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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, pTRAkc-AH and pTRAkc-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, pTRAkc-AH and pTRAkc-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 Lomonossoff, 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-HT	*Nco*I/*Xho*I	VP2	pTRAc-HT VP2co	Cytoplasm
		VP3	pTRAc-HT VP3co	
		VP5	pTRAc-HT VP5co	
		VP7	pTRAc-HT VP7co	
pTRAkc-AH	*Nco*I/*Xho*I	VP2	pTRAkc-AH VP2co	Apoplast
		VP3	pTRAkc-AH VP3co	
		VP5	pTRAkc-AH VP5co	
		VP7	pTRAkc-AH VP7co	
pTRAkc-rbcs1-cTP	*Mlu*I/*Xho*I	VP2	pTRAkc-rbcs1-cTPVP2co	Chloroplast
		VP3	pTRAkc-rbcs1-cTPVP3co	
		VP5	pTRAkc-rbcs1-cTPVP5co	
		VP7	pTRAkc-rbcs1-cTPVP7co	
pEAQ-*HT*	*Age*I/*Xho*I	VP2	pEAQ-*HT* VP2co	Cytoplasm
		VP3	pEAQ-*HT* VP3co	
		VP5	pEAQ-*HT* VP5co	
		VP7	pEAQ-*HT* VP7co	

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