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Comparative analysis of recombinant *Human Papillomavirus* 8 L1 production in plants by a variety of expression systems and purification methods

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Summary

Human papillomavirus 8 (HPV-8), one of the high-risk cutaneous papillomaviruses (cHPVs), is associated with epidermodysplasia verruciformis and nonmelanoma skin cancer in immunocompromised individuals. Currently, no vaccines against cHPVs have been reported; however, recent studies on cross-neutralizing properties of their capsid proteins (CP) have fostered an interest in vaccine production against these viruses. We examined the potential of producing HPV-8 major CP L1 in *Nicotiana benthamiana* by agroinfiltration of different transient expression vectors: (i) the binary vector pBIN19 with or without silencing suppressor constructs, (ii) the nonreplicating *Cowpea mosaic virus*-derived expression vector pEAQ-HT and (iii) a replicating *Tobacco mosaic virus* (TMV)-based vector alone or with signal peptides. Although HPV-8 L1 was successfully expressed using pEAQ-HT and TMV, a 15-fold increase was obtained with pEAQ-HT. In contrast, no L1 protein could be immune detected using pBIN19 irrespective of whether silencing suppressors were coexpressed, although such constructs were required for identifying *L1*-specific transcripts. A fourfold yield increase in L1 expression was obtained when 22 C-terminal amino acids were deleted (L1ΔC22), possibly eliminating a nuclear localization signal. Electron microscopy showed that plant-made HPV-8 L1 proteins assembled in appropriate virus-like particles (VLPs) of T = 1 or T = 7 symmetry. Ultrathin sections of L1ΔC22-expressing cells revealed their accumulation in the cytoplasm in the form of VLPs or paracrystalline arrays. These results show for the first time the production and localization of HPV-8 L1 protein *in planta* and its assembly into VLPs representing promising candidate for potential vaccine production.

Introduction

Human papillomaviruses (HPVs) are tumorigenic, nonenveloped, 55-nm-diameter viruses that contain a double-stranded circular DNA genome of approximately 8000 bp (Münger *et al.*, 2004). HPVs, which account for approximately 5% of all cancers (Parkin, 2006), are classified into mucosal and cutaneous viruses. HPV-5 and HPV-8 are high-risk cutaneous HPVs (cHPVs), which were the first HPVs suspected of human tumorigenesis as a consequence of their association with skin epidermodysplasia verruciformis (EV) (Jablonska *et al.*, 1966; Pfister *et al.*, 1981). HPV-5 and HPV-8 have also been implicated in a variety of cancerous conditions after being found in nonmelanoma skin cancer lesions, such as those occurring in basal and squamous cell carcinomas (SCC) of immuno-compromised individuals or persons infected by *Human immunodeficiency virus* (Weissenborn *et al.*, 2005; Bouwes Bavinck *et al.*, 2007; Asgari *et al.*, 2008; Gormley and Kovarik, 2009). Although different HPV types have been found in benign tumours of patients with EV, HPV-5 or HPV-8 were the predominating types of those found in malignant tumours (Pfister, 2003a). In patients with actinic keratoses, a condition that often leads to SCC, a similar variety of HPV types was identified (Harwood and Proby, 2002; Pfister *et al.*, 2003b). Serological studies supported this involvement, as antibodies against the HPV-5 and HPV-8 major capsid protein (CP) were found in SCC-affected individuals (Feltkamp *et al.*, 2003; Masini *et al.*, 2003). Interestingly, a more recent relationship between cHPVs and the development of other epithelial cancers, such as SCC of the conjunctiva, has been established (Ateenyi-Agaba *et al.*, 2010).

The HPV major CP L1 may spontaneously self-assemble *in vivo* and *in vitro* into highly immunogenic capsomers and virus-like particles (VLPs) (Kirnbauer *et al.*, 1992; Modis *et al.*, 2002). VLPs can occur in two forms, an icosahedral lattice particle with a T = 7 symmetry containing 72 pentamers, which resembles native virions, and a small T = 1 particle composed of 12 pentamers (Baker *et al.*, 1991; Chen *et al.*, 2000). As L1 VLPs are highly immunogenic, they have been utilized as prophylactic vaccines against HPV, as has been the case of yeast-made vaccine against HPV types 16, 18, 6 and 11, and the insect-cell-derived vaccine against HPV-16 and HPV-18. In spite of efficient protective capabilities, there are issues concerning these vaccines, such as the high costs of production that impede their use in poor countries, the limited number of vaccine-covered HPV types, the requirement of intramuscular delivery and a cold chain (Haug, 2008).

Plants have been piloted as biotechnological tools to produce pharmaceuticals, such as monoclonal antibodies (MAbs) and

candidate antigens including VLPs, as they offer the advantage of relative ease of production, high scalability at reduced costs and lower risk of contamination by human pathogens (Daniell *et al.*, 2001; Yusibov and Rabindran, 2008; Rybicky, 2010). For these advantages, plants have been utilized to produce immunogens (including those that can be delivered orally), some of which have already entered clinical trials (Tacket *et al.*, 1998; Kapusta *et al.*, 1999; Warzecha *et al.*, 2003; Chia *et al.*, 2010; Davoodi-Semiromi *et al.*, 2010). With regard to HPV, *in planta* produced HPV-16 and HPV-11 L1-based VLPs have been reported (Biemelt *et al.*, 2003; Varsani *et al.*, 2003a; Warzecha *et al.*, 2003), and there are also indications of highly protective immune response to plant-made L1 protein of different papillomaviruses (reviewed by Giorgi *et al.*, 2010). For example, ingestion of transgenic HPV-11 L1 potato tubers activated an anti-VLP immune response in mice that was qualitatively similar to that induced by VLP parenteral administration (Warzecha *et al.*, 2003). In a separate study, neutralizing antibodies were elicited after intraperitoneal injection of mice with plant-derived HPV-16 L1 that was codelivered with Freund's or aluminium hydroxide adjuvants (Fernández-San Millán *et al.*, 2008). Such plant-produced vaccines have also been applied against papillomaviruses other than HPV; for example, *Cottontail rabbit papillomavirus* (CRPV) L1-based vaccine showed complete protection against CRPV elicited by intramuscular injection of CRPV L1-containing concentrated plant extracts with Freund's incomplete adjuvant (Kohl *et al.*, 2006).

None of the currently available recombinant expression platforms offers the cheapness and speed of production that transient expression in plants can provide. In addition, *in planta* transient expression systems allow the production of numerous proteins in a broad range of plant species. Plant transient expression requires transfer of the T-complex containing the gene/genes of interest from *Agrobacterium tumefaciens* to the plant cell nucleus where it subsequently becomes transcriptionally and translationally active; this typically follows infiltration of agrobacteria, which contains T-complex binary vectors, into the extracellular space between plant cells. This process does not lead to stable transformation as the T-complex does not result in T-DNA integration into the chromosomes of reproductive tissues (Kapila *et al.*, 1997). A number of transient expression systems have allowed production of proteins and antibodies to yields that are suitable for industrial-scale applications (Marillonnet *et al.*, 2005; Giritch *et al.*, 2006; Lindbo, 2007a; Sainsbury and Lomonossoff, 2008). Additional advantages of this system rely on the possibility of expressing large genes and the capacity to simultaneously express several genes to produce multimeric proteins or antibodies. However, a major concern of this system is that the expression level of heterologous proteins could be influenced by posttranscriptional gene silencing, which becomes active in the agroinfiltrated tissue. This problem can be overcome via the use of silencing suppressors that combat silencing, and thus increase the yield of the transiently expressed proteins (Johansen and Carrington, 2001; Voinnet *et al.*, 2003).

Until now, there have been no reports describing the expression of cHPV L1 proteins in plants. In this work, HPV-8 was selected as a prototype of high-risk cHPVs and trailed for transient expression in *Nicotiana benthamiana* plants using different vectors. We also compared the yield of full-length L1 protein originating from a wild-type gene and its truncated version deprived of the C-terminal nuclear localization signal (NLS). We assessed whether L1 was able to spontaneously assemble in plants into potentially immunogenic forms, such as VLPs and capsomers, by analysing crude plant sap; we also directly checked particle accumulation in plant cells. By means of different purification protocols, we identified methods that enhanced concentration and stability of L1 in plant extracts.

Results

Transient expression and quantification of L1 and L1ΔC22 in plants using different vectors

Transient expression of L1 and L1ΔC22 in *N. benthamiana* plants was assessed using the binary vector pBIN19 (Bevan, 1984), the *Cowpea mosaic virus* (CPMV)-derived small binary vector pEAQ-HT (Sainsbury *et al.*, 2009) and the deconstructed *Tobacco mosaic virus* (TMV)-based vector (Icon Genetics, Halle, Germany). HPV-8 *L1* wild-type gene and *L1ΔC22* lacking the 3′-terminal sequence encoding 22 amino acids (aa), which contains a potential NLS conserved amongst the HPV L1 proteins (Zhou *et al.*, 1991; Merle *et al.*, 1999), were amplified by polymerase chain reaction and introduced into the multiple cloning site of: (i) pBIN19 (generating pBIN-L1 and pBIN-L1ΔC22), (ii) pEAQ-HT (creating pEAQ-L1 and pEAQ-L1ΔC22) and (iii) the 3′-module pICH11599 of TMV-based vector (producing pICH11599-L1 and pICH11599-L1ΔC22). pICH11599-L1 and pICH11599-L1ΔC22 were used along with pICH14011 integrase construct, and either with pICH17338, a TMV 5′-module devoid of any targeting signal; pICH17610, carrying a polygalacturonase signal peptide (ApoA) or pICH17620, with a calreticulin signal peptide (ApoC) (Figure 1). The resulting plasmids were introduced into *A. tumefaciens*; pBIN-L1, pBIN-L1ΔC22, pEAQ-L1 and pEAQ-L1ΔC22 into the strain LBA4404 and all TMV-based constructs into the strain GV3101.

An antiserum raised against the recombinant HPV-8 L1 protein fused to Glutathione S-transferase (GST-8L1) made in *Escherichia coli* and purified using Glutathione sepharose beads strongly reacted with its homologous antigen when tested in Western blot and specifically recognized a band of approximately 59 kDa obtained after thrombin digestion of the fusion product (Figure 2a), indicating that this antiserum was a suitable tool to detect whether plants could express L1 and its truncated version. To gather information about the best candidate vector to transiently express L1 and L1ΔC22 in *N. benthamiana* plants, leaves were agroinfiltrated in parallel experiments with the different constructs. After 6 days of incubation in a growth chamber, the tissue was harvested, and total proteins were extracted and analysed by Western blot using the polyclonal GST-8L1 antiserum. HPV-8 L1 and L1ΔC22 were immune detected as monomers with the expected size of 59 kDa for L1 and 55 kDa for L1ΔC22 when pEAQ-HT or TMV was used (Figure 2b). On the other hand, no reaction could be observed in extracts agroinfiltrated with pBIN19-based constructs. As it can be observed in Figure 2b, pEAQ-HT induced a higher L1 and L1ΔC22 accumulation with respect to all three versions of the TMV-based vector. Comparing the different signal peptides of the TMV vector, higher levels of L1ΔC22 were detected when no exogenous targeting signal was present. When fused to either signal peptide (ApoA and ApoC), both proteins showed a slightly slower migration pattern, suggesting possible post-translational modifications or incorrect cleavage of the signal peptides. Overall, using pEAQ-HT and the two versions of the TMV-based vector, the protein lacking the NLS (L1ΔC22) was expressed at higher levels compared with its full-length

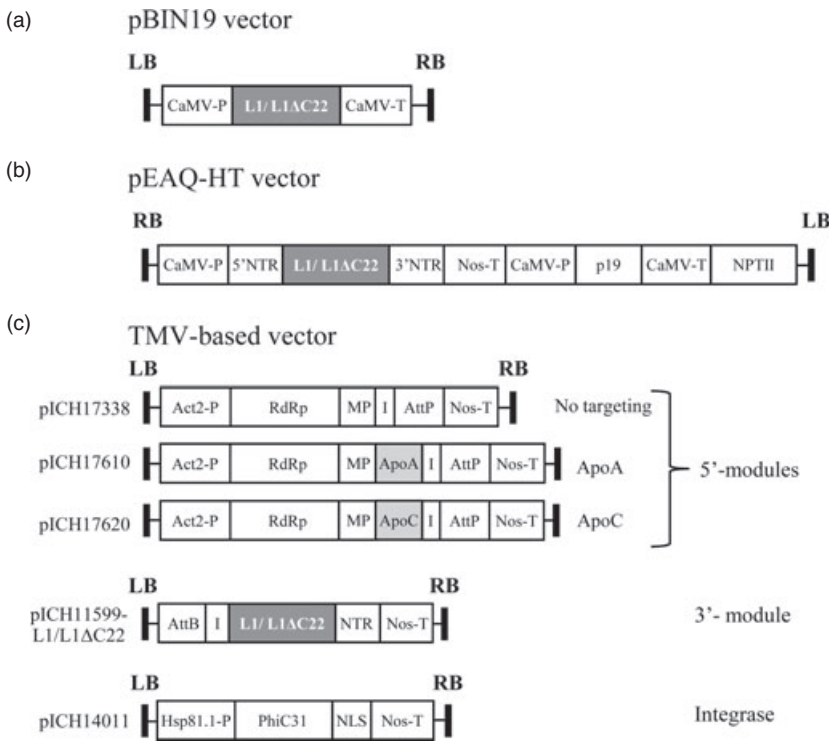

Figure 1 Schematic representation of the T-DNAs carrying the *L1* and *L1ΔC22* coding regions used in this study. (a) Derivatives of pBIN19, with the *L1* or *L1ΔC22* genes under control of the *Cauliflower mosaic virus* 35S promoter (CaMV-P) and terminator (CaMV-T). (b) Derivatives of pEAQ-HT, with the *L1* or *L1ΔC22* genes under control of the CaMV-P, 5′ and 3′ nontranslated regions (NTR) of *Cowpea mosaic virus* RNA-2 and nopaline synthase terminator (Nos-T). (c) 5′-modules of *Tobacco mosaic virus* (TMV)-based vector: pICH17338 (no signal peptide), pICH17610 (carrying the polygalacturonase signal peptide, ApoA) and pICH17620 (carrying the calreticulin signal peptide, ApoC); 3′-module pICH11599-L1 or pICH11599-L1ΔC22; integrase module pICH14011 containing the integrase gene, PhiC31. Luria-Bertani broth (LB) and RB, left and right borders of T-DNA; P19, suppressor of gene silencing; NPT, neomycin phosphotransferase; Act2-P, actin promoter; RdRp, RNA-dependent RNA polymerase; MP, movement protein; I, intron; AttP and AttB, recombination elements; Hsp81.1-P, Hsp81.1 promoter; NLS, nuclear localization signal.

equivalent (L1). This is in contrast with the results of Green Fluorescent Protein (GFP) expression driven by the same vectors, where all vectors successfully expressed it, but TMV (without signal peptides) induced the highest GFP yield (Figure 2c).

To discover the reasons of the unsuccessful detection of L1 and L1ΔC22 with the pBIN19 vector, Northern blot analysis was carried out on total RNAs extracted from leaves 6 days after agroinfiltration with pBIN-L1 and pBIN-L1ΔC22, either alone or in combination with the following silencing suppressors: P19 of *Carnation Italian ring spot virus* (CIRV; Lakatos *et al.*, 2006), P19 of *Tomato bushy stunt virus* (TBSV; Voinnet *et al.*, 1999) and HC-Pro of *Potato virus Y* (PVY; Brigneti *et al.*, 1998). *L1*-specific mRNAs were observed with both constructs, only when the binary vector was used in combination with silencing suppressors, with a stronger signal for the *L1ΔC22* transcripts compared with the *L1* transcripts (Figure 3); however, in spite of the strong impact exerted by the coexpressed silencing suppressors on transcript accumulation, L1 and L1ΔC22 proteins could not be detected (data not shown).

The identity of Ll and L1ΔC22 proteins expressed by pEAQ-HT and the TMV-based vector was confirmed using the MD2H11 MAb, which was initially developed against HPV-16 L1, and found to recognize a conserved linear epitope present in several papillomaviruses (M. Müller, pers. commun.). Transient expression of L1 and L1ΔC22 was quantified using different amounts of a purified pseudovirion preparation of HPV-5 as a reference on Western blots probed with the MD2H11 MAb (data not shown). This approach was employed as a high serological relationship between HPV-8 and -5 L1s has been previously demonstrated (Handisurya *et al.*, 2009). The highest expression was achieved with L1ΔC22 (240 mg/kg fresh leaf material) with the pEAQ-HT vector, which was about four times greater compared with the full-length polypeptide (L1) using the same vector (60 mg/kg of fresh leaf material). With TMV-based vectors, about 3.5 mg of L1 and 17 mg of L1ΔC22 per kg of fresh leaf material were obtained. As only the intact protein bands (55 or 59 kDa) were detected, the calculated expression levels took into consideration only non-degraded L1 protein. As L1 and L1ΔC22 were more highly expressed in plants using pEAQ-HT, this vector was subsequently used to detect VLP formation in crude plant sap and in purified protein products using electron microscopy.

Assessment of VLP formation in crude plant sap

The assembly of L1 and L1ΔC22 in higher-order structures such as capsomers or VLPs was investigated by Immuno-sorbent electron microscopy (ISEM) on crude sap preparations from agroinfiltrated leaves. Spherical VLPs of about 30 nm with probable T = 1 symmetry as well as capsomers (about 10–15 nm) were observed in preparations from leaves infiltrated with both constructs, indicating that spontaneous assembly of L1 and L1ΔC22 in immunogenic forms occurred in the plant tissue (Figure 4).

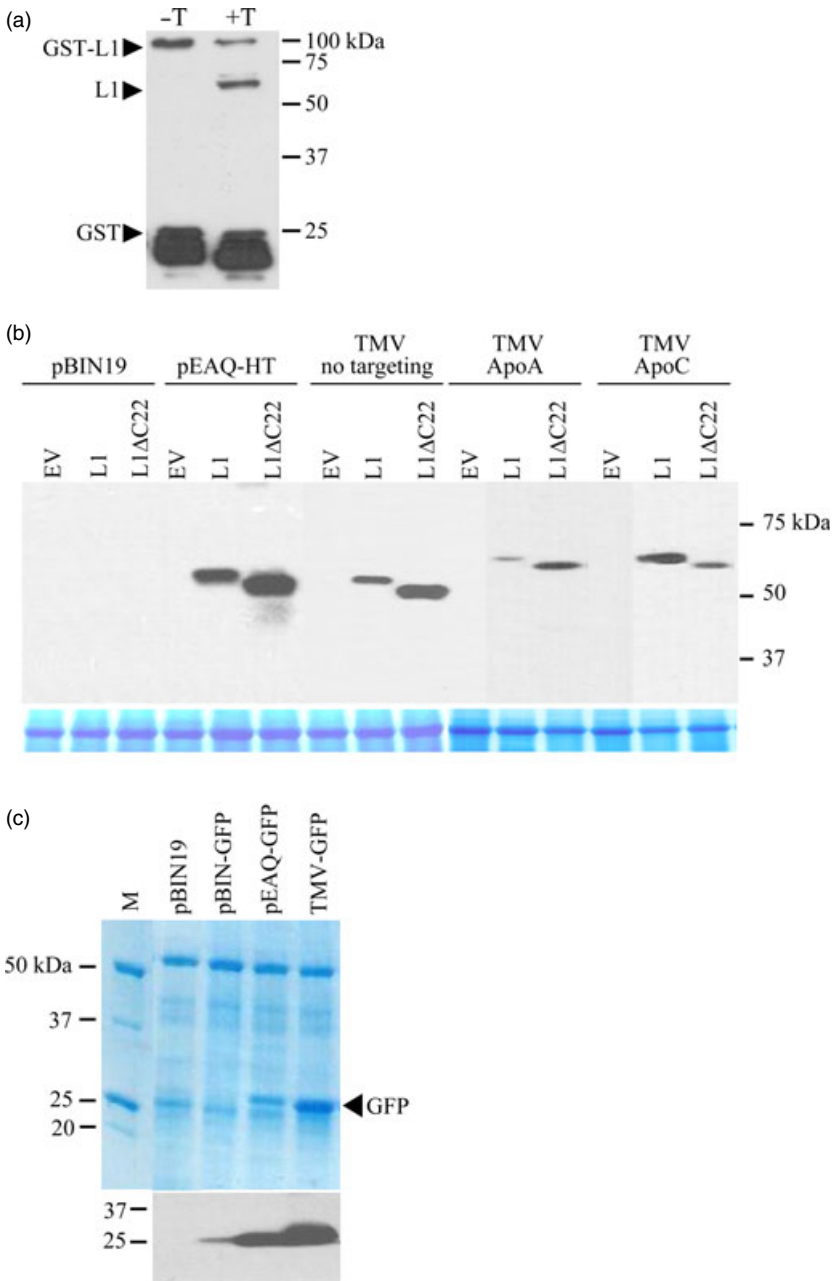

Figure 2 Immunoblot analysis of L1 and L1ΔC22 proteins. (a) Glutathione S-transferase (GST)-L1 fusion protein expressed in BL21DE3 *Escherichia coli* following IPTG induction and purification with Glutathione sepharose 4B beads was digested with thrombin (–T, uncleaved protein; +T, protein cleaved with thrombin, 1 NIH Unit per 40 μg GST-L1). (b) Total proteins extracted from *Nicotiana benthamiana* leaf tissue (corresponding to 0.4 mg of fresh tissue/lane) 6 days postagroinfiltration with the different vectors carrying either *L1* or *L1ΔC22* gene or the corresponding empty vectors (EV), that is, pBIN19, pEAQ-HT and *Tobacco mosaic virus* (TMV) used as a negative control. TMV vector was used with or without the ApoA or ApoC signal peptides. The polyclonal antiserum GST-8L1 was used at 1 : 1000 dilution. Coomassie-stained Rubisco large subunit is shown as a loading control. (c) Coomassie-stained SDS-PAGE and Western blot of protein extracts agroinfiltrated with Green Fluorescent Protein (GFP)-expressing constructs; pBIN19 was used as empty vector (negative control).

Subcellular localization of VLPs

After observing VLPs in crude sap, we investigated whether we could detect them intracellularly in glutaraldehyde-fixed sections of agroinfiltrated leaves using electron microscopy. By scanning the grids, we observed VLPs (50 nm) in the cytoplasm of the L1ΔC22-expressing cells using the pEAQ-HT vector (Figure 5a) and aggregates of spherical particles (25–30 nm) arranged into paracrystalline arrays using the TMV vector (Figure 5b). Following the observation of multiple sections, these structures could neither be viewed in other organelles (mitochondria, chloroplasts, etc.) nor in mock-infiltrated plant cells (Figure 5c). On the other hand, when the full length L1 was expressed with pEAQ-HT and TMV vector, neither VLPs nor paracrystalline arrays could be detected within the plant cell nucleus (Figure 5d,e). Analysis of tissue infiltrated with TMV vectors

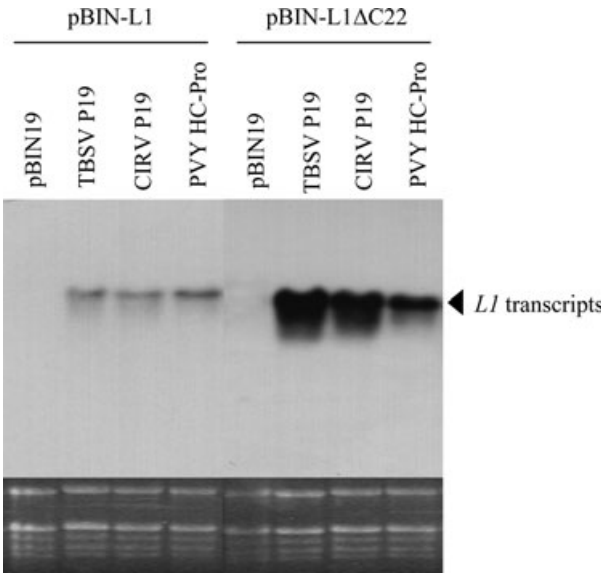

Figure 3 Northern blot analysis of *Nicotiana benthamiana* leaf extracts coagroinfiltrated with pBIN-L1 or pBIN-L1ΔC22 plus the indicated silencing suppressor construct or pBIN19, as a control. The membrane was hybridized with an L1-specific probe. Ethidium bromide-stained gel is shown as a loading control.

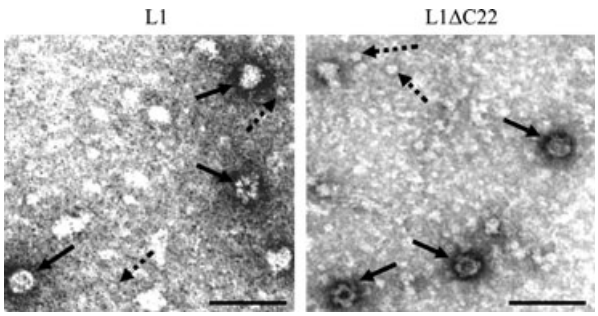

Figure 4 Immuno-sorbent electron microscopy on crude sap collected from *Nicotiana benthamiana* leaves 6 days postagroinfiltration with pEAQ-L1 and pEAQ-L1ΔC22. Immuno-trapping of L1 and L1ΔC22 proteins was carried out with the MD2H11 monoclonal antibodies (diluted 1 : 500). For both proteins, representative T = 1 virus-like particles (about 30 nm in diameter) are shown by black arrows, and capsomers (about 10 nm) by dotted arrows. Bar represents 100 nm.

expressing L1ΔC22 fused to the ApoA or ApoC signal peptides did not result in the observation of high-order protein structures within the apoplast (data not show). Overall, L1ΔC22 VLPs and paracrystalline arrays were only successfully detected within the plant cytoplasm.

Strategies for the purification of L1 and L1ΔC22

To define a method suitable for purifying L1-based VLPs from leaves, three different purification strategies were assessed. These were protocols previously developed for the purification of: (i) HPV-16 VLPs from baculovirus-infected insect cells (Protocol 1; Varsani *et al.*, 2003b); (ii) begomovirus from *N. benthamiana*-infected plants (Protocol 2; Luisoni *et al.*, 1995) and (iii) HPV pseudovirions (Protocol 3; Buck *et al.*, 2005). While the pseudovirion purification method that included a homogenization step in high-salt phosphate-buffered saline (PBS) followed by an Optiprep™ (Sigma-Aldrich, Buchs, Switzerland) gradient did not result in the isolation of recognizable VLPs or capsomers (data not shown), protocols 1 and 2 allowed us to obtain discrete visible bands following gradient centrifugations, which according to subsequent electron microscopy analysis contained high-order protein structures for both L1 and L1ΔC22. With the HPV-16 VLP protocol that is based on an extraction in a high-salt PBS, followed by a sucrose cushion and CsCl equilibrium centrifugations, VLPs of 55 nm with T = 7 symmetry, small VLPs of about 30 nm with T = 1 symmetry as well as capsomers were observed (see Figure 6a, where L1ΔC22 is shown as representative protein). Using the begomovirus protocol that included an overnight tissue extraction in phosphate buffer (PB) containing a detergent and a cell-wall-degrading enzyme, followed by Cs_2SO_4 density gradient, the concentration of VLPs was greatly increased for both proteins, as it can be appreciated in Figure 6b. Analysis of purified products obtained with the begomovirus protocol showed that L1 assembled into small T = 1 VLPs and capsomers (Figure 6c), whereas L1ΔC22 assembled in addition into large T = 7 VLPs (Figure 6d), resembling HPV-5 pseudovirions (Figure 6e); T = 7 VLPs approximately accounted to 15% of the overall VLP population.

Western blot analysis of samples observed by electron microscopy confirmed the presence of monomeric bands of the expected size, 59 and 55 kDa for L1 and L1ΔC22, respectively, with both purification methods (Figure 6f). However, in the case of the HPV-16 VLP protocol, SDS-PAGE analysis revealed a major additional band, present in relatively higher amounts in both L1 and L1ΔC22 constructs that did not react specifically with the antibody, indicating the occurrence of contaminants and the lower purification quality of these products (Figure 6f). Conversely, SDS-PAGE of samples obtained with the begomovirus protocol showed that the band detected by the L1 antibody was the major one, yet another band of about 42 kDa was present that reacted specifically in Western blot in L1ΔC22 samples. This 42-kDa band could be a degradation product of L1, as already observed for L1 of HPV-8 and other papillomaviruses (Fernández-San Millán *et al.*, 2008; Lenzi *et al.*, 2008; Handisurya *et al.*, 2009). Noticeably, the begomovirus protocol did not result in the copurification of contaminating polypeptides as in the case of the HPV-16 VLP protocol (Figure 6f). Altogether, only potentially immunogenic forms of L1 and L1ΔC22 proteins were recovered after purification confirming the appropriate self-assembly of proteins.

Discussion

In this work, we attempted the expression and purification of wild-type HPV-8 L1 from *N. benthamiana* plants. For this purpose, full-length HPV-8 L1 and its truncated version lacking a potential C-terminal NLS were used. To achieve this goal, we compared three different plant expression vectors: (i) a classical binary nonreplicating vector (pBIN19), (ii) a smaller binary nonreplicating vector (pEAQ-HT) enhanced with regulatory 5′- and 3′-untranslated regions from CPMV RNA-2 and augmented with a potent P19 silencing suppressor and (iii) a replicating TMV-based viral vector. We report the first successful expression in plants of HPV-8 L1 and L1ΔC22 by using pEAQ-HT and TMV-based vectors. This is in contrast to our attempts with pBIN19, in which neither protein could be detected.

Plant gene silencing is one of the factors governing the level of expression of foreign genes, and it was shown to be

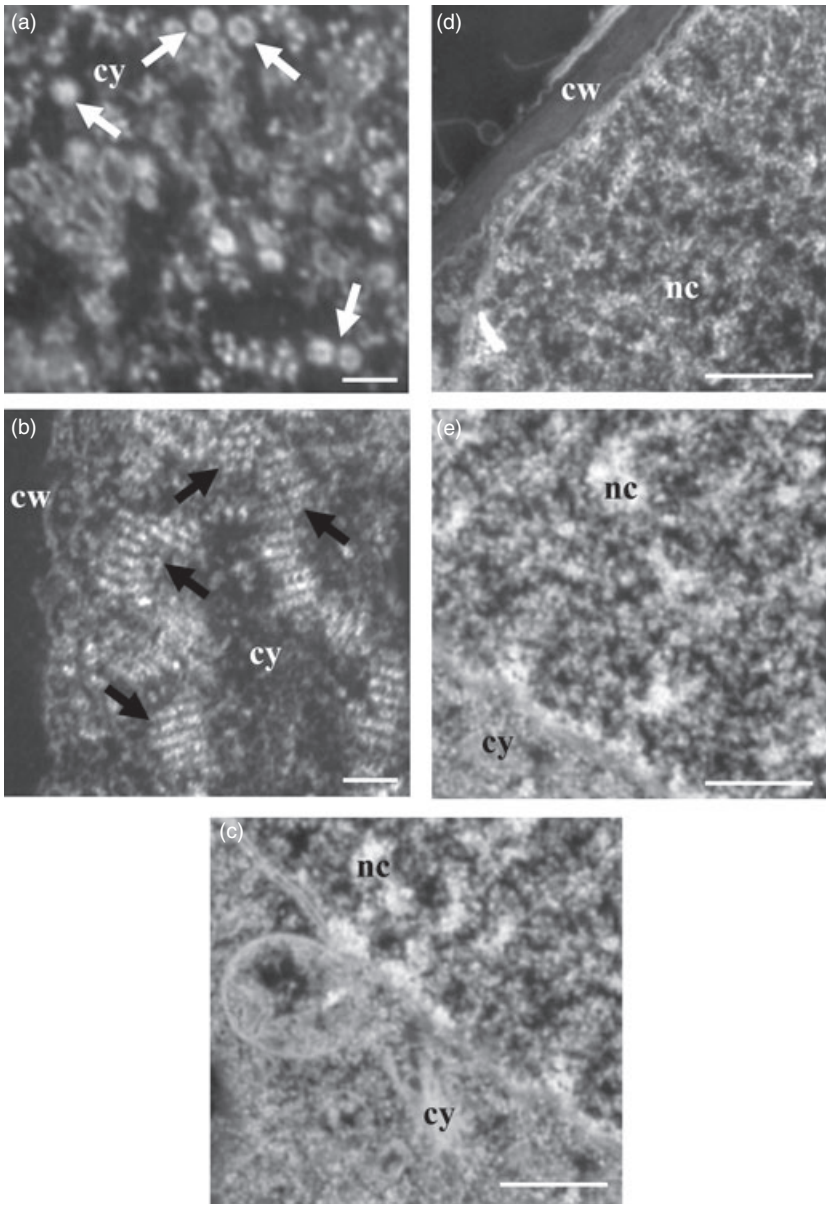

Figure 5 Intracellular localization of L1 and L1ΔC22-based virus-like particles (VLPs) by positive staining electron microscopy of ultrathin sections of *Nicotiana benthamiana* leaf tissue. pEAQ-HT and *Tobacco mosaic virus* (TMV)-based vector without signal peptides were used for transient expression of L1 and L1ΔC22. (a) VLPs of about 55 nm within the cytoplasm of the L1ΔC22-expressing cells using the pEAQ-HT vector are indicated by white arrows (b) Spherical particles (25–30 nm) in the form of paracrystalline arrays distributed in the cytoplasm of the L1ΔC22-expressing cells using the TMV vector are shown by black arrows. (c) Mock-infiltrated *N. benthamiana* plant was shown as a negative control. Recognizable protein structures were not detected within the nucleus of L1-expressing cells using (d) the pEAQ-HT vector or TMV vector (e). Bar represents 100 nm (a,b) and 500 nm (c,d,e). cw, cell wall; cy, cytoplasm; nc, nucleus.

positively counteracted by coexpressing proteins that suppress it, with the result of increasing and extending the expression (Johansen and Carrington, 2001; Voinnet *et al.*, 2003). In this report, in spite of the substantial increase in the accumulation of *L1* or *L1ΔC22* transcripts driven by pBIN19-based constructs in combination with silencing suppressors (Figure 3), no detectable protein expression was achieved. The successful expression of HPV8-L1 proteins by the enhanced binary vector pEAQ-HT can be explained through its improvements with respect to the original pBIN vector, such as the presence of silencing suppressor within the same T-DNA, the insertion of CPMV regulatory sequences or the reorientation of the T-DNA borders, as already explained by Sainsbury *et al.* (2009). Furthermore, as we have observed similar transcript accumulation levels with constructs based on pBIN19 (plus silencing suppressor) and pEAQ-HT (data not shown), it is possible that the CPMV regulatory sequences have a further role of stabilizing *L1* transcripts, slowing RNA decay or improving translation through the attraction of translation initiation complex elements.

Overall, comparing the three different vectors, pEAQ-HT induced the highest expression of HPV-8 L1 proteins, not only compared with pBIN-based constructs, but also to the TMV

system. Besides, the absence of CPMV regulatory sequences, the lower expression of HPV-8 L1 achieved with TMV could also be attributed to the replicative nature of these vectors, as already described for another filamentous viral vector undergoing genetic instability when engineered with large inserts (Avesani *et al.*, 2007).

The results of a higher expression of L1 by pEAQ-HT (about 15-fold) differ from those achieved with GFP, where TMV behaved as the best performing vector (see Figure 2c). While attempting to enhance GFP expression by a TMV vector deprived of a CP gene through the suppression of post-transcriptional silencing, no effect was observed by coexpressing a silencing suppressor (Lindbo, 2007b), leading to the hypothesis that the TMV CP behaved as an inducer of gene silencing. In that way, a foreign gene sequence such as a coat protein of another virus could itself stimulate/interfere with the induction of silencing in a manner that cannot be counteracted by the endogenous TMV silencing suppressor (Csorba *et al.*, 2007). These data prove that for obtaining large amounts of a foreign gene product for biotechnological purposes, the choice of the gene and expression strategy are of crucial importance. Furthermore, this demonstrates that gene expression is subjected to multiple layers of controls requiring a specific optimization of the transcriptional or translational context.

Considering that the deconstructed TMV vector used in this study offers additional advantages of modular protein targeting, we tested whether L1 accumulation could be enhanced by targeting it to the apoplast, as previously shown for another HPV-8 protein (Noris *et al.*, 2011). However, among the three different variants of TMV used in this study, the higher accumulation of L1ΔC22 was obtained when no signal peptides were introduced. A reduced accumulation in the apoplast could derive from a possible proteolytic degradation in the extracellular space (Faye *et al.*, 2005; Doran, 2006) or the introduction of post-translational modifications that might also negatively impact protein turnover in that tissue location.

In our work, the removal of the putative NLS of L1 led to a four to fivefold yield increase in comparison with full-length equivalent L1. This indicates that ensuring the cytoplasmic localization of HPV-8 L1 proteins (via NLS removal) likely promotes its stability and accumulation. Previous results point to a contradictory influence of the C-terminal NLS on L1 accumulation in plants. In the case of HPV-16, while the presence of the NLS did not greatly modify expression in transgenic plants (Varsani *et al.*, 2003a), its removal had a detrimental impact on a chloroplast-targeted transiently expressed protein, resulting in a fivefold yield decrease (Maclean *et al.*, 2007). On the contrary, NLS removal of HPV-11 L1 enhanced its expression in potato (Warzecha *et al.*, 2003). These contrasting results might reflect either the different properties of each papillomavirus L1 variant or the different approaches the studies have taken with regard

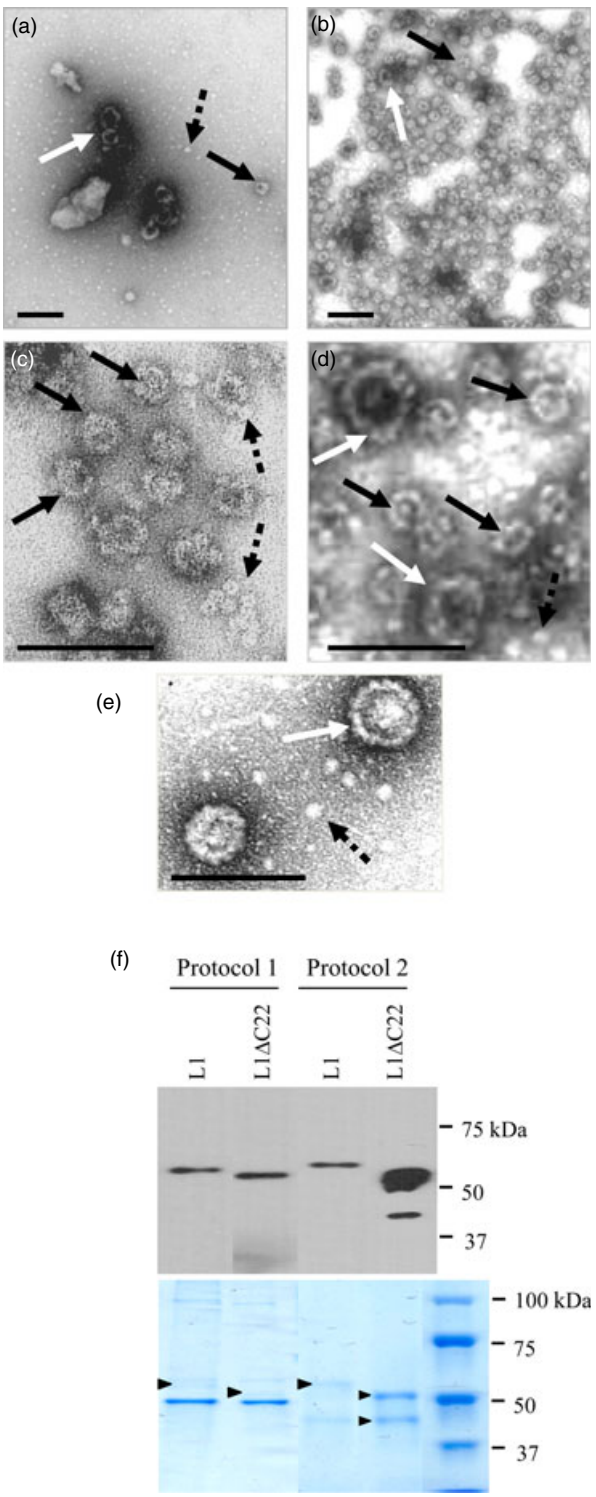

Figure 6 L1 and L1ΔC22-made particles purified with two different protocols from *Nicotiana benthamiana* tissue infiltrated with pEAQ-L1 or pEAQ-L1ΔC22. Electron micrographs of L1ΔC22 particles (as a representative protein) purified by (a) the *Human papillomavirus* (HPV)-16 virus-like particles (VLP) method (Protocol 1) or (b) the begomovirus method (Protocol 2). Higher magnification images of (c) L1 and L1ΔC22-based particles (d), obtained by the begomovirus protocol, were shown. (e) Pseudovirions of HPV-5 were used as a positive control. T = 7 VLPs (about 55 nm), and T = 1 VLPs (about 30 nm) are shown by white or black arrows, respectively; capsomers are indicated by dotted arrows. Bar represents 100 nm in all electron micrographs. (f) Immunoblot and Coomassie-stained SDS-PAGE analysis of purified preparations of L1 and L1ΔC22 proteins obtained with protocols 1 and 2, using the MD2H11 monoclonal antibodies (1 : 10 000 dilution). The bands that gave a positive reaction in Western blot are indicated by triangles on the Coomassie-stained SDS-PAGE.

to expression strategy, presence or absence of cellular targeting signals or codon optimization methodology.

Previous work on the expression of wild-type L1 of other papillomaviruses in plants reported yields ranging from 0.4 mg/kg of CRPV L1 in TMV-infected *N. benthamiana* plants to 1 mg/kg in transgenic *Nicotiana tabacum* plants (Kohl *et al.*, 2006). For HPV-16 L1 transiently expressed in its native form in combination with the *Tomato spotted wilt virus* silencing suppressor, 10 mg per kg of *N. benthamiana* tissue was obtained when the expression was targeted to the cytoplasm, whereas 137 mg/kg of L1 was detected when expression was targeted to the chloroplasts (Maclean *et al.*, 2007). In addition, for HPV-11, 2 and 12 mg of L1ΔC21 per kg of transgenic *N. tabacum* and *Arabidopsis thaliana* tissue, respectively, were reported (Kohl *et al.*, 2007). Compared with these yields, we obtained between two and 600-fold higher amounts of HPV-8 L1ΔC22. *N. benthamiana* is thus a suitable host for obtaining significant yields for both HPV-8 L1 proteins. Hence, transient expression system proved its usefulness for rapid and efficient production of these proteins. Considering that L1 transient expression of HPV-16 in the same plant host was increased up to 10 000-fold when the gene was optimized with human codon usage and the expression vector was changed (Varsani *et al.*, 2006; Maclean *et al.*, 2007), we envisage that HPV-8 L1 yield could be raised by further optimization steps, including the use of different codons and expression strategies. Noteworthy, even with our current level of expression (60 and 240 mg/kg), we are above the required amount expected for commercially viable antibody production (reviewed by Giorgi *et al.*, 2010).

Electron microscopy of the crude plant sap showed that both proteins, L1 and L1ΔC22, self-assembled into appropriate VLPs of about 30 nm with probable T = 1 symmetry. Small VLPs were also observed when other papillomaviruses were expressed in plants (Kohl *et al.*, 2006, 2007; Maclean *et al.*, 2007). The NLS of HPV-11 and -16 L1 is not necessary for appropriate VLP assembly, as VLPs made of full-length or NLS-deleted version of L1 are morphologically similar (Merle *et al.*, 1999; Varsani *et al.*, 2003a). Our work thus agrees with these reports, as no morphological difference was observed between T = 1 VLPs made of L1 or L1ΔC22.

Few groups have reported results of the direct visualization of HPV L1-based particles in heterologous expression systems, such as prokaryotic or insect cells. While in *E. coli* only insoluble inclusion bodies were observed (Zhang *et al.*, 1998), VLPs and electron-dense bodies containing VLP aggregates could be detected intracellularly in *Lactobacillus casei* (Aires *et al.*, 2006). VLPs were also identified by electron microscopy in the nucleus and cytoplasm of Sf-9 cells infected with recombinant baculoviruses (Rose *et al.*, 1993). In plants, there is only one report of indirect localization of HPV L1 proteins using fluorescence microscopy where HPV-11 L1 fused to GFP concentrated essentially within the nucleus and the truncated form lacking the C-terminal NLS spread throughout the tobacco cell (Warzecha *et al.*, 2003). However, to our knowledge, no ultrastructural studies concerning the direct localization of HPV L1 in the plant tissue were reported. Our ultrathin section results showed not only that HPV-8 L1ΔC22 localized in the cytoplasm of *N. benthamiana* cells, but also, for the first time, that it self-assembled into VLPs or paracrystalline aggregates, confirming that the C-terminal NLS removal indeed influenced L1 intracellular accumulation. On the contrary, when NLS is present in the L1 protein, no recognizable structures could be detected within the plant nucleus. Recombinant L1 protein can spontaneously assemble into VLPs, but its assembly is enhanced when L2 is co-expressed (Hagensee *et al.*, 1993). Perhaps, for its assembly/stability in the plant nuclear compartment, L1 would require the presence of a stabilizing protein, the minor CP L2. When L1ΔC22 had a signal peptide (ApoA or ApoC), following examination of multiple sections, we failed to recognize VLPs and/or their crystalline arrays in the apoplast. As previous reports have demonstrated that glycosylated HPV-16 L1 is retained in the endoplasmic reticulum, and not transported into the Golgi complex or to the cell surface in CV-1 monkey kidney cell (Zhou *et al.*, 1993), it is possible that our inability to detect L1ΔC22 in the apoplast is because of its endoplasmic reticulum retention. Further immunogold-labelling experiments will be useful to discover the location of the L1 protein in the cell.

Analyses of purified protein products revealed the presence of T = 7 VLPs intermingled with small T = 1 VLPs and capsomers. However, T = 7 VLPs were found only in tissue expressing L1ΔC22. Therefore, it is possible that higher L1 expression and the structural stability of VLPs in the cytoplasm are conducive to T = 7 particle formation. Comparative analysis of three purification protocols demonstrated that a begomovirus extraction methodology was the most effective in yielding high concentrations of VLPs. This protocol might be more effective as it is specifically designed for purifying icosahedral virus particles of similar size to HPV from *N. benthamiana* tissue and thus is likely more conducive for VLP isolation and stability than the other methods. This perhaps shows that choosing an extraction protocol based on plant viruses, which have size and shape similar to HPV VLPs, may be an appropriate way of achieving high VLP yields. Furthermore, the VLP purification from plants must take into consideration the pH conditions, the presence of antioxidants, ion concentrations, chelating agents and detergents (Paintsil *et al.*, 1998). In conclusion, we plan to test the suitability of this protocol for purified L1-based particles of other papillomaviruses expressed in plants.

Currently, no vaccines against cHPVs have been produced, but recent cross-neutralizing studies on their major CPs have fostered an interest in vaccine production against these viruses (Handisurya *et al.*, 2009). Now, it would be interesting to check the immunogenic potential of the HPV-8 L1 produced in plants with the final goal of producing a prophylactic vaccine. The main concerns with an L1-based vaccine against mucosal HPVs are the limited number of vaccines available for the different HPVs, and also that these vaccines are exclusively prophylactic with no therapeutic efficacy. As plants offer the possibility to express several proteins simultaneously, a likely future perspective is that different proteins of diverse types of HPV could be expressed together to produce vaccines with broad prophylactic and therapeutic activities.

Experimental procedures

Construction of plasmids for transient expression *in planta*

To perform cloning in the common binary expression vector pBIN19, the native full-length *L1* and *L1ΔC22* were amplified using *Pfu* DNA polymerase from a complete HPV-8 clone (GenBank accession no. M12737, provided by H. Pfister, Institute of Virology, Cologne, Germany). Primers for the amplification were pBIN-L1-5851F x pBIN-L1-7379R, and pBIN-L1-5851F x

pBIN-L1ΔC22-7326R, respectively. The amplicons were cloned into pJIT60 (provided by P. Mullineaux, University of Essex, Colchester, United Kingdom) restricted with *Bam*HI/*Sma*I. The expression cassette of pJIT60 containing either *L1* or *L1ΔC22*, *Cauliflower mosaic virus* 35S promoter and partial 35S terminator was then transferred into *Kpn*I/*Bgl*II-restricted pBIN19 to obtain pBIN-L1 and pBIN-L1ΔC22.

For cloning into the CPMV-derived small binary expression vector pEAQ-HT (Sainsbury *et al.*, 2009), *L1* or *L1ΔC22* was amplified, cloned into pEAQ-HT and digested with *Nru*I/*Xho*I, generating pEAQ-L1 and pEAQ-L1ΔC22.

To prepare constructs in the TMV-based vectors (Icon Genetics), *L1* or *L1ΔC22* was amplified, digested with *Nco*I/*Hind*III and *Nco*I/*Bam*HI, respectively, and cloned into the TMV 3′-module pICH11599, creating pICH11599-L1 and pICH11599-L1ΔC22. All primers used for cloning are listed in Table 1.

Expression of recombinant L1 in bacteria

L1 was *Pfu*-amplified using the primers shown in Table 1 and cloned into *Bam*HI/*Sma*I-digested pGEX-2T (GE Healthcare, Piscataway, NJ) fused to the *GST* gene. This way, pGEX-L1 was generated and used to transform competent *E. coli* BL21DE3, which would subsequently be used to express L1 fused with 25.6 kDa GST. The recombinant strain was grown overnight in 2 mL of Luria-Bertani broth (LB), containing ampicillin (Amp, 100 μg/mL) and chloramphenicol (Cam, 10 μg/mL) at 225 rpm at 37 °C. Five hundred microlitres of overnight culture were transferred into Erlenmeyer flasks containing 50 mL of LB-Amp-Cam and incubated at 225 rpm at 37 °C until the optical density (OD_{600}) reached 0.6. L1 expression was induced with 0.2 mM of isopropyl-β-D-thiogalactopyranoside with subsequent incubation at 225 rpm for 3.5 h at 25 °C. A noninduced control was grown in parallel. After incubation, the bacterial cells were harvested by centrifugation at 4000 ***g*** for 10 min. The above HPV-8 L1 amplicon was also directly cloned into pGEM®-T-Easy vector (Promega, Madison, WI), and the obtained pGEM-L1 was used to generate gene specific RNA probes.

Extraction and purification of L1 from *Escherichia coli*

Bacteria were lysed by freeze-thaw cycles and resuspended in 1.5 mL buffer [50 mM Tris–HCl (pH 8.0), 0.2 M NaCl, 1 mM DTT, 1 mM EDTA]. After sonication, urea was slowly added to the lysate to a final concentration of 3.5 M. The mixture was incubated at 4 °C for 1 h with gentle shaking and then dialyzed against three changes of buffer [50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 1 mM DTT, 1 mM EDTA] overnight. The solution was clarified by centrifugation at 25 000 ***g*** for 75 min at 4 °C. GST-L1 was purified from soluble proteins using Glutathione sepharose 4B (GE Healthcare) and eluted with 30 mM glutathione. L1 was cleaved from GST using thrombin (approximately 1 NIH unit every 40 μg GST-L1).

Production of polyclonal antiserum against L1

Approximately, 250 μg of purified recombinant L1 were subcutaneously injected into New Zealand rabbits four times at weekly intervals. Following two boosters (at weeks 9 and 11 after the first immunization), bleedings were made at 20 and 30 days after the last injection. To reduce nonspecific binding to bacterial proteins, antisera were preabsorbed with extracts from *E. coli*, prior to IgG purification on protein A-Sepharose column according to Clark and Bar-Joseph (1984).

Agroinfiltration

Agrobacterium tumefaciens LBA4404 was transformed with plasmids pBIN-L1, pBIN-L1ΔC22, pEAQ-L1 and pEAQ-L1ΔC22. Bacterial cultures were grown for 2 days at 28 °C in YEB medium containing kanamycin (50 μg/mL) and rifampicin (Rif, 50 μg/mL) and pelleted. Following resuspension in 10 mM MES 2-(N-morpholino) ethansulfonic acid, pH 5.6, 10 mM $MgCl_2$ and 100 μM acetosyringone to an OD_{600} of 0.8 and incubation of 3 h at room temperature, bacterial suspensions were syringe infiltrated into the leaves of 6–8-week-old *N. benthamiana* plants. Control infiltration included the empty vectors. Constructs in the pBIN19 vectors were also coagroinfiltrated with constructs expressing different silencing suppressors mixed at equal volumes. The following constructs were used: CIRV-P19 encoding the P19 gene of CIRV (provided by J. Burgyàn, Agricultural Biotechnology Center, Gödöllõ, Hungary), TBSV-P19, encoding the P19 gene of TBSV and PVY-HC-Pro, coding for the HC-Pro protein of PVY (both constructs provided by D. Baulcombe, University of Cambridge, Cambridge, United Kingdom).

In the case of TMV-based vectors, all clones were introduced into *A. tumefaciens* strain GV3101 and prepared as described earlier under carbenicillin and Rif selection (50 μg/mL each). Leaves were agroinfiltrated with a tripartite bacterial mixture (1 : 1 : 1), each at OD_{600} = 0.2, consisting of (i) either the TMV 5′-module pICH17338 (no signal peptide), or –17610

Table 1 List of primers used in this work

Primer name	Primer sequence (5′-3′)	Destination vector	Restriction sites
pBIN-L1-5851F	TT**GGATCC**GAGCTCATGGCAGTGTGG	pBIN19	*Bam*HI
pBIN-L1-7379R	GCAAGCTT**CCCGGG**ATCTAATTTTTCCGTTTTC	pBIN19	*Sma*I
pBIN-L1ΔC22-7326R	TAC**GGATCCGCGCGC**CTACGTCTGTTGCAAACC	pBIN19	*Bam*HI, *Bss*HII
pEAQ-L1-5851F	ATT**TCGCGACCCGGG**ATGGCAGTGTGGCAATCG	pEAQ-HT	*Nru*I, *Sma*I
pEAQ-L1-7379R	TAC**CCCGGGCTCGAG**CTAATTTTTCCGTTTTC	pEAQ-HT	*Sma*I, *Xho*I
pEAQ-L1ΔC22-7326R	TAC**CCCGGGCTCGAG**CTACGTCTGTTGCAAACC	pEAQ-HT	*Sma*I, *Xho*I
TMV-L1-5851F	CTAACA**GAGCTCCATGG**CAGTGTGGCAATCGG	TMV	*Sac*I, *Nco*I
TMV-L1-7379R	CA**CTCGAGAAGCTT**GCTAATTTTTCCGTTTTC	TMV	*Xho*I, *Hind*III
TMV-L1ΔC22-7326R	TAC**GGATCCGAGCTC**CTACGTCTGTTGCAAACC	TMV	*Bam*HI, *Sac*I
pGEX2T-L1-5851F	TT**GGATCC**GAGCTCATGGCAGTGTGGCAATCG	pGEX2T	*Bam*HI
pGEX2T-L1-7379R	GCAAGCTT**CCCGGG**ATCTAATTTTTCCGTTTTC	pGEX2T	*Sma*I

TMV, *Tobacco mosaic virus.* Restriction sites are shown in bold.

(containing the apple polygalacturonase signal peptide, GenBank accession no. P48798, ApoA) or −17620 (carrying the calreticulin signal peptide, ApoC) (Borisjuk *et al.*, 1998), together with (ii) either the 3′-module creating pICH11599-L1, pICH11599-L1ΔC22 or the empty vector pICH11599 (negative control) and (iii) the integrase construct pICH14011.

For GFP expression, a pBIN-based GFP clone (provided by D. Baulcombe), pEAQ-HT-GFP (Sainsbury *et al.*, 2009) and pICH7410 (3′ module of TMV vector provided by Icon Genetics) were used as control constructs according to the aforesaid procedures.

In all cases, plants were maintained in a growth chamber at 23 °C (16 : 8 h light : dark), and leaf tissue was harvested 6 days after agroinfiltration.

Western blot analysis

Agroinfiltrated leaf samples homogenized in nine volumes of Laemmli sample buffer were separated on 7.5% Mini-PROTEAN® TGX™ gel (Bio-Rad, Richmond, CA) in Tris-glycine SDS-PAGE buffer and either stained with Coomassie brilliant blue or electroblotted to polyvinylidene difluoride membranes (Millipore, Billerica, MA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% ethanol). Membranes were incubated overnight at 4 °C in blocking solution (5% nonfat dry milk in PBS with 0.05% Tween-20) and incubated for 1 h at room temperature with primary antibody. Following three washings with PBS-0.05% Tween-20, the membranes were incubated for 1 h at room temperature with the anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich), depending on the primary antibody, both at a 1 : 10 000 dilution. The reaction was detected by supersignal west pico chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL). Purified pseudovirions of HPV-5 (provided by D. Lembo, University of Torino, Torino, Italy) were used as a standard for quantification of L1 and L1ΔC22.

RNA analysis

Total RNAs were extracted with TRIzol® (Invitrogen, Carlsbad, CA) from agroinfiltrated plant tissue, separated in 1% agarose gel (10 μg/lane) after glyoxal denaturation (McMaster and Carmichael, 1977) and transferred to nylon membrane (Roche Applied Science, Mannheim, Germany). Membranes were hybridized with a digoxygenin-labelled L1 probe (50 ng/mL) obtained by T7 RNA polymerase transcription of the *Spe*I-linearised pGEM-L1 clone and subjected to chemiluminescent detection.

Electron microscopy of crude plant sap

Proteins from agroinfiltrated leaves with pEAQ-L1 and pEAQ-L1ΔC22 were extracted using PB to perform ISEM assay (Milne and Luisoni, 1977). Samples were incubated with the MD2H11 MAb (provided by M. Müller, German Cancer Research Center, Heidelberg, Germany) on carbon-coated grids. The grids were negatively stained with 0.5% uranyl acetate and observed using a CM 10 electron microscope (Philips, Eindhoven, The Netherlands).

Subcellular localization of VLPs

Fragments of leaves agroinfiltrated with L1 and L1ΔC22 using pEAQ-HT and TMV-based vector (either with or without signal peptides) were fixed in PB containing 2.5% glutaraldehyde and washed in PB following a postfixation with 1% osmium tetroxide. Fixed samples were embedded in Epon resin, and ultrathin layers were placed on a grid. After positive staining with 5% uranyl acetate and 0.2% lead citrate, the grids were observed by electron microscope.

Purification of VLPs and capsomers

Virus-like particles and capsomers were purified from leaves agroinfiltrated with pEAQ-L1 and pEAQ-L1ΔC22 and collected 6 days postagroinfiltration essentially following three different protocols.

The protocol developed by Varsani *et al.* (2003b) for HPV-16 VLPs purification was carried out with slight modifications. The harvested leaves were ground with liquid N_2, homogenized in PBS/0.5 M NaCl (pH 7.4) and incubated for 2 h at 4 °C. The homogenate was centrifuged for 20 min at 8000 ***g*** (Sorvall SS-34 rotor; Du Pont, Bristol CT). The supernatant was overlaid onto a 40% sucrose cushion and centrifuged at 100 000 ***g*** (Beckman SW28 rotor; Beckman, Brea, CA) for 3 h. The pellet was resuspended in CsCl buffer (PBS with 0.4 g/mL CsCl), and the solution was centrifuged at 100 000 ***g*** at 10 °C for 24 h (Beckman SW41 Ti rotor). Fractions were collected and dialyzed against PBS at 4 °C overnight.

We applied also a protocol for begomovirus purification, essentially according to Luisoni *et al.* (1995). After grinding with liquid N_2, leaves were homogenized in five volumes of extraction buffer (0.5 M PB, pH 6.0 containing antioxidants, 2.5 mM EDTA, 1% Triton X-100 and 0.1% Driselase) and incubated overnight at 4 °C. The homogenate was emulsified with 15% chloroform and centrifuged for 15 min at 8000 ***g*** (Sorvall SS-34 rotor). The aqueous phase was collected and centrifuged for 2 h at 205 000 ***g*** (Beckman 55.2 Ti rotor). The resulting pellet was resuspended in 0.5 M PB (pH 7.0) containing 2.5 mM EDTA and centrifuged for 15 min at 8000 ***g***. The supernatant was then loaded onto 20%–50% Cs_2SO_4 density gradient in 0.5 M PB (pH 7.0) with 2.5 mM EDTA, and centrifuged for 5 h at 160 000 ***g*** (Beckman SW41 Ti rotor). Visible bands were collected, diluted in 0.1 M PB (pH 7.0) and ultracentrifuged for 40 min at 390 000 ***g*** (Beckman TL100 rotor). Final pellets were resuspended in 0.1 M PB (pH 7.0).

Finally, we adapted the protocol for HPV pseudovirion purification (Buck *et al.*, 2005) to the plant tissue, as follows. The leaves were ground up with liquid N_2, homogenized in PBS/0.8 M NaCl and incubated for 1 h at 4 °C. The homogenate was filtered and centrifuged for 20 min at 8000 ***g*** (Sorvall SS-34 rotor). The supernatant was ultracentrifuged for 2 h at 205 000 ***g*** (Beckman 55.2 Ti rotor). The resulting pellet was resuspended in PBS/0.8 M NaCl buffer and loaded on top of a density gradient of 27% to 33% to 39% Optiprep™ (Sigma-Aldrich) at room temperature for 4 h. The material was centrifuged at 160 000 ***g*** for 4 h at 16 °C (Beckman SW41 Ti rotor) and collected by puncturing the bottom of the tubes.

Electron microscopy of purified protein products

The above described final fractions and pellets were adsorbed onto carbon-coated grids for approximately 1–3 min. Excess fluid was removed with filter paper, and grids were negatively stained with 0.5% uranyl acetate. The grids were then observed and photographed by electron microscope.

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References

Aires, K.A., Cianciarullo, A.M., Carneiro, S.M., Villa, L.L., Boccardo, E., Pérez-Martinez, G., Perez-Arellano, I., Oliveira, M.L. and Ho, P.L. (2006) Production of human papillomavirus type 16 L1 virus-like particles by recombinant *Lactobacillus casei* cells. *Appl. Environ. Microbiol.* **72**, 745–752.

Asgari, M.M., Kiviat, N.B., Critchlow, C.W., Stern, J.E., Argenyi, Z.B., Raugi, G.J., Berg, D., Odland, P.B., Hawes, S.E. and de Villiers, E.M. (2008) Detection of human papillomavirus DNA in cutaneous squamous cell carcinoma among immunocompetent individuals. *J. Invest. Dermatol.* **128**, 1409–1417.

Ateenyi-Agaba, C., Franceschi, S., Wabwire-Mangen, F., Arslan, A., Othieno, E., Binta-Kahwa, J., van Doorn, L.J., Kleter, B., Quint, W. and Weiderpass, E. (2010) Human papillomavirus infection and squamous cell carcinoma of the conjunctiva. *Br. J. Cancer*, **102**, 262–267.

Avesani, L., Marconi, G., Morandini, F., Albertini, E., Bruschetta, M., Bortesi, L., Pezzotti, M. and Porceddu, A. (2007) Stability of *Potato virus X* expression vectors is related to insert size: implications for replication models and risk assessment. *Transgenic Res.* **16**, 587–597.

Baker, T.S., Newcomb, W.W., Olson, N.H., Cowsert, L.M., Olson, C. and Brown, J.C. (1991) Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys. J.* **60**, 1445–1456.

Bevan, M. (1984) Binary agrobacterium vectors for plant transformation. *Nucleic Acids Res.* **12**, 8711–8721.

Biemelt, S., Sonnewald, U., Galmbacher, P., Willmitzer, L. and Müller, M. (2003) Production of human papillomavirus type 16 virus-like particles in transgenic plants. *J. Virol.* **77**, 9211–9220.

Borisjuk, N., Sitailo, L., Adler, K., Malysheva, L., Tewes, A., Borisjuk, L. and Manteuffel, R. (1998) Calreticulin expression in plant cells: developmental regulation, tissue specificity and intracellular distribution. *Planta*, **206**, 504–514.

Bouwes Bavinck, J.N., Euvrard, S., Naldi, L., Nindl, I., Proby, C.M., Neale, R., Abeni, D., Tessari, G.P., Feltkamp, M.C., Claudy, A., Stockfleth, E. and Harwood, C.A. (2007) Keratotic skin lesions and other risk factors are associated with skin cancer in organ-transplant recipients: a case–control study in The Netherlands, United Kingdom, Germany, France, and Italy. *J. Invest. Dermatol.* **127**, 1647–1656.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W. and Baulcombe, D.C. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* **17**, 6739–6746.

Buck, C.B., Pastrana, D.V., Lowy, D.R. and Schiller, J.T. (2005) Generation of HPV pseudovirions using transfection and their use in neutralization assays. *Methods Mol. Med.* **119**, 445–462.

Chen, X.S., Garcea, R.L., Goldberg, I., Casini, G. and Harrison, S.C. (2000) Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol. Cell*, **5**, 557–567.

Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F. and Jeng, C.R. (2010) Immunogenicity of recombinant GP5 protein of porcine reproductive and respiratory syndrome virus expressed in tobacco plant. *Vet. Immunol. Immunopathol.* **135**, 234–242.

Clark, M.F. and Bar-Joseph, M. (1984) Enzyme immunosorbent assays in plant virology. In *Methods in Virology* (Maramorsch, K. and Koprowski, H., eds), pp. 51–85, New York: Academic Press.

Csorba, T., Bovi, A., Dalmay, T. and Burgyán, J. (2007) The p122 subunit of *Tobacco mosaic virus* replicase is a potent silencing suppressor and compromises both small interfering RNA- and microRNA-mediated pathways. *J. Virol.* **81**, 11768–11780.

Daniell, H., Streatfield, S.J. and Wycoff, K. (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci.* **6**, 219–226.

Davoodi-Semiromi, A., Schreiber, M., Nalapalli, S., Verma, D., Singh, N.D., Banks, R.K., Chakrabarti, D. and Daniell, H. (2010) Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. *Plant Biotechnol. J.* **8**, 223–242.

Doran, P.M. (2006) Foreign protein degradation and instability in plants and plant tissue cultures. *Trends Biotechnol.* **24**, 426–432.

Faye, L., Boulaflous, A., Benchabane, M., Gomord, V. and Michaud, D. (2005) Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. *Vaccine*, **23**, 1770–1778.

Feltkamp, M.C., Broer, R., di Summa, F.M., Struijk, L., van der Meijden, E., Verlaan, B.P., Westendorp, R.G., ter Schegget, J., Spaan, W.J. and Bouwes Bavinck, J.N. (2003) Seroreactivity to epidermodysplasia verruciformis-related human papillomavirus types is associated with nonmelanoma skin cancer. *Cancer Res.* **63**, 2695–2700.

Fernández-San Millán, A., Ortigosa, S.M., Hervás-Stubbs, S., Corral-Martínez, P., Seguí-Simarro, J.M., Gaétan, J., Coursaget, P. and Veramendi, J. (2008) Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnol. J.* **6**, 427–441.

Giorgi, C., Franconi, R. and Rybicki, E.P. (2010) Human papillomavirus vaccines in plants. *Expert Rev. Vaccines*, **9**, 913–924.

Giritch, A., Marillonnet, S., Engler, C., van Eldik, G., Botterman, J., Klimyuk, V. and Gleba, Y. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. *Proc. Natl Acad. Sci. USA*, **103**, 14701–14706.

Gormley, R.H. and Kovarik, C.L. (2009) Dermatologic manifestations of HPV in HIV-infected individuals. *Curr. HIV/AIDS Rep.* **6**, 130–138.

Hagensee, M.E., Yaegashi, N. and Galloway, D.A. (1993) Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J. Virol.* **67**, 315–322.

Handisurya, A., Gambhira, R., Schellenbacher, C., Shafti-Keramat, S., Forslund, O., Favre, M. and Kirnbauer, R. (2009) Serological relationship between cutaneous human papillomavirus types 5, 8 and 92. *J. Gen. Virol.* **90**, 136–143.

Harwood, C.A. and Proby, C.M. (2002) Human papillomaviruses and non-melanoma skin cancer. *Curr. Opin. Infect. Dis.* **15**, 101–114.

Haug, C.J. (2008) Human papillomavirus vaccination—reasons for caution. *N. Engl. J. Med.* **359**, 861–862.

Jablonska, S., Fabjanska, L. and Formas, I. (1966) On the viral etiology of epidermodysplasia verruciformis. *Dermatologica*, **132**, 369–385.

Johansen, L.K. and Carrington, J.C. (2001) Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiol.* **126**, 930–938.

Kapila, J., De Rycke, R., Van Montagu, M. and Angenon, G. (1997) An Agrobacterium- mediated transient gene expression system for intact leaves. *Plant Sci.* **122**, 101–108.

Kapusta, J., Modelska, A., Figlerowicz, M., Pniewski, T., Letellier, M., Lisowa, O., Yusibov, V., Koprowski, H., Plucienniczak, A. and Legocki, A.B. (1999) A plant-derived edible vaccine against hepatitis B virus. *FASEB J.* **13**, 1796–1799.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D.R. and Schiller, J.T. (1992) Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl Acad. Sci. USA*, **89**, 12180–12184.

Kohl, T., Hitzeroth, I.I., Stewart, D., Varsani, A., Govan, V.A., Christensen, N.D., Williamson, A.L. and Rybicki, E.P. (2006) Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. *Clin. Vaccine Immunol.* **13**, 845–853.

Kohl, T.O., Hitzeroth, I.I., Christensen, N.D. and Rybicki, E.P. (2007) Expression of HPV-11 L1 protein in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. *BMC Biotechnol.* **7**, 56.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.P., Dolja, V.V., Calvino, L.F., López-Moya, J.J. and Burgyán, J. (2006)

Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J.* **25**, 2768–2780.

Lenzi, P., Scotti, N., Alagna, F., Tornesello, M.L., Pompa, A., Vitale, A., De Stradis, A., Monti, L., Grillo, S., Buonaguro, F.M., Maliga, P. and Cardi, T. (2008) Translational fusion of chloroplast-expressed human papillomavirus type 16 L1 capsid protein enhances antigen accumulation in transplastomic tobacco. *Transgenic Res.* **17**, 1091–1102.

Lindbo, J.A. (2007a) High-efficiency protein expression in plants from agroinfection-compatible Tobacco mosaic virus expression vectors. *BMC Biotechnol.* **7**, 52.

Lindbo, J.A. (2007b) TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant Physiol.* **145**, 1232–1240.

Luisoni, E., Milne, R.G. and Vecchiati, M. (1995) Purification of tomato yellow leaf curl geminivirus. *New Microbiol.* **18**, 253–260.

Maclean, J., Koekemoer, M., Olivier, A.J., Stewart, D., Hitzeroth, I.I., Rademacher, T., Fischer, R., Williamson, A.L. and Rybicki, E.P. (2007) 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**, 1460–1469.

Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V. and Gleba, Y. (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotechnol.* **23**, 718–723.

Masini, C., Fuchs, P.G., Gabrielli, F., Stark, S., Sera, F., Ploner, M., Melchi, C.F., Primavera, G., Pirchio, G., Picconi, O., Petasecca, P., Cattaruzza, M.S., Pfister, H.J. and Abeni, D. (2003) Evidence for the association of human papillomavirus infection and cutaneous squamous cell carcinoma in immunocompetent individuals. *Arch. Dermatol.* **139**, 890–894.

McMaster, G.K. and Carmichael, G.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl Acad. Sci. USA*, **74**, 4835–4838.

Merle, E., Rose, R.C., LeRoux, L. and Moroianu, J. (1999) Nuclear import of HPV11 L1 capsid protein is mediated by karyopherin alpha 2/beta 1 heterodimers. *J. Cell. Biochem.* **74**, 628–637.

Milne, R.G. and Luisoni, E. (1977) Rapid immune electron microscopy of virus preparations. *Methods Virol.* **6**, 265–281.

Modis, Y., Trus, B.L. and Harrison, S.C. (2002) Atomic model of the papillomavirus capsid. *EMBO J.* **21**, 4754–4762.

Münger, K., Baldwin, A., Edwards, K.M., Hayakawa, H., Nguyen, C.L., Owens, M., Grace, M. and Huh, K. (2004) Mechanisms of human papillomavirus-induced oncogenesis. *J. Virol.* **78**, 11451–11460.

Noris, E., Poli, A., Cojoca, R., Rittà, M., Cavallo, F., Vaglio, S., Matic, S. and Landolfo, S. (2011) A human papillomavirus 8 E7 protein produced in plants is able to trigger the mouse immune system and delay the development of skin lesions. *Arch. Virol.* **156**, 587–595.

Paintsil, J., Müller, M., Picken, M., Gissmann, L. and Zhou, J. (1998) Calcium is required in reassembly of bovine papillomavirus in vitro. *J. Gen. Virol.* **79**, 1133–1141.

Parkin, D.M. (2006) The global health burden of infection-associated cancers in the year 2002. *Int. J. Cancer*, **118**, 3030–3044.

Pfister, H. (2003a) Chapter 8: human papillomavirus and skin cancer. *J. Natl. Cancer Inst. Monogr.* **31**, 52–56.

Pfister, H., Nurnberger, F., Gissmann, L. and zur Hausen, H. (1981) Characterization of a human papillomavirus from epidermodysplasia verruciformis lesions of a patient from Upper-Volta. *Int. J. Cancer*, **27**, 645–650.

Pfister, H., Fuchs, P.G., Majewski, S., Jablonska, S., Pniewska, I. and Malejczyk, M. (2003b) High prevalence of epidermodysplasia verruciformis-associated human papillomavirus DNA in actinic keratoses of the immunocompetent population. *Arch. Dermatol. Res.* **295**, 273–279.

Rose, R.C., Bonnez, W., Reichman, R.C. and Garcea, R.L. (1993) Expression of human papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of viruslike particles. *J. Virol.* **67**, 1936–1944.

Rybicky, E.D. (2010) Plant-made vaccines for humans and animals. *Expert Rev. Vaccines*, **8**, 620–637.

Sainsbury, F. and Lomonossoff, G.P. (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol.* **148**, 1212–1218.

Sainsbury, F., Thuenemann, E.C. and Lomonossoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol. J.* **7**, 682–693.

Tacket, C.O., Mason, H.S., Losonsky, G., Clements, J.D., Levine, M.M. and Arntzen, C.J. (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat. Med.* **4**, 607–609.

Varsani, A., Williamson, A.L., Rose, R.C., Jaffer, M. and Rybicki, E.P. (2003a) Expression of human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. *Xanthi. Arch. Virol.* **148**, 1771–1786.

Varsani, A., Williamson, A.L., de Villiers, D., Becker, I., Christensen, N.D. and Rybicki, E.P. (2003b) Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. *J. Virol.* **77**, 8386–8393.

Varsani, A., Williamson, A.L., Stewart, D. and Rybicki, E.P. (2006) Transient expression of human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. *Virus Res.* **120**, 91–96.

Voinnet, O., Pinto, Y.M. and Baulcombe, D.C. (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl Acad. Sci. USA*, **96**, 14147–14152.

Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D. (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the P19 protein of *tomato bushy stunt virus*. *Plant J.* **33**, 949–956.

Warzecha, H., Mason, H.S., Lane, C., Tryggvesson, A., Rybicki, E., Williamson, A.L., Clements, J.D. and Rose, R.C. (2003) Oral immunogenicity of human papillomavirus-like particles expressed in potato. *J. Virol.* **77**, 8702–8711.

Weissenborn, S.J., Nindl, I., Purdie, K., Harwood, C., Proby, C., Breuer, J., Majewski, S., Pfister, H. and Wieland, U. (2005) Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J. Invest. Dermatol.* **125**, 93–97.

Yusibov, V. and Rabindran, S. (2008) Recent progress in the development of plant-derived vaccines. *Expert Rev. Vaccines*, **7**, 1173–1183.

Zhang, W., Carmichael, J., Ferguson, J., Inglis, S., Ashrafian, H. and Stanley, M. (1998) Expression of human papillomavirus type 16 L1 protein in *Escherichia coli*: denaturation, renaturation and self-assembly of virus-like particles in vitro. *Virology*, **243**, 423–431.

Zhou, J., Doorbar, J., Sun, X.Y., Crawford, L.V., McLean, C.S. and Frazer, I.H. (1991) Identification of the nuclear localization signal of human papillomavirus type 16 L1 protein. *Virology*, **185**, 625–632.

Zhou, J., Sun, X.Y. and Frazer, I.H. (1993) Glycosylation of human papillomavirus type 16 L1 protein. *Virology*, **194**, 210–218.