQ-VP7*HT* (Figure 1a). Expression cassettes of these vectors were excised using AscI/PacI sites and transferred to the compatible AsiSI/MluI or AscI/PacI sites of pEAQexpress to yield pEAQex-VP5*HT*-VP2*HT*, pEAQex-VP7*HT*-VP3*HT*. A vector for expression of VP3 from a construct lacking the *HyperTrans* mutations of the CPMV-*HT* system was made by substitution of the modified CPMV RNA-2 leader with an amplified wild-type leader. This vector's expression cassette was used to make pEAQex-VP7*HT*-VP3wt, containing codon-optimized genes of VP7 and VP3, but without the *HyperTrans* mutation in the VP3 expression cassette.

All expression constructs were transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation and propagated at 28 °C in Luria–Bertani media containing 50 μg/mL kanamycin and 50 μg/mL rifampicin.

Plant growth and expression

Transient expression of the BTV-8 constructs was carried out by agroinfiltration of *Nicotiana benthamiana* leaves. Plants were grown in a greenhouse maintained at 23–25 °C and infiltrated 3–4 weeks after the seedlings were pricked out. The first three mature leaves of each plant were selected for infiltration. *Agrobacterium tumefaciens* strains were subcultured and grown overnight, pelleted and resuspended to OD_{600} = 0.3 in MMA (10 mM MES buffer, pH 5.6; 10 mM magnesium chloride; 100 μM acetosyringone) and then infiltrated into leaf intercellular spaces using a blunt-ended syringe. When *n* constructs were coinfiltrated, they were prepared to have an overall final OD = 0.3 * *n*, with equal concentrations of each strain. Tissue was harvested 8–9 days postinfiltration, a time span found to achieve expression of all four structural proteins of BTV10 in earlier experiments (data not shown). Leaf tissue was immediately extracted in three volumes of

VLP extraction buffer (50 mM Bicine, pH 8.4; 20 mM sodium chloride (NaCl), 0.1% (w/v) NLS sodium salt; 1 mM dithiothreitol; Complete Protease Inhibitor Cocktail (Roche, Welwyn Garden City, UK)) or CLP extraction buffer (as VLP extraction buffer but with 140 mM NaCl) by homogenization using a Waring (Torrington, CT) blender. Crude extracts were filtered through two layers of Miracloth (Merck Millipore, Darmstadt, Germany) and then centrifuged for 10 min at 4200 × ***g***, 10 °C to remove cell debris.

Purification

Particles were purified by density gradient centrifugation using either OptiPrep iodixanol (Axis-Shield, Oslo, Norway) or sucrose step gradients. Iodixanol solutions (20%–50%) and sucrose solutions (30%–60%) were prepared to contain 50 mM Tris–HCl, pH 8.4, and 20 mM NaCl (for VLPs) or 140 mM NaCl (for CLPs). Step gradients of 3 mL 10% incrementing steps were overlayed with 24 mL of clarified leaf extract and centrifuged at 21,500 rpm (85,800 × ***g*** max.) and 10 °C for 3 h in a Surespin 630 rotor (Thermo Scientific Sorvall, Asheville, NC). Fractions of 1 mL were collected from the bottom, and 10 μL of each was analysed by denaturing SDS-PAGE (4%–12% NuPage, Life Technologies, Grand Island, NY) followed by protein staining with Instant Blue (Expedeon, Harston, UK). Particles were generally found in the densest fractions (45%–55% sucrose or 30%–40% iodixanol).

Particles produced for animal experiments were further purified and concentrated in a second round of gradient centrifugation by pooling the best fractions of the first gradient, gradually diluting them (50 mM Tris–HCl, pH 8.4, 20/140 mM NaCl) and reapplying to a second, smaller gradient. This was centrifuged at 21,500 rpm (79,100 × ***g*** max.) and 10 °C for 3 h in an SW-41Ti rotor (Beckman Coulter, High Wycombe, UK) and fractionated from the bottom.

Transmission electron microscopy

Particles from density gradient fractions were adsorbed onto plastic and carbon-coated copper grids, washed successively by floating on three droplets of water and then stained with 2% (w/v) uranyl acetate for 20 s. Grids were imaged using a FEI Tecnai G2 20 Twin TEM with bottom-mounted digital camera.

Particle sizing

Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA) was used to measure particle diameters on images obtained from TEM using the 'Measure Tool'. Twelve particles of each type were measured and the mean diameter and standard deviation determined.

Mass spectrometry

Gel pieces were washed, treated with trypsin and extracted according to standard procedures adapted from the study by Shevchenko *et al.* (2007). The peptide solution resulting from the digest was spotted onto a PAC plate (Prespotted AnchorChip™ MALDI target plate, Bruker Daltonics; Coventry, UK), and the spots were washed briefly with 10 mM ammonium phosphate and 0.1% TFA according to the manufacturer.

After drying, the samples were analysed by MALDI-TOF on a Bruker Ultraflex TOF/TOF. The instrument was calibrated using the prespotted standards (ca. 200 laser shots). Samples were analysed using a method optimized for peptide analysis, and spectra were summed from ca. 30 × 15 laser shots. Data were processed in FlexAnalysis (Bruker) and submitted for a database search using an in-house Mascot Server 2.2 (Matrixscience; London, UK) on the sptrembl20100119 or sptrembl20090901 database with taxonomy set to Viruses. For the search, the enzyme was set to trypsin with maximum one missed cleavage using a peptide mass tolerance of 30 ppm. Carbamidomethyl (C) was used as fixed and oxidation (M) as variable modification. Protein scores >72 were considered significant ($P < 0.05$).

Seroconversion of sheep injected with plant-produced VLPs

One milliliter of blood was taken from each of two BTV-free sheep before they were each injected subcutaneously with 20 μg plant-produced VLPs in a ratio of 1 : 1 with Freund's incomplete adjuvant. They were boosted with 20 μg VLPs at days 21 and 42 and bled 14 days after each inoculation with the final bleed taken at day 56. Serum was tested for BTV antibodies using a Bluetongue virus antibody test kit, cELISA (VMRD Inc., Pullman, WA). Dilutions of the BTV-8 VLPs originally used for the inoculations were separated on a 10% denaturing SDS polyacrylamide gel and blotted onto nylon membranes (transblotter) for Western blotting. Membranes were probed with 1/2000, 1/5000 and 1/10 000 dilutions of sheep serum from the final bleed and subsequently with a 1/10 000 dilution of anti-goat/anti-sheep alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich, Gillingham, UK). BTV-8 proteins were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Roche, Welwyn Garden City, UK) substrate. A similar Western blot membrane was probed with prebleed sheep serum diluted 1 : 5000 and subsequently treated as described above.

Immunization and challenge of sheep

The immunization and challenge experiments were approved by the Onderstepoort Biological Products Animal Ethics Committee.

Twenty 1-year-old, BTV-naive merino sheep were kept in insect-proof isolation stables and divided into 4 groups and subcutaneously injected into the inner thigh with 1 mL of the following: 50 μg VLP with 50% (v/v) Montanide ISA70 VG (Seppic; SEPPIC, Puteaux, France) as an adjuvant, 200 μg CLP with 50% (v/v) Montanide ISA70 VG, PBS with 50% (v/v) Montanide ISA70 VG and 5×10^4 $TCID_{50}$/mL live, attenuated, monovalent BTV-8 (commercial vaccine seed stock for BTV-8, Onderstepoort Biological Products; South Africa). On day 28, each group was boosted with the same. Serum samples were taken on days 0, 7, 14, 21 and 28 postvaccination as well as after booster vaccination. Animals were monitored for reactions at the injection site, and temperature reactions were monitored for 14 days after injection. This was carried out to ensure that the inoculum was safe and secondly to monitor for possible other infections that could affect the results. On day 56, each group was challenged with an intravenous 1-mL injection of virulent BTV-8 sheep blood. The clinical reactions were monitored for the first 14 days postchallenge. The severity of clinical bluetongue after challenge with the virulent virus was expressed in a numerical form as the clinical reaction index (CRI; Huismans *et al.*, 1987b), which was obtained by adding the following three scores (a + b + c):

- Febrile reaction – The cumulative total fever readings above 40 °C from days 3 to 14.
- Clinical lesion score – Lesions of the nose, mouth and feet are scored on a scale of 0–4.
- Fatality – an additional four points are added if death occurs within 14 days.

This CRI represents an estimate of bluetongue disease that takes into account the duration of any temperature increase

(>40 °C) and the extent, types and location of lesions (nose, mouth, hoof, etc.).

Bluetongue virus (BTV)-specific neutralizing antibodies were measured according to the procedure of the serum neutralisation test as described in the Office International des Epizooties (OIE) Manual of diagnostic tests and vaccines for terrestrial animals (2009 version; http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.03_BLUETONGUE.pdf). Antibody titres are expressed as the reciprocal of the serum dilution causing a 50% reduction in cytopathic effect and are calculated using the Spearman–Karber method.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Some BTV8 structural proteins cause symptoms when expressed in plants individually, but not when expressed in combination with their structural neighbouring proteins.

Figure S2 Mass spectrometry analysis of 4 main protein bands recovered from SDS-PAGE analysis of a density gradient fractions from leaf tissue coinfiltrated with constructs for coexpression of BTV-8 VP2, VP3, VP5 and VP7.

Figure S3 Clarified extract from leaf tissue expression four BTV structural proteins, separated on two consecutive step sucrose gradients.

Figure S4 Densitometric analysis of Coomassie-stained gel of Figure 3b (inset).

