

# ***In planta* production of a candidate vaccine against bovine papillomavirus type 1**

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## **Abstract**

Bovine papillomavirus type 1 (BPV-1) is an economically important virus that induces tumorigenic pathologies in horses and cows. Given that the BPV-1 L1 major coat protein can self-assemble into highly immunogenic higher-order structures, we transiently expressed it in *Nicotiana benthamiana* as a prelude to producing a candidate vaccine. It was found that plant-codon optimization of L1 gave higher levels of expression than its non-optimized counterpart. Following protein extraction, we obtained high yields (183mg/kg fresh weight leaf tissue) of relatively pure L1, which had self-assembled into virus like particles (VLPs). We found that these VLPs elicited a highly specific and strong immune response, and therefore they may have utility as a potential vaccine. This is the first report demonstrating the viable production of a candidate BPV vaccine protein in plants.

**Keywords:** Bovine papillomavirus • Codon optimization • Plant expression • Vaccine protein • Virus-like particles

## **Abbreviations:**

BPV	Bovine papillomavirus
CPMV	Cowpea mosaic virus
CRPV	Cottontail rabbit papillomavirus
ELISA	Enzyme-linked immunosorbent assay
HPV	Human papillomavirus
Mes	2-morpholinoethanesulphonic acid

PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UTRs	Untranslated regions
VLP	Virus-like particle

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## Introduction

The use of plants as protein expression platforms to produce pharmacologically important molecules has evolved beyond the proof of concept stage. In comparison to bacterial-, insect cell-, Chinese Hamster Ovary- and yeast-based expression systems, plant platforms offer the prospect of better product safety, increased scalability, and simpler, faster and cheaper production (Ma et al. 2005; Rybicki 2010; Penney et al. 2011). Moreover, in many cases the functionality of *in planta* expressed proteins has been shown to be at least as good as those produced using conventional methods (Strasser et al. 2009).

Since production of transgenic plants for protein expression is very time-consuming, it is increasingly common to induce rapid but transient expression of vaccine candidates in wild-type plants using *Agrobacterium tumefaciens* harbouring an expression cassette. This is achieved by infiltrating suspensions of *Agrobacterium* into leaf material, which results in the delivery of the cassette directly into the plant cell nucleus (Fischer et al. 1999). Following nuclear localization, the expression cassette becomes transcriptionally active, leading to expression of the target protein three to five days post infiltration.

In recent years improvements made to binary vectors or expression cassettes have been essential for the *in planta* production of high yields of vaccine proteins. Many of these newer expression cassettes which either carry self-replicating plant viruses or components of viruses, have the advantage of inducing very high levels of protein expression at the site of *Agrobacterium* infiltration (Canizares et al. 2005; Lindbo 2007), without the issues of genetic instability, biocontainment and size limits of the protein to be expressed. Recently a pEAQ HT vector series has been produced by Sainsbury et al. (2009) in which the traditional non-viral expression cassette has been augmented by inclusion of cowpea mosaic virus (CPMV) 5' and 3' untranslated regions (UTRs), which abut the gene to be expressed. This system also delivers P19 (derived from tomato bushy stunt virus), an RNA silencing suppressor that promotes higher expression levels in plants. Taken together, these modifications facilitate extremely high level production of proteins without viral replication. In this paper we have used *Agrobacterium* to deliver this novel expression vector into plant cells in order to produce high yields of a candidate vaccine against Bovine papillomavirus type 1 (BPV-1).

BPV-1 and BPV-2, members of the genus *Deltapapillomavirus*, are 55-60nm diameter non-enveloped icosahedral viruses with 8kbp circular double stranded DNA genomes. These

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3 encode genes for replication (Mohr et al. 1990; Kurg et al. 2009; Liu et al. 2010), genome  
4 encapsidation (L1 and L2 are the major and minor capsid proteins, respectively) and oncogenesis  
5 in animals (Tong et al. 1997; Zimmermann et al. 2000; Ashrafi et al. 2002; Zago et al. 2004).  
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7 Unlike the other eight characterized types of BPV (Nasir and Campo 2008), BPV-2 and to a  
8 greater extent BPV-1 are not as species-specific, and have been reported to infect and cause  
9 disease in a variety of bovids and equids (Nasir and Campo 2008). BPV is endemic in many parts  
10 of the world and can be spread by direct or indirect contact between infected cattle and may also  
11 be disseminated via insect transmission. BPV-1 typically induces benign teat, facial and penile  
12 tumours of both the epithelium and underlying derma (Nasir and Campo 2008), symptoms which  
13 substantially impair milk duct and reproductive function, and often lead to slaughter (Campo  
14 1995; Borzacchiello and Roperto 2008). Moreover, BPV infections are associated with an  
15 increased risk of carcinogenesis and immunosuppression, leading to an insidious decline in  
16 animal health, which often goes unreported (Bogaert et al. 2008). The high incidence and  
17 economic impact of BPV disease (Campo 1995; Borzacchiello and Roperto 2008) has galvanized  
18 the development of therapeutic vaccines. Initial prophylactic vaccine trials involved vaccinating  
19 calves with tumours and purified virus particles taken from BPV-2 and BPV-4 infected sites  
20 (Jarrett et al. 1990). This provided some level of virus-type specific immunity against the  
21 particular viral challenge, and also indicated that the structural proteins L1 and L2 likely play a  
22 key role in this immunity (Jarrett et al. 1990). This was later confirmed by treatment of calves  
23 with *E. coli* expressed L1 and L2 fused to  $\beta$ -galactosidase. In this case the L1 vaccine prevented  
24 BPV-2-induced tumour formation in calves only if it was given before the challenge, whereas the  
25 L2 promoted tumour rejection irrespective of whether it was administered before or after the  
26 challenge (Jarrett et al. 1991). Since it is not possible to grow BPV in culture for the preparation  
27 of traditional killed or attenuated live vaccines (Campo 2003), yeast and insect cell expression  
28 systems have been used to produce L1 and L2 based vaccines. However, these are very  
29 expensive.

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31 It has previously been demonstrated that BPV L1 expressed in insect cells initially self-  
32 assembles into pentamers, which may in turn unite to form higher order structures such as T=1 or  
33 T=7 virus like particles (VLPs), composed of 12 and 72 pentamers, respectively (Kirnbauer et al.  
34 1992; Campo 2003). These VLPs and their pentameric subunits were found to be highly  
35 immunogenic and have been shown to confer protection against future infection by BPV types  
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3 from which they are derived (Kirnbauer et al. 1992; Campo 2003). Consequently, L1 VLPs and  
4 their precursors are regarded as suitable prophylactic vaccines.  
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7       Until now BPV vaccine proteins have not been expressed in plants; however components  
8 of other members of the *Papillomaviridae* family have. For example, Human papillomavirus  
9 (HPV) type 16 L1 (which shares 47% protein sequence identity to BPV-1 L1) VLPs were  
10 expressed in transgenic (Varsani et al. 2003) and transplastomic plants (Lenzi et al. 2008;  
11 Waheed et al. 2011), and also in wild-type plants using transient expression (Maclean et al.  
12 2007). In this paper we report for the first time the successful expression and isolation of intact  
13 BPV L1 VLPs from plants, made possible via the use of *Agrobacterium* delivery of a novel  
14 CPMV enhanced binary vector carrying L1. The VLP yield we obtained (183 mg/kg fresh plant  
15 weight) exceeds the basic level (>40mg of purified protein per kg) required for economical  
16 production of a vaccine (Fischer et al. 2004). Moreover, we demonstrate that the VLPs can elicit  
17 a strong and highly specific immune response, which make them excellent candidates for a  
18 potential vaccine.  
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## 34 **Materials and methods**

### 35 Cloning of BPV1 L1

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39 The BPV-1 genome, which had been extracted and amplified from sarcoid tissue and cloned into  
40 pcDNA3.1(-) using HindIII sites, was provided by Prof Lubna Nasir (Glasgow University,  
41 Glasgow, Scotland, UK). The plasmid vector lies within the HindIII site of the BPV-1 L1 gene,  
42 thus making direct PCR amplification of L1 impossible. This was rectified by HindIII digestion  
43 and self-ligation of the BPV genome prior to PCR amplification of L1. BPVL1 L  
44 (CACCATGGCGTTGTGGCAACA) and BPVL1 R  
45 (CCTTCTGTTTTACATTTACAGAACATT) oligonucleotides were used to produce a blunt-  
46 ended PCR product which was subsequently integrated into the pENTR DTOPO vector  
47 (Invitrogen, Paisley, Scotland, UK). Following transformation of this vector into *E.coli*, three  
48 colonies were picked and the extracted plasmids were sent for stringent sequencing (The James  
49 Hutton Institute sequencing service, Invergowrie, Scotland, UK), whereby sequencing reads  
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covered the complete gene at least twice. The sequences from the independent clones were identical; thus eliminating the possibility of PCR or sequencing errors. The L1 sequence we obtained was optimized for expression in *Nicotiana tabacum*, and synthesized using the gene synthesis service of GenScript (Piscataway, NJ, USA). Our sequence was optimized to account for appropriate GC content, cryptic splicing sites, premature PolyA sites, regions prone to gene silencing (CpG dinucleotide and negative CpG islands), mRNA secondary structure and stability, and codon usage (as provided by GenScript). Using BPV L1 CO L (CACCATGGCTCTCTGGCAACAAG) and BPV L1 CO R (TCACTTCTTTTTCTTCTTTGCTG) oligonucleotides, L1 CO was PCR amplified and cloned into pENTR DTOPO. The integrity of the sequence was confirmed as before. The pENTR DTOPO constructs containing codon optimized or non-optimized L1 sequences were BspHI digested and purified in order to remove the kanamycin resistance gene, thus allowing appropriate selection with kanamycin after LR clonase recombination of the L1 gene into the binary pEAQ-HT-DEST GATEWAY destination vector (Sainsbury et al. 2009). The pEAQ-HT-DEST vector series facilitates very high *in planta* expression and contains epitope tags; allowing us to generate L1 with and without a N-terminal 6xHis tag fusion if required. In addition, this vector also delivers P19, a potent silencing suppressor which enhances transient expression. A schematic of the pEAQHT vector T-DNA region containing L1 is shown in Supplementary Fig. S1. Binary plasmids were transformed into and maintained in *Agrobacterium tumefaciens* strain LBA4404.

#### *In planta* transient expression of L1

LBA4404 containing the pEAQ-HT-DEST L1 binary expression vectors were grown in LB medium containing appropriate antibiotics, until the stationary phase was reached. Cultures were centrifuged and the pellets were resuspended in infiltration medium (10mM Mes, 10mM MgCl<sub>2</sub>, 20μM acetosyringone) to an OD 600 of 0.5. The cultures were incubated in darkness at room temperature for 4h before syringe infiltrating (Sainsbury et al. 2009) into *Nicotiana benthamiana* Domin leaves (obtained from The James Hutton Institute), which were grown to the five leaf stage under long day, 25°C conditions.

#### Western blot detection of L1 protein

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3 *N. benthamiana* leaves were harvested for Western blotting five days after infiltration.  
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5 Approximately 30mg of leaf material was ground in liquid nitrogen, combined with 100µl of 2X  
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7 Laemmli loading buffer and then boiled for 5min to denature the proteins. After centrifugation at  
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9 16,000g, 20µl was loaded onto 10% SDS-PAGE gels, along with 15µl of Novex prestained  
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11 ladder (Invitrogen). Gels were either stained in a Coomassie-based staining solution (Instant  
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13 Blue; Expedeon, Harston, UK) or were electroblotted onto Immobilon-P membrane (Millipore,  
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15 Watford, UK). The Immobilon-P membrane was blocked by incubating in 1XPBS, 1% BSA and  
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17 0.05% Tween, for 1h. Anti-L1 (BPV-1-1H8; Abcam, Cambridge, UK) mouse monoclonal  
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19 primary antibodies were added to the blocking solution at a dilution of 1:3000. An anti-mouse  
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21 horseradish peroxidase conjugated secondary antibody (NXA931; Amersham Biosciences, Little  
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23 Chalfont, UK) at a dilution of 1:5000 was used in conjunction with ECL plus (RPN2132;  
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25 Amersham Biosciences) for signal detection.

#### 26 VLP purification and quantification

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28 Six grams of *N. benthamiana* leaf material was harvested 5 days post infiltration and VLPs were  
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30 extracted using protocols established for begomovirus purification (Caciagli et al. 2009). Frozen  
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32 and powderized leaf material was combined 1:5 (w/v) with extraction buffer (0.5M KPO<sub>4</sub>, pH6,  
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34 10mM Na<sub>2</sub>SO<sub>3</sub>, 2mM EDTA, 0.1% β-mercaptoethanol, 1% triton X100, 0.1% driselase (w/v))  
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36 and a protease inhibitor (Complete EDTA-free; Roche Diagnostics, Mannheim, Germany). After  
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38 overnight agitation at 4°C the mixture was emulsified with 15 % chloroform and spun for 15min  
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40 at 8,000g. The supernatant was removed and spun at 205,000g for 2h. Pellets were resuspended  
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42 in 3ml of 0.5M KPO<sub>4</sub>, pH7, 2.5mM EDTA, using a glass rod. These were loaded onto 20%-50%  
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44 Cs<sub>2</sub>SO<sub>4</sub>, 0.5M KPO<sub>4</sub>, pH7 gradients and then centrifuged for 4h at 160,000g. Bands containing  
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46 VLPs were removed and diluted 1:5 in 0.1M KPO<sub>4</sub>, pH7 (or 1X PBS for samples that are to be  
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48 used in immunization studies). The VLPs were pelleted after a 40min 390,000g centrifugation  
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50 step and resuspended in 0.1M KPO<sub>4</sub>, pH7 (or 1X PBS). The concentration of the purified L1 VLP  
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52 fractions were estimated spectrophotometrically using the wavelengths and formula: mg/ml=  
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54 (1.55 x A<sub>280</sub>) – (0.76 x A<sub>260</sub>). This formula is typically used to estimate protein concentrations of  
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56 unknowns in which there may be nucleic acids (Layne 1957).

#### 57 Electron microscopy of virus particles

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3 For EM analysis, purified samples were adsorbed to carbon-coated copper grids. Grids were  
4 stained with 2% (w/v) uranyl acetate and examined in a JEOL 1400 transmission electron  
5 microscope at 80kV.  
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## 8 9 10 Immunogenicity trials and ELISA

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13 Purified VLPs in PBS and Freund's incomplete adjuvant were administered to four rabbits such  
14 that each would receive a 150µg dosage. Three weeks after the initial vaccination, a booster was  
15 given and blood samples were taken a further two weeks later. The antisera from this bleed were  
16 used in the ELISA.  
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20 Purified VLPs in PBS were serially diluted two-fold in coating buffer (12.8mM Na<sub>2</sub>CO<sub>3</sub>,  
21 34.8mM NaHCO<sub>3</sub>) to produce concentrations ranging from 500ng to 1ng per ELISA plate  
22 (439454; SLS, East Riding of Yorkshire, UK) well. After incubating overnight at 4 °C, plates  
23 were rinsed four times with PBS containing 0.05% Tween 20. Plates were then blocked by  
24 adding 200ul of PBS with 0.5% Non-fat Dried Milk to each well and incubating for 1h at room  
25 temperature. Antisera were serially diluted in blocking buffer, to produce a dilution series ranging  
26 from 1/250 to 1/32000, of which 200µl of each was added to the appropriate wells of the ELISA  
27 plate. The plate was incubated at 37 °C for 2h, after which it was washed in PBS- Tween as  
28 before. To each well 100 µl of antirabbit IgG AP conjugate (A8025; Sigma, Dorset, UK) diluted  
29 1/1000 in PBS-milk was added. After 2h incubation at 37 °C, plates were washed as before and  
30 100 µl of p-nitrophenol (pNPP) substrate, prepared according to manufacturers instructions  
31 (S0942; Sigma), was added to each well. In the presence of alkaline phosphatase the pNPP is  
32 hydrolyzed to a yellow coloured product which can be detected spectrophotometrically at an  
33 absorbance of 405nm.  
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## 47 48 Results

### 49 50 Expression of L1 in *Nicotiana benthamiana* and the effect of codon optimization

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52 Since codon optimization is known to influence the amount of protein produced, we compared  
53 the *in-planta* expression level of *N. tabacum* codon optimized L1 with its non-optimized  
54 counterpart in western blots. Using a BPV antibody which is specific for L1 VLPs we were able  
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3 to detect in both codon-optimized and non-optimized samples a ~55kDa band corresponding to  
4 L1 and also a lower band which likely represents a degradation product (Fig. 1). This banding  
5 pattern is completely consistent with earlier studies in which BPV L1 was expressed in insect  
6 cells (Shafti-Keramat et al. 2009). The signal in the western blot was much stronger in the codon  
7 optimized sample in comparison to the non-optimized, thus indicating the importance of such an  
8 optimization for substantially improved plant expression of L1 (Fig. 1). Following successful *in*  
9 *planta* expression of L1, it was of importance that our candidate vaccine protein could form  
10 VLPs and also that these could be purified to a high purity and concentration. Since the non-  
11 optimized L1 is very poorly expressed we decided to use the codon-optimized L1 for these  
12 studies.  
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#### 22 Purification of L1 VLPs from *N. benthamiana*

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26 L1 VLPs were purified from plant leaf material using a methodology which was previously used  
27 to isolate high purity begomovirus particles (Luisoni et al. 1995; Caciagli et al. 2009) and HPV  
28 VLPs from plants (Matic et al. 2011). The concentration of the VLP preparation was quantified  
29 spectrophotometrically and 50ng was loaded onto SDS-PAGEs for Coomassie and western blot  
30 analysis (Fig. 2). The bands observed in the Coomassie of the L1 VLPs (Fig. 2a) correspond to  
31 the bands detected in the western blot probed with the L1 antibody (Fig. 2b); thus indicating that  
32 we successfully isolated VLPs from plant material to a high purity. Again, the banding pattern we  
33 observed matches that of earlier reports (Shafti-Keramat et al. 2009) and is consistent with that  
34 we obtained in Fig. 1.  
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42 Following successful detection of purified VLP components, electron microscopy was  
43 used to analyse particle formation. Particles with a diameter of ~30nm were observed in VLP  
44 preparations, which is consistent (Matic et al. 2012) with them representing T=1 particles of BPV  
45 (Fig. 3). In addition some capsomer (pentamer) aggregates were also detected in the preparations  
46 (Fig. 3). This indicates that BPV L1 retains its functionality to assemble into virus like structures  
47 in plants, and also that their integrity is preserved during the purification procedure.  
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#### 56 Immunogenicity test of plant produced L1 VLPs

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3 The level of immune response invoked by our plant produced L1 VLPs was tested in rabbits. A  
4 pre-immune bleed was taken from four rabbits prior to immunization with 150µg of L1 VLPs in  
5 PBS and incomplete Freund's adjuvant. A booster was administered three weeks after the initial  
6 immunization, and blood was collected a further two weeks after this for ELISA analysis. Plant  
7 derived VLPs were adsorbed on to the surface of ELISA wells at concentrations ranging from  
8 5ng/µl to 1pg/µl (determined by spectrophotometry), and pre- and post- immunization antisera  
9 from four rabbits were subsequently applied to these wells at dilutions ranging from 1/250 to  
10 1/32000. The IgG specific cross reactivity was measured using an assay based on alkaline  
11 phosphatase, the results of which are shown in Fig. 4a. As expected, the antisera collected before  
12 immunization did not cross-react with the VLPs, thus illustrating that the rabbits were naïve to  
13 BPV L1 (Fig. 4a). In contrast, antisera collected after the immunization was strongly cross-  
14 reactive against the plant produced VLPs, indicating that rabbits had produced IgGs against the  
15 VLPs (Fig. 4a). The level of immune response generated in the rabbits was strong since a  
16 significant signal in the ELISA was still detected even after the antisera were diluted 1 in 32000.  
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28 To confirm that the immune response generated by the plant produced L1 VLPs is highly  
29 specific, a comparison of cross-reactivity was made between *N. benthamiana* total protein  
30 extracts which either express or do not express L1, the plant-derived VLPs, and a known quantity  
31 of pure VLPs produced using insect cells (Shafti-Keramat et al. 2009). One-, two- and four-fold  
32 dilutions of VLPs were assayed, which corresponds to 2.5ng/µl, 1.25ng/µl and 0.625ng/µl,  
33 respectively. The same dilutions were performed on plant extracts, which corresponds to 100µl of  
34 total protein from 1mg, 0.5mg and 0.25mg of plant material. These were assayed against a  
35 1:16000 dilution of rabbit antisera in order to reach the linear range of the ELISA which would  
36 be conducive to accurate comparison amongst samples. The results demonstrate that antisera  
37 from the immunized rabbits can cross react with the insect cell and plant produced L1 VLPs to a  
38 similar level (Fig. 4b). In contrast, the extracts from plants which do not express L1 fail to cross  
39 react with the rabbit antisera, thus indicating that any trace plant contaminants in the VLPs used  
40 for immunization are not responsible for the strong cross reactivity of the plant produced VLPs  
41 with the rabbit antisera (Fig. 4b). This therefore indicates that our plant produced VLPs can  
42 invoke a specific immune response in rabbits.  
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54 As expected, cross-reactivity occurred with the extracts from plants that express L1 (Fig.  
55 4b), and these signals were used in conjunction with the insect cell VLP standard curves (not  
56 shown) in order to estimate the level of L1 expressed in plants. We estimated that from 1mg of  
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3 leaf material around 224ng of L1 was detected, this equates to 224mg/kgfw (per kilogram fresh  
4 leaf weight). A similar procedure was applied to the data of the purified plant produced VLP  
5 samples, and we calculated that our starting concentration of plant derived VLPs were around  
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7 0.38mg/ml (this is consistent with our earlier spectrophotometric estimates of yield, and indicates  
8 that spectrophotometry can be used reliably to measure VLP concentration). Since we extracted  
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10 3ml of this (1.1mg) from 6g of leaf material, this suggests that we can purify to yields of  
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12 183mg/kgfw. This yield is around 4-fold higher than that required for economically viable  
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14 production (Fischer et al. 2004).  
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## 21 Discussion

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24 In our study we found that plant codon optimization of the BPV-1 L1 gene substantially  
25 increased expression of the protein in *N. benthamiana*. Interestingly, previous work has  
26 illustrated that optimization for plant codon usage does not always give the highest level of  
27 expression of papillomavirus L1. For example, Biemelt et al. (2003) tried to express plant or  
28 human codon optimized or non-optimized HPV type 16 L1 in transgenic potato and tobacco, and  
29 found very small amounts of L1 in plants transformed with the human codon optimized sequence,  
30 whereas no L1 protein was detected in plants transformed with the unmodified or plant codon  
31 enhanced sequence. A similar result was obtained by Maclean et al. (2007), who found that HPV-  
32 16 L1 optimized for plant codon usage gave poor levels of expression in *N. benthamiana*,  
33 compared to its non-optimized counterpart. Thus, although appropriate codon optimization is  
34 important for the enhancement of L1 expression in plants, it is not safe to predict what type of  
35 optimization facilitates higher yields of the various L1 proteins in different plants (Giorgi et al.  
36 2010; Rybicki 2010).  
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48 In addition to codon optimization, plant choice is an important factor to consider when  
49 expressing L1. For example, changing from *N. tabacum* cv. Xanthi to SR1 resulted in a 100-fold  
50 increase in expression of native HPV-16 L1 to 0.5mg/kgfw (Rybicki 2010). It has been found that  
51 HPV-11 L1 degrades badly in *N. tabacum* but not in *Arabidopsis thaliana* (Kohl et al. 2007);  
52 yields of L1 were 2mg/kgfw and 12mg/kgfw, respectively, for these plants. Levels of *in planta*  
53 L1 expression are also dependent on the virus strain from which the L1 is derived. For example  
54 previous work has shown that unmodified cottontail rabbit papillomavirus (CRPV) L1 and HPV-  
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3 16 L1 genes are expressed in transgenic tobacco to levels of 1mg/kgfw (Kohl et al. 2006) and  
4 4µg/kgfw (Varsani et al. 2003), respectively. There are clearly a number of basic variables which  
5 can influence the yield of L1 in plants in unpredictable ways, and consequently the optimization  
6 of L1 expression needs to be empirically determined through experimentation. In addition to  
7 addressing these variables, some other groups have also experimented with either targeting or  
8 expressing L1 in different cellular locations. Maclean et al. (2007) found that putting a  
9 chloroplast export signal on to the HPV-16 L1 enhanced transient expression four-fold more than  
10 cytoplasmic localized L1; yields of 533mg/kgfw were obtained, one of the highest reported for  
11 transiently expressed HPV L1. They suggested that the chloroplast may have reduced protease  
12 activity or that the export signal may improve the expression level or stability of the protein.  
13 Although we obtained high yields (224mg/kgfw) of BPV L1 in plants through transient  
14 expression of a plant codon optimized gene, it is possible that improvements to this yield may be  
15 made by considering different codon optimization schemes (for example human codon  
16 optimization), plant variety and cellular targeting.  
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30 Expression of L1 monomers typically leads to a mixed population of T=1 VLPs, T=7  
31 VLPs and other higher order structures (Waheed et al. 2011). Previous studies on HPV-16 L1,  
32 have tried to elucidate the level of immunogenicity evoked by these various structures. In  
33 summary, it was found that while pentamers were potent elicitors of the immune response, T=1  
34 and T=7 VLPs induced even stronger reactions (Thones and Muller 2007). Consequently, we  
35 were interested in trying to extract and enrich for VLPs in our plant expression system; this was  
36 achieved by following a begomovirus purification method (Caciagli et al. 2009). This method  
37 was chosen since its acidity (pH6) and ionic strength is conducive to maintaining BPV VLP  
38 stability (Paintsil et al. 1998; Wolf et al. 2010).  
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46 In our EM analysis we found high levels of T=1 but no T=7 VLPs, which is in contrast to  
47 previous reports that either demonstrated a range of particle sizes when HPV-16 L1 was  
48 expressed *in planta* (Biemelt et al. 2003; Varsani et al. 2003; Maclean et al. 2007), or no VLP  
49 formation, in the case of plant expressed CRPV L1 (Kohl et al. 2006). These authors suggested  
50 that high concentrations of L1 in plant tissues are required to drive the equilibrium from free  
51 protein towards the formation of large VLPs. Moreover, Kohl et al. (2006) suggested that the  
52 level of L1 expression in plants required to form large VLPs varies according to the L1 type. In  
53 addition, large VLP formation may also be dependent on the cellular location of self assembly.  
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3 For example, Maclean et al. (2007) observed that chloroplast targeted L1 produced a high  
4 proportion of large VLPs, whereas L1 targetted to the cytoplasm led chiefly to small VLPs. In  
5 contrast, Fernandez-San Millan et al. (2008) found that expression of HPV-16 L1 in chloroplasts  
6 produced even higher yields but much lower proportions of T=7 particles. Fernandez-San Millan  
7 et al. (2008) suggested that the light-inducible promoter used in their study would have led to the  
8 synthesis of L1 during the light cycle, a period when the redox potential could be inadequate for  
9 the assembly of the VLPs in the chloroplast. Appropriate redox status would likely be crucial for  
10 the formation of the ~360 disulphide bonds required to produce the T=7 VLPs. It is therefore  
11 possible that constitutive L1 expression or expression during darkness may facilitate production  
12 of T=7 morphologies in the chloroplast. The predominance of lower order L1 structures in the  
13 cytoplasm (compared to the higher proportion of T=7 particles observed in the chloroplast) may  
14 be due to the lower levels of expression/stability of L1, or perhaps might be linked to the copious  
15 amounts antioxidants in the cytosol or its capacity to change its redox homeostasis in response to  
16 environmental or experimental conditions. It is known that abiotic and biotic stress responses can  
17 modulate plant cell redox status and this can result in reduction of disulphide bonds (Foyer 2005).  
18 Therefore it is possible that if such pathways are activated (perhaps by wounding and injection of  
19 *Agrobacterium* for example), they may affect VLP formation in the cytosol. Given that pentamers  
20 and small VLPs have been reported to be highly immunogenic, it is not necessary to obtain T=7  
21 VLPs for immunization. Moreover, if large VLPs are required, it is possible to dissociate L1  
22 structures and reassemble them in vitro into the T=7 morphology (Zhao et al. 2012).  
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40 Although we demonstrated that plant produced BPV L1 T=1 VLPs can induce a specific  
41 and highly immunogenic response in rabbits, it was important that we could obtain high  
42 concentrations which may be amenable for economical up-scaling of production. Using transient  
43 expression, we were able to express in plants L1 to a level of 224mg/kgfw. After purification of  
44 the VLPs from this material we obtained yields around 183mg/kgfw, a concentration which is  
45 four-fold higher than the basic level required for economical production (Fischer et al. 2004). The  
46 yield we obtained is of similar magnitude to the highest levels reported for other *in planta*  
47 transiently expressed Papillomavirus (HPV) L1s (533 mg/kgfw in the case of a chloroplast-  
48 targeted HPV L1 protein; Maclean et al. 2007). In summary, our study is the first report of a  
49 successful plant produced candidate vaccine against BPV-1.  
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## Figure legends

**Fig. 1** Transient expression in *N. benthamiana* of non- optimized (L1n) and *Nicotiana tabacum* codon- optimized L1 (L1o). C (control) represents plant material that does not express L1. Each well of the Western blot contains total protein extracts from 3mg of infiltrated leaf material. The blot was probed with a 1:3000 dilution of BPV-1-1H8, an L1 specific antibody. L1 has an expected size of 55kDa and the cross reacting band is indicated. L1\* denotes a putative degradation product of L1 protein. Large Rubisco subunit stained with Ponceau is shown as a loading control

**Fig. 2a** Coomassie stained SDS-PAGE of purified plant produced VLPs. **b** Corresponding Western blot of purified VLPs probed with the BPV L1 antibody. Fifty nanograms of protein was loaded per well. L1 has an expected size of 55kDa and the cross reacting band is indicated. L1\* denotes a putative degradation product of L1 protein

**Fig. 3** Electron microscopy of VLPs purified from plant tissue expressing codon optimized L1. White arrows highlight 30nm diameter T1 VLPs. Black arrow indicates capsomers.

**Fig. 4a** ELISA data demonstrating cross reactivity of 5ng/μl of plant produced BPV VLPs to dilutions of pre-immunization and post-immunization antisera. Data are averages of four biological replicates (antisera from four rabbits), with corresponding standard deviations shown as error bars. **b** The level of cross reactivity of purified plant and insect cell produced VLPs and leaf extracts of total protein (from plants which express or do not express L1) to immunized rabbit antisera. One-, two- and four-fold dilutions of VLPs were assayed, which corresponds to 2.5ng/μl, 1.25ng/μl and 0.625ng/μl, respectively. The same dilutions were used on plant extracts, which correspond to total protein isolated from 1mg, 0.5mg and 0.25mg of plant material. The assay was conducted against a 1:16000 dilution of antisera, and thus the data shown is in the linear range of the ELISA. Data are averages of four biological replicates (antisera from four rabbits), with corresponding standard deviations shown as error bars

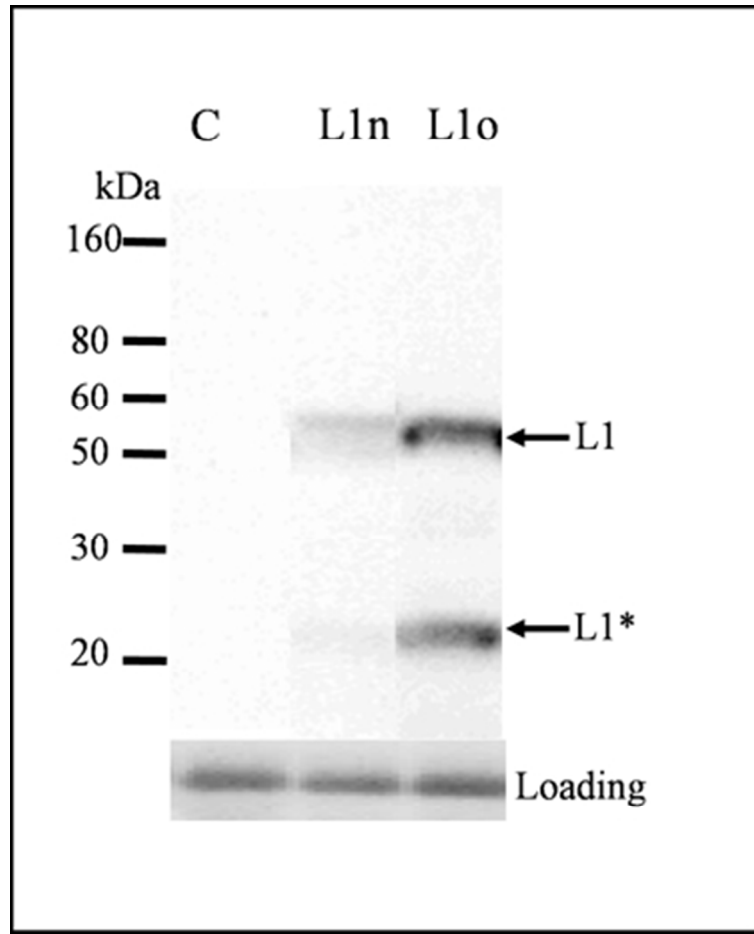


Fig. 1 Transient expression in *N. benthamiana* of non- optimized (L1n) and *Nicotiana tabacum* codon- optimized L1 (L1o). C (control) represents plant material that does not express L1. Each well of the western blot contains total protein extracts from 3mg of infiltrated leaf material. The blot was probed with a 1:3000 dilution of BPV-1-1H8, an L1 specific antibody. L1 has an expected size of 55kDa and the cross reacting band is indicated. L1\* denotes a putative degradation product of L1 protein. Large Rubisco subunit stained with Ponceau is shown as a loading control  
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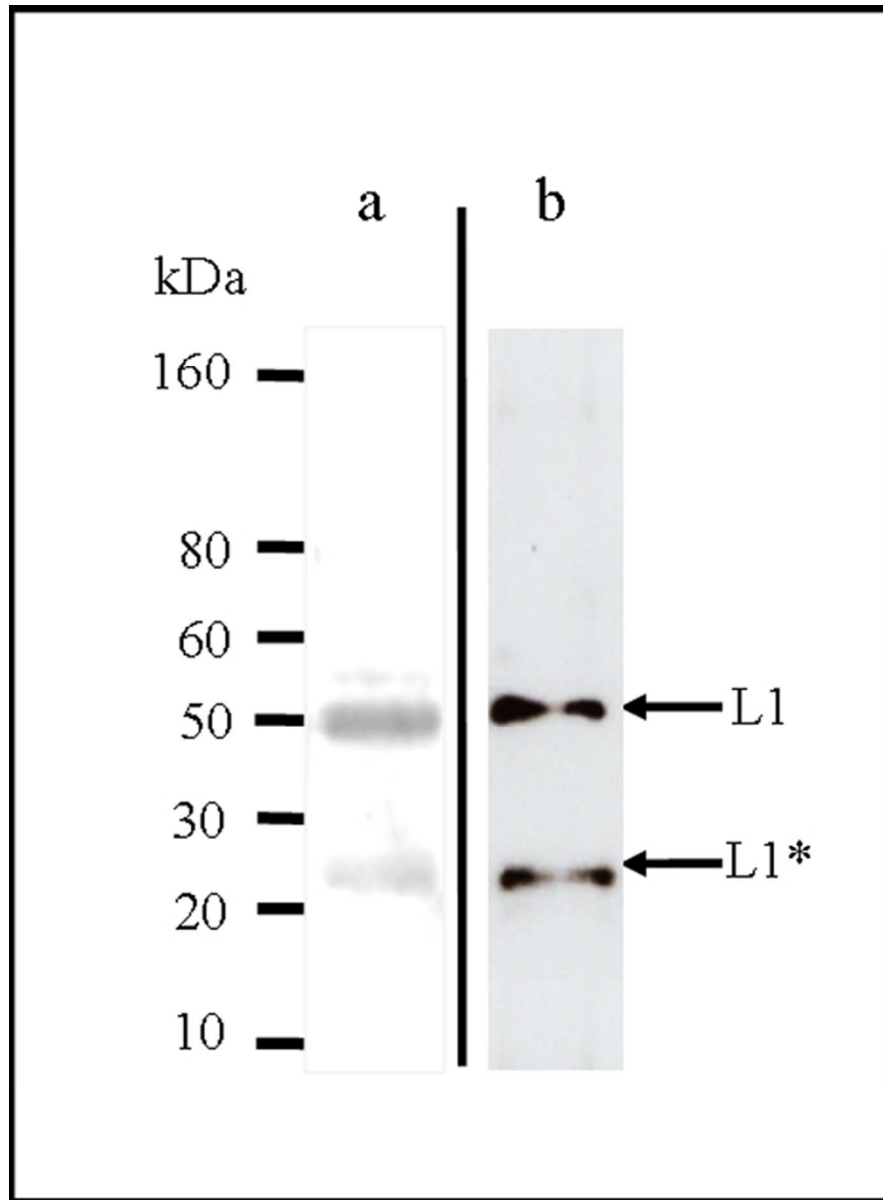


Fig. 2 a Coomassie stained SDS-PAGE of purified plant produced VLPs;  
b Corresponding western blot of purified VLPs probed with the BPV L1 antibody. Fifty nanograms of protein was loaded per well. L1 has an expected size of 55kDa and the cross reacting band is indicated. L1\* denotes a putative degradation product of L1 protein

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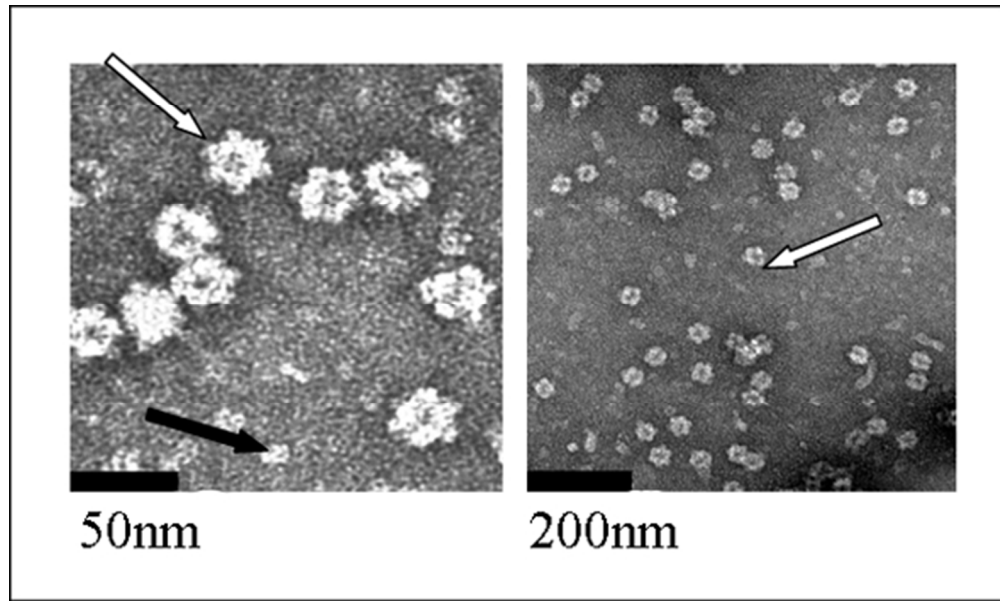


Fig. 3 Electron microscopy of VLPs purified from plant tissue expressing codon optimized L1. White arrows highlight 30nm diameter T1 VLPs. Black arrow indicates capsomers. Scale bars are shown 100x59mm (150 x 150 DPI)

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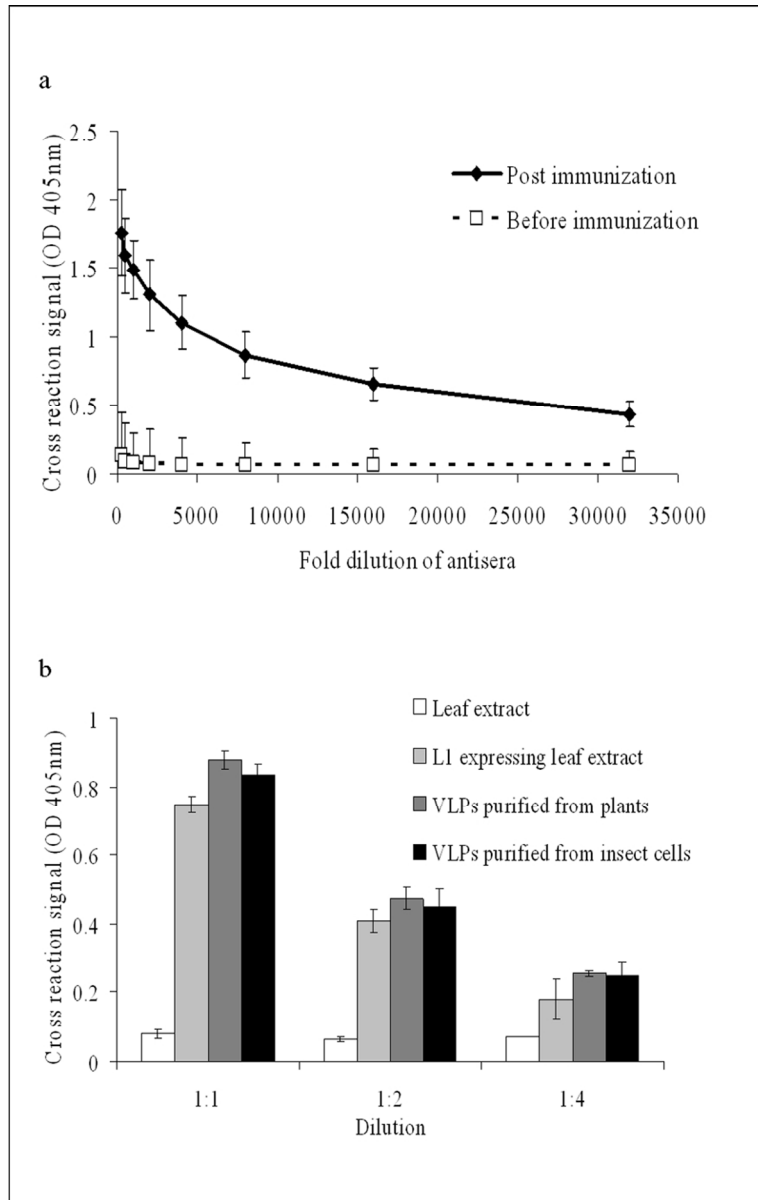
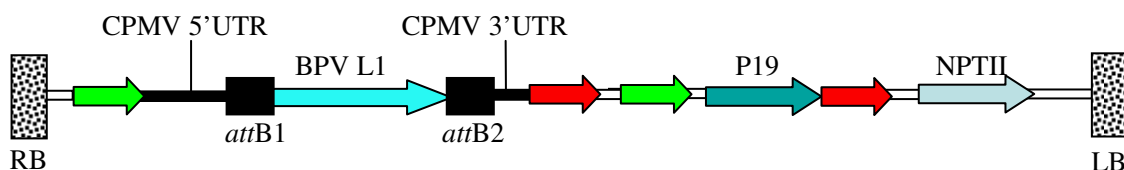


Fig. 4 a ELISA data demonstrating cross reactivity of 5ng/ $\mu$ l of plant produced BPV VLPs to dilutions of pre-immunization and post-immunization antisera. Data are averages of four biological replicates (antisera from four rabbits), with corresponding standard deviations shown as error bars; b The level of cross reactivity of purified plant and insect cell produced VLPs and leaf extracts of total protein (from plants which express or do not express L1) to immunized rabbit antisera. One-, two- and four-fold dilutions of VLPs were assayed, which corresponds to 2.5ng/ $\mu$ l, 1.25ng/ $\mu$ l and 0.625ng/ $\mu$ l respectively. The same dilutions were used on plant extracts, which correspond to total protein isolated from 1mg, 0.5mg and 0.25mg of plant material. The assay was conducted against a 1:16000 dilution of antisera, and thus the data shown is in the linear range of the ELISA. Data are averages of four biological replicates (antisera from four rabbits), with corresponding standard deviations shown as error bars  
150x236mm (150 x 150 DPI)



**Supplementary Fig. S1** Schematic of pEAQHT T-DNA region with L1 insertion (adapted from Sainsbury et al. 2009). BPV L1 (shown as turquoise arrow) was cloned into the pEAQ-HT destination vector 1 using LR recombination (recombination sites *attB1* and *attB2* are denoted by black boxes). Green and red arrows represent 35s promoter and Nos terminator sequences, respectively. The P19 silencing suppressor is denoted by a dark turquoise arrow and the NPTII region is shown as a pale blue arrow. UTRs are indicated by a solid black line, and left and right T-DNA borders are denoted as speckled boxes.