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Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic

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Summary

Cervical cancer is the second most prevalent cancer in women worldwide. It is linked to infection with human papillomavirus (HPV). As the virus cannot be propagated in culture, vaccines based on virus-like particles have been developed and recently marketed. However, their high costs constitute an important drawback for widespread use in developing countries, where the incidence of cervical cancer is highest. In a search for alternative production systems, the major structural protein of the HPV-16 capsid, L1, was expressed in tobacco chloroplasts. A very high yield of production was achieved in mature plants (~3 mg L1/g fresh weight; equivalent to 24% of total soluble protein). This is the highest expression level of HPV L1 protein reported in plants. A single mature plant synthesized ~240 mg of L1. The chloroplast-derived L1 protein displayed conformation-specific epitopes and assembled into virus-like particles, visible by transmission electron microscopy. Furthermore, leaf protein extracts from L1 transgenic plants were highly immunogenic in mice after intraperitoneal injection, and neutralizing antibodies were detected. Taken together, these results predict a promising future for the development of a plant-based vaccine against HPV.

Introduction

Cervical cancer is the second most prevalent cancer in women worldwide and the most common cancer in developing countries. Every year, approximately 500 000 women develop cervical cancer, 80% of whom live in poor countries where screening programmes are inadequate or absent (Franceschi, 2005). Every year, this disease causes about 200 000 deaths. In 1995, the World Health Organization declared some types of human papillomavirus (HPV) to be carcinogenic in humans and to be responsible for cervical cancer (IARC, 1995). Nearly 120 HPV strains have been identified so far, and two-thirds of cervical cancer cases are associated with infection by either HPV-16 (51%) or HPV-18 (16%). Taken together, these data led the scientific community to increase their interest in developing an HPV vaccine. This virus cannot be propagated in culture, hindering the generation of attenuated or dead HPV particles for vaccine purposes.

The major structural protein of the HPV capsid, L1, is the most highly conserved of all papillomavirus proteins. When expressed in a variety of heterologous systems, L1 protein self-assembles into non-infectious virus-like particles (VLPs) that closely resemble the native spherical virion of ~50–55 nm in diameter. A single VLP is composed of 72 pentameric capsomers. Each capsomer contains five monomers of the 55-kDa L1 protein (Sapp *et al.*, 1998). Papillomavirus VLPs have been reported previously to efficiently induce both

humoral and cellular responses. In addition, it has been demonstrated that immunization with HPV VLPs provides protection against challenge with the infectious virus in animal models (Breitburd *et al*., 1995; Suzich *et al*., 1995). Therefore, VLPs are the prime candidates for the development of prophylactic vaccines against HPV.

Recently, two subunit vaccines against HPV have been marketed as prophylactic cervical cancer vaccines. In both cases, the selected antigen is the L1 protein. One of them, the tetravalent vaccine, covers HPV types 16, 18, 6 and 11 and the expression system is yeast. The other, a bivalent vaccine, includes L1 from HPV-16 and HPV-18 produced in insect cells. However, despite the high probability for these two vaccines to promote immunity against high-risk HPV infection, their high cost prohibits their widespread use in developing countries (Sanders and Taira, 2003; Crum *et al*., 2006; Kane *et al*., 2006; Schiller and Nardelli-Haefliger, 2006), where the highest cervical cancer mortality rates have been reported, especially in sub-Saharan Africa (Pisani *et al*., 1999). Therefore, a primary challenge is to develop cost-effective HPV vaccines, available for the developing world.

Transgenic plants are potentially one of the most economical systems for the production of recombinant vaccine proteins. Studies in this field have indicated that transgenic plants can produce recombinant proteins 10–100 times cheaper than cell culture systems (Kusnadi *et al*., 1997; Mison and Curling, 2000). Cost reductions would be even higher if plant material could be used directly as an oral vaccine (e.g. capsules of dried powder), thus avoiding purification steps. It has also been demonstrated that plant-derived antigens can induce immune responses against important human or animal pathogens (Streatfield and Howard, 2003; Floss *et al*., 2007). However, one of the main drawbacks of antigen production in plants is the low level of recombinant protein expression when the transgene is integrated into the nuclear genome. Average antigen expression levels are in the range of 0.01%–0.4% of total soluble protein (TSP) (Daniell *et al*., 2001b).

HPV-16 L1 has been expressed constitutively in tobacco and potato by nuclear transformation (Biemelt *et al*., 2003; Varsani *et al*., 2003; Liu *et al*., 2005) and transiently in *Nicotiana benthamiana* using a viral vector (Varsani *et al*., 2006). HPV-11 L1 has also been reported to be produced in potato (Warzecha *et al*., 2003). All of these publications reported very low levels of antigen expression (< 1% of TSP). In contrast, transgenic tobacco plants expressing a human codon-optimized *L1* gene linked to a chloroplast-targeting signal expressed L1 protein at levels up to 11% of TSP (Maclean *et al*., 2007). Furthermore, by agroinfiltration of the same genetic construct, L1 protein expression increased to up to 17% of TSP (Maclean *et al*., 2007). Concentrated protein extracts containing this antigen were reported to be immunogenic in mice, and elicited neutralizing antibodies. Agroinfiltration, however, is a complex method to scale up, and is therefore not the most useful choice for mass production.

An alternative expression system to nuclear transformation is plastid genetic engineering. Transformation of chloroplasts allows for very high levels of foreign protein because of the high copy number of the transgene per cell (for a review, see Daniell *et al*., 2002; Bock and Khan, 2004; Maliga, 2004). Additional advantages of this recombinant protein expression system include transgene containment via maternal inheritance of plastids, lack of gene silencing, multigene expression in a single transformation step and expression of foreign proteins lacking methionine as the N-terminal amino acid (Fernández-San Millán *et al*., 2007). This system has been demonstrated to be effective for the expression of vaccine antigens against several human and animal diseases (Daniell, 2006). Average expression levels are in the range of 4%–20% of TSP, with maximum values of 31% in the case of canine parvovirus antigen expression (Molina *et al*., 2004). Mice and rabbits injected with chloroplast-derived vaccines developed specific immune responses and neutralizing antibodies against the pathogens (Molina *et al*., 2005; Chebolu and Daniell, 2007). In the case of anthrax and tetanus, mice immunized with tobacco-derived vaccines were protected against lethal toxin challenge (Koya *et al*., 2005; Tregoning *et al*., 2005). Together, these data indicate that chloroplast-transformed plants can be an effective source of purified antigens for prophylactic vaccines.

In this study, the expression of the HPV-16 L1 antigen in tobacco chloroplasts was analysed. The ability of this molecule to self-assemble into VLPs when expressed in the chloroplast was also investigated. Finally, the immunogenicity of plant-derived L1 was verified after intraperitoneal administration in mice.

Results

Vector construction

The pAF vector was engineered for tobacco plastid transformation. This vector (5913 bp in length) includes the following sequences (Figure 1a): the *trnI* and *trnA* border sequences, homologous to the inverted repeat regions of the tobacco plastid genome; the aminoglycoside 3′-adenylyltransferase gene (*aadA*) from *Escherichia coli*, conferring resistance to both spectinomycin and streptomycin, driven by the constitutive

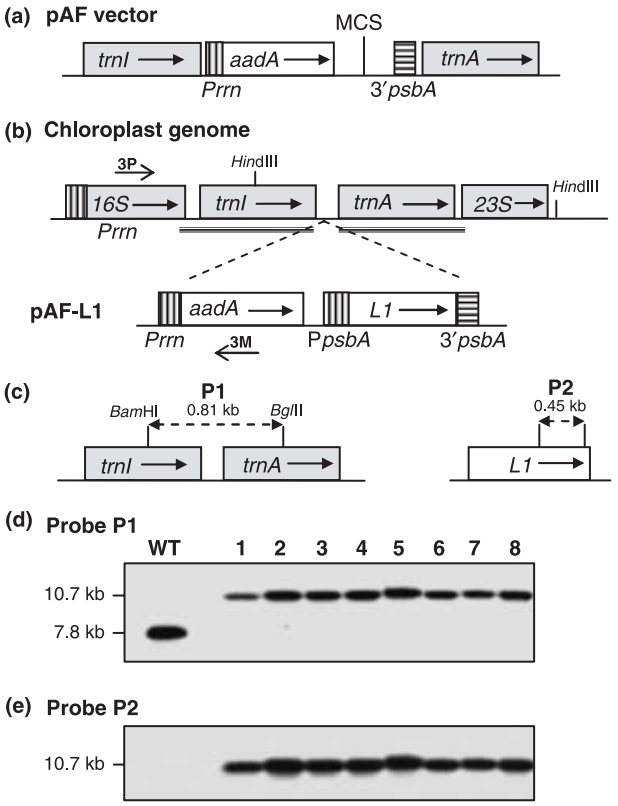

Figure 1 Schematic representation of plastid genome transformation with the human papillomavirus *L1* gene (constructs not drawn to scale). (a) Main components of the pAF vector for transformation of tobacco chloroplasts. (b) Map of the wild-type and L1-transformed genomes. Regions for homologous recombination are underlined in the native chloroplast genome. The *L1* sequence is driven by the *psbA* promoter. The annealing sites of the primers (3P and 3M) used to test the integration of the cassette into the chloroplast genome are shown. (c) The 0.8-kb fragment (P1) of the targeting region for homologous recombination and the 0.45-kb *L1* sequence (P2) were used as probes for Southern blot analysis. (d, e) Southern blot analysis of eight independent transgenic lines. The blots were probed with P1 (d) and P2 (e). *aadA*, aminoglycoside 3′-adenylyltransferase; MCS, multicloning site; *Prrn*, 16S rRNA promoter; 3′*psbA*, terminator region of the *psbA* gene; *trnI*, *trnA*, original sequences of the chloroplast genome; WT, wild-type.

promoter of the rRNA operon (*Prrn*), and the consensus GGAGG ribosome binding site; a multiple cloning site between the end of the *aadA* coding sequence and the *psbA* terminator with the following unique sites: *Eco*RV, *Kpn*I, *Sal*I, *Hin*cII, *Not*I, *Xba*I and *Spe*I.

The *L1* gene of HPV-16 was polymerase chain reaction (PCR) amplified, fused to the promoter and 5′-untranslated region (5′-UTR) of the *psbA* gene and finally introduced into the multiple cloning site of pAF, rendering the final vector pAF-L1 (Figure 1b). The *L1* gene encodes an aspartate residue at amino acid position 202 instead of the histidine residue of the prototype. This change allows the efficient assembly of HPV-16 L1 molecules (Kirnbauer *et al*., 1993). The regulatory sequences of the *psbA* gene were chosen because of the high levels of heterologous gene expression they confer in transplastomic plants (Fernández-San Millán *et al*., 2003; Molina *et al*., 2004).

Determination of chloroplast integration and homoplasmy

Shoots developed after the second round of selection on spectinomycin were analysed for transgene integration by PCR. Primer 3P binds on the chloroplast genome upstream of the *trnI* gene outside the vector integration site, and primer 3M binds to the *aadA* gene (Figure 1b). The resulting PCR product is specific for transformed genomes and cannot be obtained from wild-type or nuclear transgenic plants. An analysis of 22 independent shoots showed that all were PCR positive (data not shown). Southern blot analysis was performed to verify site-specific integration and to confirm homoplasmy. Total plant DNA was digested with *Hin*dIII. The flanking region probe (P1, Figure 1c) identified a 7.8-kb fragment in the wild-type plant, and a 10.7-kb fragment in the chloroplast transgenic plants (Figure 1d). The absence of 7.8-kb bands in the transformed lines indicated homoplasmy. To confirm that the 10.7-kb fragment contained the *L1* sequence, the same membrane was re-probed with the P2 probe (Figure 1c). Hybridization was observed in the eight transgenic lines, but was absent in the wild-type control plant (Figure 1e). Southern blot analysis of the T_1 generation of three transgenic lines confirmed that homoplasmy was maintained (data not shown).

Expression and quantification of the L1 protein

The recombinant protein in transformed chloroplasts was detected by western blot with the Cam Vir-1 monoclonal antibody (mAb), which is specific for HPV-16 L1 protein. A 55-kDa band of the expected size for the L1 protein was detected in the three independent transgenic lines analysed (Figure 2a). This band showed an electrophoretic mobility similar to that of the L1 protein purified from insect cells, which was used as a control. Higher molecular weight signals indicated the presence of multimeric structures, despite the denaturing conditions during sample preparation and electrophoresis. The foreign protein was present in both the soluble and insoluble fractions, suggesting the presence of insoluble aggregates or the association of L1 protein with the thylakoid membranes of grana. No signal was present in protein samples extracted from the wild-type tobacco control plant. All of the three transgenic lines analysed showed

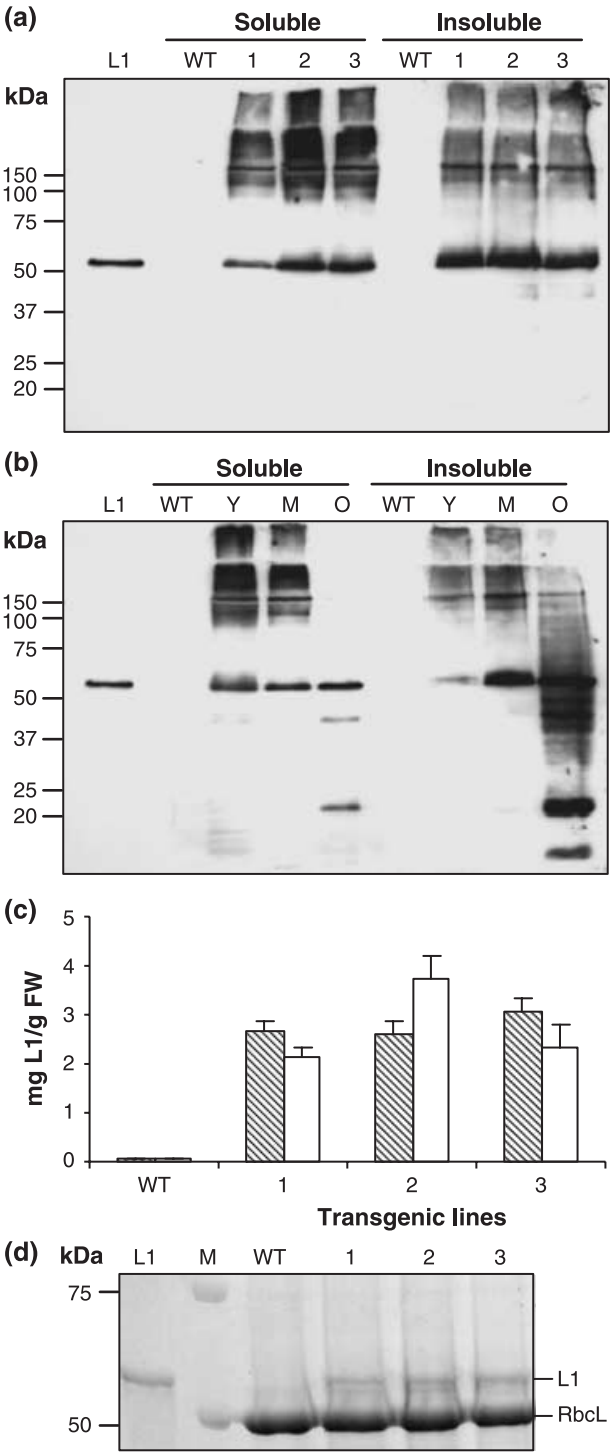

Figure 2 (a, b) Immunoblot analysis of L1 protein expression in transgenic tobacco chloroplasts. Soluble and insoluble proteins extracted from 1.5 mg leaf fresh weight/well were loaded. (a) Three independent transgenic lines and a wild-type (WT) plant used as a negative control were analysed. (b) Young (Y), mature (M) and old (O) leaves from line 1 and WT plants were analysed. Blots were detected using Cam Vir-1 anti-L1 as primary antibody. Purified 6 × His-L1 expressed in insect cells (L1) was used as a positive control (150 ng). (c) Recombinant protein quantification in transgenic tobacco chloroplasts. L1 accumulation in three independent transgenic lines and wild-type plants was analysed by enzyme-linked immunosorbent assay (ELISA), using Cam Vir-1 (striped) or H16.V5 (white) as primary antibody. The data are presented as the means and standard errors (n = 8, four plants/line) of samples from young and mature leaves. (d) Coomassie blue-stained sodium dodecylsulphate-polyacrylamide gel electrophoresis of plant samples equivalent to those shown in (b). Total protein extracted from 6 mg leaf fresh weight/well was loaded. L1, 3 μg of insect cell-derived and purified L1 protein; M, molecular weight marker; WT, wild-type; RbcL, Ribulose bisphosphate carboxylase large subunit.

similar expression levels relative to leaf fresh weight (FW). The effect of leaf age on L1 protein expression was analysed in plants grown in the glasshouse for 60 days. Monomers of 55 kDa and higher molecular weight structures were observed in young and mature leaves (Figure 2b). Two bands of approximately 39 and 20 kDa, probably L1 degradation products, were clearly detected in the soluble fraction of old leaves (Figure 2b). Accordingly, aggregates of high molecular weight were not observed. This pattern was more pronounced in the insoluble fraction, with multiple bands of degradation.

The amount of L1 protein was quantified by enzyme-linked immunosorbent assay (ELISA) using Cam Vir-1 or H16.V5 mAbs. Cam Vir-1 recognizes linear and conformational L1 epitopes, whereas H16.V5 recognizes a conformational epitope only present in non-denatured HPV-16 VLPs (Christensen *et al*., 1996). Both antibodies recognized the chloroplast-derived L1 protein in an equivalent manner. This shows that chloroplast-derived L1 protein displays mostly conformation-specific epitopes. The three independent transgenic lines expressed the recombinant protein at high levels (Figure 2c), ranging from 2.1 to 3.7 mg L1/g FW. This is equivalent to 20%–26% of TSP. The high levels of L1 protein were confirmed when total protein extracts from chloroplast transgenic plants were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. A 55-kDa band corresponding to the L1 expected molecular size was observed in transgenic lines, but was absent in the wild-type plant (Figure 2d). The relatively low intensity of the detected band was probably a result of the presence of multimeric structures of higher molecular weight, only detected by western blot (Figure 2a). In order to quantify the yield of L1 protein per plant and the relative contributions of different leaves, recombinant protein levels were calculated by taking into account the average number and weight of leaves. Old (bleached and senescing) leaves were discarded from this calculation because of the high L1 protein degradation process observed by western blotting (Figure 2b). A total of 242.2 mg of L1 antigen was produced by a single plant (Table 1).

Table 1 Yield per plant of the human papillomavirus L1 protein expressed in tobacco chloroplasts (n = 4 plants)

Leaf age	Average number of leaves per plant	Fresh weight (FW) per leaf (g)	Average amount of L1 (mg/g FW)	Average amount of L1 (total mg)
Young	3.1	5.8	2.5	45.1
Mature	6.6	9.9	3.0	197.1
Total recombinant protein (mg) per plant				242.2

Young: small, dark-green leaves from the upper part of the plant; mature: large, well-developed leaves from the middle part of the plant.

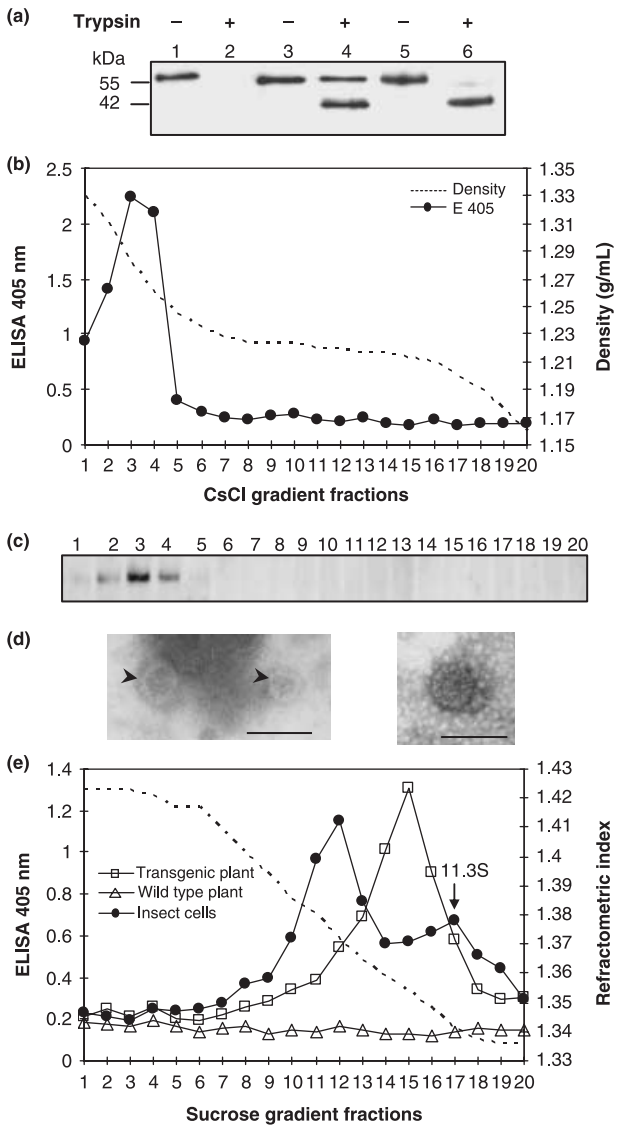

Figure 3 Purification of virus-like particles (VLPs) from transgenic tobacco chloroplasts. (a) Trypsin digestion of purified L1 proteins. western blot of insect cell-derived denatured and monomeric L1 (1, 2), insect cell-derived VLPs (3, 4) and chloroplast-derived VLPs (5, 6). Protein samples were untreated (1, 3, 5) or trypsin digested (2, 4, 6). (b) Detection of L1 by enzyme-linked immunosorbent assay (ELISA) in fractions of a CsCl gradient. The conformational H16.V5 anti-L1 monoclonal antibody (mAb) was used as primary antibody. The broken line indicates the density of the different fractions. (c) Western blot of the different fractions. The band corresponds to the L1 monomeric form. (d) Electron microscopy of negatively stained chloroplast-derived VLPs from fraction 3 of the CsCl gradient. Bar, 100 nm. (e) Sucrose sedimentation analysis of L1 derived from transgenic tobacco chloroplasts, detected by ELISA with the conformational H16.V5 mAb. Soluble proteins from tobacco leaves and insect cells were fractionated by sucrose gradient centrifugation (fraction 1 corresponds to the tube bottom). Wild-type plant was used as a negative control. The broken line indicates the refractive index of the different fractions. The arrow indicates the fraction in which the standard (corresponding to capsomer size) migrated.

L1 protein synthesized in the chloroplast self-assembles into VLPs

Data from western blots and ELISA with the conformational H16.V5 mAb (Figure 2) demonstrated the successful expression of L1, and suggested that the recombinant protein was present as higher order molecular structures in tobacco chloroplasts. The sensitivity of viral proteins to protease digestion is an informative method to assess their native conformation. Purified L1 capsomers (viral pentamers of L1) and VLPs treated with trypsin yielded a 42-kDa product resulting from cleavage at R412 (87 amino acid residues from the carboxy terminus) (Li *et al.*, 1997). Insect cell- and chloroplast-derived L1 proteins treated with trypsin were reduced to the expected 42-kDa species (Figure 3a). By contrast, the monomeric and denatured L1 protein produced in insect cells was totally digested with trypsin. This result confirms that capsomers are correctly assembled.

To determine whether L1 assembles into VLPs, plant extracts were sedimented through a sucrose cushion, followed by a CsCl equilibrium density gradient. L1 protein was detected by ELISA in the different fractions using the conformational H16.V5 mAb. Most of the L1 protein concentrated in fractions 1–4 at a density of 1.27–1.32 g/mL (Figure 3b). A western blot also detected the 55-kDa L1 protein in fractions 1–4, as expected (Figure 3c). Fraction 3 (density of 1.28 g/mL) of the CsCl gradient was analysed by electron microscopy to determine whether VLPs were actually assembled within

tobacco chloroplasts. Spherical particles of approximately 55–65 nm in diameter (Figure 3d) were observed in samples from transgenic plants. The assembly of particles of different sizes has been observed previously when HPV-16 L1 was expressed in the cytoplasm of tobacco cells (Biemelt *et al.*, 2003; Maclean *et al.*, 2007). The morphology of the chloroplast-derived VLPs was qualitatively identical to that of VLPs produced in insect cells by recombinant baculovirus infection (data not shown).

To determine the proportion of L1 protein that is in an assembled form, crude extracts of tobacco leaves and insect cells (as a control) were subjected to sucrose sedimentation analysis. After sucrose gradient centrifugation, insect cell-derived VLPs were detected with the conformational H16.V5 mAb in fractions 10–14 (Figure 3e). Chloroplast-derived L1 was mainly present in fractions 12–17, displaying a maximum peak in fraction 15 (a similar pattern was observed when sucrose gradient fractions were detected with Cam Vir-1 mAb; data not shown). VLPs of different sizes were detected in these fractions by electron microscopy (data not shown). The shift to lower sedimentation coefficients indicates that approximately 30% of chloroplast-synthesized VLPs are similar in size to true virion particles (55 nm in diameter), whereas 70% are particles of smaller size. The treatment of insect cell-derived VLPs with β-mercaptoethanol led to disassembly into capsomers with a lower sedimentation coefficient, which were concentrated in fractions 17 and 18 (data not shown). Catalase, used as capsomer sedimentation marker, also migrated in fraction 17 (Figure 3e). Only a small proportion of capsomers was present in VLPs purified either from insect cells or plant extracts (fraction 17). This demonstrates that chloroplast-derived L1 protein mostly self-assembles into structures higher than capsomers.

A further analysis to confirm the assembly of VLPs was performed. The ELISA reactivity of chloroplast-derived L1 protein and VLPs purified from insect cells was investigated using H31.D24 and H16.V5 mAbs. H31.D24 recognizes a conserved linear epitope present in both denatured and non-denatured L1 proteins of high-risk HPV types, and H16.V5 recognizes a type-specific conformational epitope. L1 protein was not detected in extracts from the wild-type tobacco control plant (Figure 4). A similar reactivity of insect cell- and plant-derived VLPs was observed with both mAbs, thus confirming again that chloroplast-derived L1 was assembled into capsomers or VLPs. The optical density at 492 nm ($OD_{492\ nm}$) in the first dilution (20-fold) from plant extracts was lower than that in the 40- and 80-fold dilutions. This could be the result of the higher concentration of inhibitory compounds at the lower dilution.

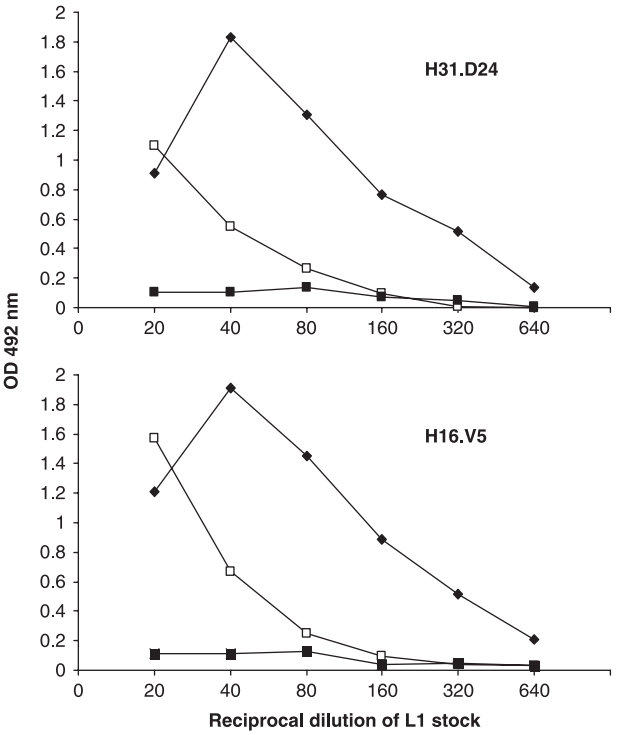

Figure 4 Comparative analysis of insect cell-derived and chloroplast-derived virus-like particle (VLP) binding by enzyme-linked immunosorbent assay (ELISA). L1 protein was detected using H31.D24 and H16.V5 monoclonal antibodies (mAbs). H31.D24 binds a linear epitope present in denatured and native VLPs, and H16.V5 binds a conformational epitope. Crude extracts (100 mg of leaf tissue in 50 μL of phosphate-buffered saline) from transgenic and wild-type plants were analysed. VLPs purified from insect cells were used as a control. ■, wild-type extract; ◆, chloroplast-derived extract; □, insect cell-produced VLPs.

In situ electron microscopy and immunogold labelling

Palisade mesophyll cells from leaves of both transgenic and wild-type tobacco plants presented a highly ordered disposition and an elongated, cylinder-like morphology. In cross-sectional views (Figure 5a), a large vacuole could be seen occupying most of the cell volume and displacing the abundant chloroplasts to a peripheral location. Grana in chloroplasts looked apparently normal in both transgenic and wild-type plants. However, several electron-dense, rounded particles could be observed dispersed in the stroma of transgenic plants (Figure 5b), often in close proximity to the grana. At higher magnification, these particles frequently appeared as ~30 nm-diameter, electron-dense rings enclosing a lighter central region (inset in Figure 5b). Such particles were never observed in chloroplasts of wild-type tobacco plants (Figure 5c).

Immunogold labelling of transgenic mesophyll cells with anti-L1 Cam Vir-1 mAb presented a high signal-to-noise ratio, with gold particles decorating chloroplasts almost exclusively

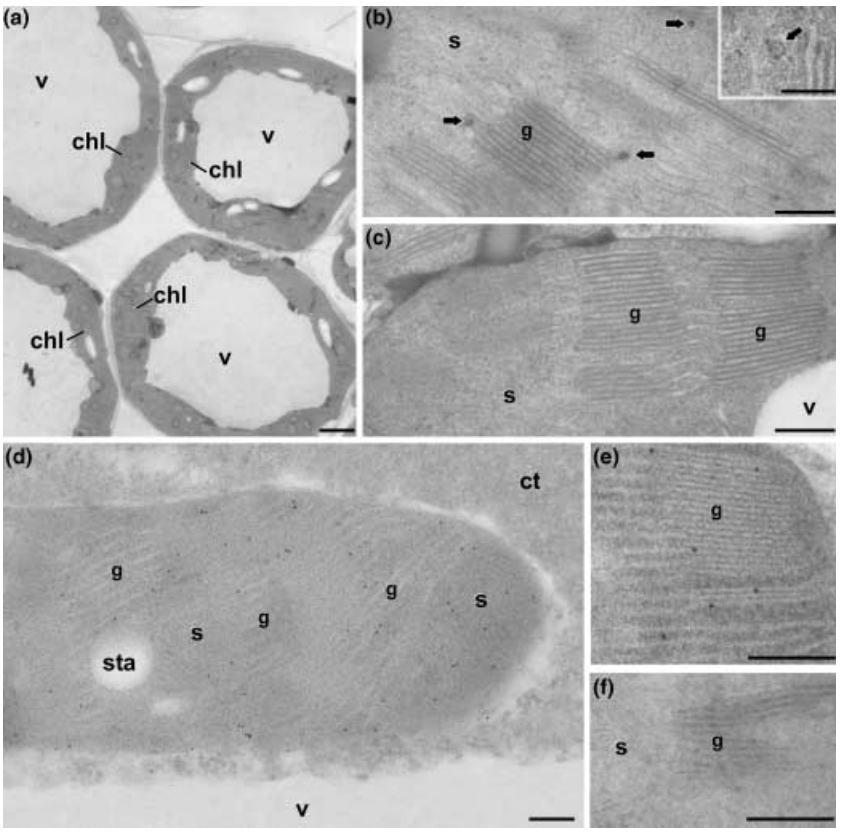

Figure 5 *In situ* electron microscopy and immunogold labelling. (a) Cross-section of mesophyll cells of a transgenic tobacco leaf showing the large central vacuole (v) and the peripheral belt of chloroplasts (chl). (b) Chloroplast of a transgenic plant showing the presence of round, electron-dense particles (arrows) in the stroma (s) or associated with grana stacks (g). Inset in (b) shows a magnification of one particle in which an outer dense ring can be discerned. (c) Chloroplast of a wild-type plant; no dense particles are present. (d, e) Immunogold labelling of L1 protein in chloroplasts of a representative L1 transgenic plant; sta, starch granule. (f) Control excluding primary antibody. Bars: (a) 2 μm; (b–f) 200 nm; inset in (b) 100 nm.

(Figure 5d). Isolated gold particles were very occasionally observed in the cytoplasm, but never in the nucleus, vacuoles, cell walls, starch or lipid deposits within chloroplasts or any other cell compartments. Within chloroplasts, gold particles were observed dispersed throughout the stroma and occasionally formed small clusters of two or three particles (Figure 5d). Individual gold particles were also observed at the boundaries of grana stacks (Figure 5d), and also within stacks, over thylakoid membranes (Figure 5e). No clusters were observed over thylakoids. Controls performed in parallel were almost devoid of labelling (Figure 5f).

Plant-derived HPV-16 L1 VLPs are immunogenic

In order to study the immunogenicity of plant-produced VLPs, Balb/c mice were immunized intraperitoneally with partially purified leaf extracts from L1-transformed plants or wild-type plants (control). Freund's adjuvant or aluminium hydroxide was used as adjuvant. Anti-L1 antibodies were detected in both groups after the first immunization (Figure 6a). Titres of antibodies increased with both boosters. No antibodies (titre < 10) were detected in the group immunized with wild-type plant extracts. Both adjuvants led to a similar L1 immune response, with final antibody titres of 20 000 (Freund's) and 42 000 (aluminium) after the second booster (Figure 6a). When mice sera were analysed against denatured VLPs, the titres were negligible (titre < 10). In accordance with our results, similar titres were obtained using plant protein extracts from tobacco plants in which L1 protein was targeted to the chloroplast (Maclean *et al.*, 2007).

To characterize the L1-induced immune response, mice sera were analysed after the second booster. Denatured and non-denatured VLPs were blotted on to nitrocellulose filters and incubated with H16.V5 conformational mAb, Cam Vir-1 or the pooled sera of mice immunized with aluminium as an adjuvant. None of the antibodies or sera reacted with bovine serum albumin (BSA) used as negative control (Figure 6b). Although Cam Vir-1 equally recognized both denatured and non-denatured VLPs, H16.V5 reacted only with correctly assembled VLPs. Mice sera displayed a similar behaviour to H16.V5 mAb, identified by a strong spot signal when it reacted with the non-denatured VLP sample and a very weak signal with denatured VLPs (Figure 6b). This result and the negligible titres against denatured VLPs strongly suggest that antibodies elicited with L1 plant protein extracts are mostly directed against L1 conformational epitopes, and, in addition, that chloroplast-derived L1 protein is preferentially assembled into higher order structures.

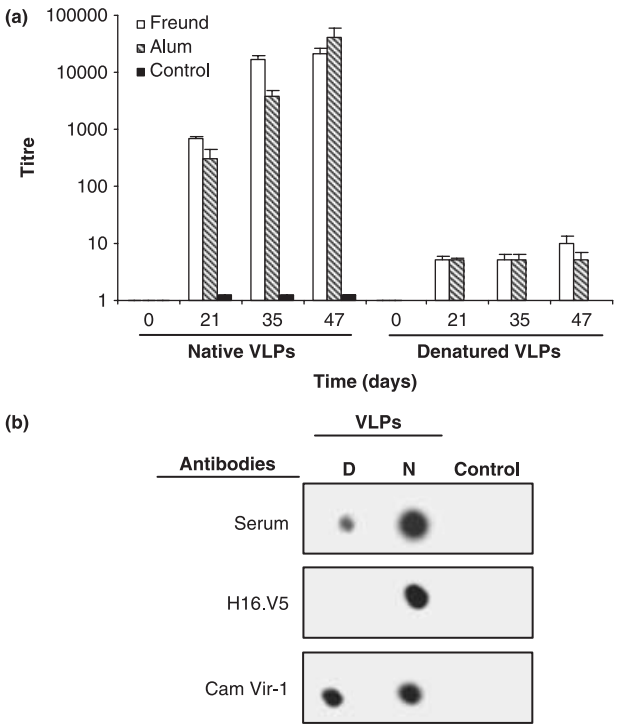

Figure 6 Analysis of the L1-induced antibody response in mice. (a) Titres of antibodies induced by chloroplast-derived L1 protein against native or denatured insect cell-derived virus-like particles (VLPs). Balb/c mice were intraperitoneally immunized with partially purified leaf extracts. Each mouse received 30 μg of L1. Animals were boosted at days 21 and 35. Freund, mice group immunized with complete Freund's adjuvant and boosted with incomplete Freund's adjuvant; Alum, mice group immunized and boosted with aluminium hydroxide gel; Control, mice group immunized with leaf extracts of wild-type plants. Data are presented as means and standard errors (n = 5–6). (b) Characterization of sera derived from mice immunized with tobacco chloroplast-derived VLPs (serum). Denatured (D) and native (N) insect cell-derived VLPs (120 ng/blot) were dot-blotted and incubated with H16.V5 or Cam Vir-1 monoclonal antibody (mAb) or mouse serum as primary antibody. Bovine serum albumin was used as a negative control. A representative result from three independent experiments is shown.

In addition, the presence of neutralizing antibodies was investigated in sera from mice immunized with plant extracts. Neutralization was not detected in sera from mice immunized with wild-type tobacco extracts, used as negative control. In contrast, neutralizing antibodies were detected in sera from mice immunized with plant extracts from L1-transformed plants at a dilution of 1 : 100 (aluminium adjuvant) and 1 : 400 (Freund's adjuvant). As a positive control, mice immunized with VLPs purified from insect cells elicited a neutralizing antibody titre of 1 : 400.

Discussion

Transgenic tobacco plants expressing the major structural protein L1 of HPV-16 in their chloroplasts have been developed. High expression levels of L1 foreign protein (~3 mg/g FW; equivalent to 24% of TSP) have been achieved. This is the highest expression level of HPV L1 protein reported in plants. Several antigenic proteins have been expressed previously in tobacco chloroplasts, with accumulation levels in the range of 4%–20% of TSP (Daniell, 2006). It is assumed that the transgene hyperexpression obtained in the chloroplast is a result of the high gene copy number in the plastid genome (up to 10 000 copies/cell) and of the higher stability of proteins in this organelle compared with that of proteins in the cytoplasm or other subcellular compartments.

HPV-16 L1 has been expressed previously in potato and tobacco plants by nuclear transformation, either transiently or stably, with yields lower than 0.1 mg/g FW. Recently, chloroplast targeting of L1 using a transit peptide fused to the L1 sequence allowed for significantly higher levels of accumulation of L1 protein (up to 0.65 mg/g FW) (Maclean *et al.*, 2007). This increase in foreign protein accumulation, in contrast with that achieved in the cytoplasm, could be attributed to the different protein hydrolysing machinery to which the recombinant protein was subjected. A second possibility could be a higher protein stability achieved through the correct folding of the L1 protein by the assistance of plastid-specific chaperones. These molecular processes are thought to be equal for any foreign protein present in the stroma of the chloroplast, irrespective of its point of origin (a nuclear gene whose protein is transported to the chloroplast or a plastid gene). Therefore, the approximately fivefold higher expression obtained by chloroplast transformation (0.65 vs. 3 mg/g FW) can only be explained by the high copy number of the transgene in the plastid genome and the high translation rate driven by the 5′-UTR of the *psbA* gene.

In this study, it was estimated that a single transgenic tobacco plant is able to synthesize ~240 mg of L1 protein. Old leaves were excluded from this calculation because of high proteolytic degradation of the recombinant protein. This point is relevant if the process is to be scaled up, and must be analysed for each recombinant protein. Despite the fact that the L1 protein content in old leaves is relatively high, the mixture of intact L1 molecules and multiple degradation products may be difficult to eliminate, and could be detrimental for the final use of the protein.

Despite the high accumulation of L1 protein in tobacco chloroplasts, transformed plants grew normally in the glasshouse, with no alteration of the phenotype. Ultrastructural analysis of mesophyll cells by transmission electron microscopy indicated that chloroplasts were normal in shape and size. Immunogold labelling showed a homogeneous distribution of L1 protein within the chloroplast. The presence of L1

protein in the stroma, as well as in the membranous fraction of the chloroplast, explains the observation that, after extraction, the foreign protein is present in both the soluble and insoluble fractions. Unlike L1 protein, when human serum albumin was expressed in tobacco chloroplasts, inclusion bodies were observed within the chloroplasts, and the organelles increased in size to accommodate the aggregates (Fernández-San Millán *et al*., 2003). It is probable that human serum albumin was not properly folded in the chloroplast, and hence aggregated to finally form inclusion bodies. In contrast, in the case of L1, with higher expression levels than those of human serum albumin, it is assumed that L1 monomers assemble into higher order structures that probably prevent the formation of inclusion bodies.

In this study, it was demonstrated that L1 protein expressed in tobacco chloroplasts self-assembles into VLPs. The first step of this process is the formation of the pentameric capsomer. It has been reported previously that the protection conferred by the capsomer prevents the full-length L1 protein from being completely digested by trypsin, yielding a discrete 42-kDa product detected by western blot (Li *et al*., 1997). As expected, this 42-kDa product was observed after L1 plant protein extracts were treated with trypsin, indicating the correct formation of capsomers. To detect the formation of higher order structures, plant extracts were subjected to CsCl density gradient centrifugation. This is a standard method for the purification of correctly assembled VLPs and has been used in different protein expression systems, such as insect cells (Kirnbauer *et al*., 1993), yeast (Kim *et al*., 2007), mammalian cells (Heino *et al*., 1995) and plants (Biemelt *et al*., 2003). In our studies, chloroplast-synthesized L1 protein was present in CsCl gradient fractions at a density of 1.27–1.31 g/mL, which indicates the presence of VLPs. Such fractions immunoreacted with the conformation-specific anti-HPV antibody H16.V5 mAb. The electron microscopy by negative staining of these fractions confirmed the presence of VLPs, which were also detected *in situ* by electron microscopy of leaf chloroplasts.

Sucrose sedimentation analysis of transgenic leaf soluble proteins revealed that a significant proportion of the L1 antigen assembled into structures of smaller size than insect cell-derived VLPs. The formation of either small VLPs or only capsomers has been observed previously in plants (Biemelt *et al*., 2003; Kohl *et al*., 2006; Maclean *et al*., 2007). Based on the model proposed by Zhao *et al*. (2005) on VLP assembly, smaller particles of 20 or 40 nm, with 12 or 24 capsomers, respectively, can be formed. Nevertheless, irregular VLPs of 30–50 nm in diameter have been shown to be immunogenic in humans (Koutsky *et al*., 2002). The reason for the preferential assembly of these smaller particles is unknown. Biemelt *et al*. (2003) hypothesized that relatively high concentrations of L1 protein are required for the correct formation of VLPs. Maclean *et al*. (2007) targeted the L1 protein to the chloroplast and observed that approximately 60% of the VLPs were 55–60 nm. The L1 expression level detected in our plants is around fivefold higher than that mentioned above. However, approximately 70% of the observed viral structures were smaller than 55 nm. Therefore, this result contrasts with the hypothesis of the importance of a specific L1 protein concentration to achieve a correct self-assembly into VLPs. A possible explanation for the assembly of smaller VLPs could be the difference in redox potential during day/night cycles, controlled by the ferredoxin/thioredoxin system. In our study, the *L1* gene was driven by the light-inducible *psbA* 5′-UTR. We believe that L1 is preferentially synthesized in the chloroplast during the light cycle, when the redox potential could be inadequate for the assembly of VLPs. It must be taken into account that the formation of disulphide bonds is essential for the assembly of papillomavirus capsids. Sapp *et al*. (1998) demonstrated by specific mutations that two highly conserved cysteine residues at positions 175 and 428 are involved in the trimerization of L1 proteins. As the complete capsid consists of 360 copies of the L1 protein, it is expected from the atomic model (Modis *et al*., 2002) that at least 360 disulphide bonds are formed per VLP. Several reports have confirmed the formation of disulphide bonds in the chloroplast: human somatotropin (Staub *et al*., 2000), cholera toxin B (Daniell *et al*., 2001a), immunoglobulin A (IgA) (Mayfield *et al*., 2003) and human interferon (Arlen *et al*., 2007) have been expressed in their functional forms. However, all of these proteins have a rather simple structure with less disulphide bonds (< 10) relative to the full-sized VLP.

Immunization experiments have shown that chloroplast-derived VLPs are highly immunogenic by the intraperitoneal route, inducing a specific humoral response. This result is equivalent to previous reports of the parenteral (Biemelt *et al*., 2003; Varsani *et al*., 2003; Maclean *et al*., 2007) or oral (Warzecha *et al*., 2003) administration of plant-produced L1 antigen. Importantly, neutralizing antibodies were detected in the sera of mice immunized with plant extracts, confirming that the L1 protein self-assembles into immunogenic VLPs in tobacco chloroplasts. However, the neutralization titres were much lower than the ELISA titres observed in the immunogenicity analysis. This could be the result of the difference in sensitivity between the ELISA and neutralization tests performed. A similar pattern has been observed previously by Maclean *et al*. (2007) using a neutralization assay based on the production of pseudovirions within mammalian cells (Pastrana *et al*., 2004). Neutralizing titres observed in our

experiments were approximately 10-fold lower than those reported by Maclean *et al*. (2007). However, this could be explained by a lower sensitivity of tests using pseudovirions produced in acellular systems relative to cellular systems (Fleury *et al*., 2007), as only a small proportion of disassembled VLPs reassemble into pseudovirions in acellular systems.

Interestingly, aluminium hydroxide showed a similar adjuvant effect to Freund's adjuvant, which is the most commonly used adjuvant in animal models. Despite its good immunostimulatory capacity, the use of Freund's adjuvant is not allowed for human vaccination because of its toxicity. However, aluminium-based compounds have been widely used as human vaccine adjuvants for more than 70 years. Currently, they are the most common adjuvants used for human application (e.g. both commercial vaccines against HPV include aluminium derivatives). It should be noted that Maclean *et al*. (2007) observed that non-adjuvant plant-derived VLPs elicited higher neutralizing titres than Freund's adjuvant VLPs, indicating that the adjuvant could be dispensable or even deleterious in this case.

In conclusion, the present work provides a new and economically viable system for HPV vaccine production in plants. First, L1 proteins from the most carcinogenic types of HPV can be easily expressed together in the chloroplast because of the ability of this organelle to process polycistrons. Second, high expression levels of antigens in chloroplasts will reduce the amount of plant material required for a single vaccine dose. Third, the use of aluminium as an adjuvant may facilitate approval by legal authorities for human clinical trials. It can be argued that there is no need for a new HPV vaccine when two vaccines have just arrived on the market. This is true in developed countries where the high cost of the vaccine is not an obstacle. However, the high production costs of current HPV vaccines will hinder their distribution in developing countries, where they are needed most. In the present world, the access of developing countries to these vaccines and other pharmaceuticals will only take place after a general access in rich countries.

Experimental procedures

Construction of the chloroplast expression vector

For the construction of the pAF chloroplast transformation vector, the homologous recombination regions *trnI–trnA* (104 095–106 205 of the GenBank Accession Z00044) were amplified by PCR from tobacco DNA with primers 5′-CGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCAGGGGCCTTGTACACAC-3′ and 5′-AAGCTTTGTATCGGCTAAGTTCACGAGTTGG-3′, and introduced into a pUC vector by *Pvu*I-*Hin*dIII digestion. The resulting vector was opened with *Pvu*II, which cuts the intergenic spacer between *trnI* and *trnA*. After making blunt ends, the plastidial tobacco *Prrn* promoter was introduced. This sequence (102 539–102 675) was previously cloned by PCR from tobacco DNA with the following primers: 5′-GCGGCCGCAATGTGAGTTTTTGTAGTTGG-3′ and 5′-GCGGCCGCGAATTCGATGCGCTTCATATTCGCCC-3′. The resulting vector was digested with *Eco*RI, and the *aadA* sequence was introduced. This sequence was cloned by PCR from the pFaadAII vector (provided by Dr Christian Eibl, Ludwig-Maximilians-Universität, Munich, Germany) with the following primers: 5′-GCGAATTCTAGGGAGGCAACCATGGCTCGTGAAGCGG-3′ and 5′-GGTTACCTGAATTCGTCGACACGCGTGGTACCGATATCTTATTTGCCGACTACC-3′. The resulting vector was digested with *Sal*I-*Bst*EII, and the 3′-terminator region of the *psbA* gene was introduced (536–141, GenBank Accession Z00044). This region was previously amplified by PCR from tobacco DNA with the following primers: 5′-GTCGACGAATTCTCTAGAGATCCTGGCCTAGTCTATAGG-3′ and 5′-GGTTACCTCGAATATAGCTCTTCTTTCTTATTTC-3′.

HPV-16 *L1* cDNA (GenBank Accession AF472508) was provided by Dr Robert D. Burk (Albert Einstein College of Medicine, New York, USA). For cloning into the pAF vector, *L1* was amplified by PCR with the primers 5′-CCACATGTCTCTTTGGCTGCCTAGCG-3′ and 5′-GCGGCCGCTCGAGTTACAGCTTACGTTTTTTGC-3′ in order to introduce the *Afl*III and *Not*I sites, and cloned into the pGEM-T vector (Promega, Madison, WI, USA). *L1* was digested with *Afl*III/*Not*I and fused to the promoter and 5′-UTR of the tobacco *psbA* gene in a pKS intermediate vector. Finally, the fusion was digested by *Eco*RV and *Not*I and introduced into the pAF vector. The final clone was sequenced, and its functionality was tested by western blotting of soluble extracts of *E. coli* cultures.

Bombardment and regeneration of chloroplast transgenic plants

Gold microprojectiles (0.6 μm) coated with pAF-L1 plasmid DNA were bombarded into tobacco (*Nicotiana tabacum* var. Petit Havana) *in vitro*-grown leaves using the biolistic device PDS1000/He (Bio-Rad, Hercules, CA, USA), as described previously (Daniell, 1997). After bombardment, the leaves were cut into small pieces and cultured in Magenta vessels (Sigma, St. Louis, MO, USA) containing 500 mg/L spectinomycin dihydrochloride as a selection agent (Molina *et al*., 2004). Resistant shoots were subjected to a second round of selection under the same conditions. Regenerated plants were transplanted and grown in a phytotron or glasshouse for further experiments.

Southern blot analysis

Total plant DNA was extracted from leaves of 2-month-old plants using the cetyltrimethylammonium bromide (CTAB) procedure (Murray and Thompson, 1980). Ten micrograms of total DNA were digested with *Hin*dIII, separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane. The chloroplast vector DNA digested with *Bgl*II and *Bam*HI generated a 0.8-kb probe (P1) homologous to the flanking sequences. A 0.45-kb fragment (P2 probe) of the *L1* gene was amplified by PCR with the following primers: FintL1 (5′-CGACATGGGGAGGAATATGATTTACAGTTTATTTTTC-3′) and RevL1 (5′-GCGGCCGCTCGAGTTACAGCTTACGTTTTTTGC-3′). Hybridization was performed using the chemiluminescent AlkPhos direct labelling-detection system (GE Healthcare, Buckinghamshire, UK). After

Southern blot confirmation, the plants were transferred to soil. Seeds from the T_0 generation were germinated *in vitro* on spectinomycin selection medium. The T_1 seedlings were isolated and cultured for 4 weeks in Magenta vessels. Finally, the plants were transferred to pots. Plants from the T_0 and T_1 generations were analysed for homoplasmy.

Western blot analysis

Leaf discs (100 mg) were ground in liquid nitrogen, homogenized in phosphate-buffered saline (PBS; 0.5 M NaCl, pH 7.4) and sonicated for 10 s. After centrifugation at 20 000 ***g*** for 5 min, the supernatant was considered as the soluble fraction. The pellet was resuspended in 200 μL of loading buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 5 min. After centrifugation at 20 000 ***g*** for 5 min, the supernatant was considered as the insoluble fraction. Proteins were separated on 10% SDS (w/v)-polyacrylamide gels. Proteins were transferred on to nitrocellulose membranes (Hybond C, GE Healthcare), blocked overnight in PBS with 0.1% (v/v) Tween-20 (PBS-T) and 5% skimmed milk (PBS-TM), and incubated for 1 h with Cam Vir-1 primary antibody (Abcam, Cambridge, UK) diluted 1 : 25 000 in PBS-TM. After two washes of 10 min in PBS-T, membranes were incubated for 1 h with a 1 : 25 000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Sigma) diluted in PBS-TM. After washing, the specific signal was detected using the Advanced ECL system (GE Healthcare), according to the manufacturer's instructions.

Dot blot analysis

Native and urea-denatured VLPs from insect cell cultures were applied to a nitrocellulose membrane (Hybond C, GE Healthcare) at 1.5 μL per dot at a concentration of 80 ng/μL. As a negative control, 1.5 μL of 80 ng/μL BSA (Sigma) per dot was used. After drying at room temperature for 10 min, the membrane was blocked overnight with 5% PBS-TM. After washing with PBS-T, the dot blot was incubated for 1 h at room temperature with the following antibodies and sera diluted in PBS-TM: H16.V5 conformational antibody 1 : 5000 (provided by Dr Neil Christensen, Penn State College of Medicine, Hershey, PA, USA), Cam Vir-1 antibody 1 : 20 000, and serum (diluted 1 : 1000) taken 47 days post-primary immunization from mice immunized with transgenic plant extracts and aluminium hydroxide. After four washes with PBS-T, the membranes were incubated for 1 h with a 1 : 20 000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Sigma) diluted in PBS-TM. For detection of the chemiluminescent signal, the procedure was the same as for western blotting.

ELISA quantification of HPV-16 L1 protein

Transformed and untransformed leaves (100 mg) from plants grown in a glasshouse were ground in liquid nitrogen, resuspended in 500 μL of carbonate buffer (100 mM Na_2CO_3, pH 10.5), sonicated for 10 s and centrifuged at 20 000 ***g*** for 5 min at 4 °C to remove cellular debris. The samples were incubated in a 96-well polyvinyl chloride microtitre plate (Costar Corning, Corning, NY, USA) overnight at 4 °C. The wells were blocked with PBS-TM (1% skimmed milk) for 1 h at room temperature, washed three times with PBS-T and incubated with anti-L1 H16.V5 mAb at 1 : 500 or Cam Vir-1 mAb at 1 : 1500 in PBS-TM (1 h at 37 °C). The wells were washed three times with PBS-T and incubated with a 1 : 2000 dilution of rabbit anti-mouse IgG-peroxidase conjugate in PBS-TM (1 h at 37 °C). The plates were developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid (ABTS) (Roche, Mannheim, Germany). The reaction was read at 405 nm in a microtitre plate reader (Multiskan Ex, Labsystems, Helsinki, Finland). The standard curve for calculating the amount of recombinant protein with H16.V5 mAb was obtained by plating purified VLPs produced in insect cells by baculovirus infection, as described below. VLPs were used in the range of 30–150 ng per well in 100 mM PBS (pH 7.4). For the standard curve with Cam Vir-1 mAb, insect cell-derived 6 × His-tagged L1 protein was used, purified by affinity chromatography with TALON® resin (Clontech Laboratories, Inc., Mountain View, CA, USA), as described by the manufacturer. The His-tagged L1 was used in the range of 60–125 ng per well in 100 mM carbonate buffer. Transgenic leaf extracts were diluted (1 : 1000–1 : 8000) to fit within the linear range of the standards.

Production of HPV-16 L1 VLPs via baculovirus expression in insect cells

This protein was obtained using the Bac-to-Bac© Baculovirus Expression System (Invitrogen Life Technologies, Carlsbad, CA, USA). Briefly, the *L1* gene described above was subcloned into the pFastBac™1 vector, and the resulting DNA was used to transfer DH10Bac™ *E. coli* cells for transposition into the recombinant bacmid. One microgram of this bacmid was used to transfect 1×10^6 *Spodoptera frugiperda* Sf21 cells using Cellfectin® Reagent (Invitrogen Life Technologies). The P1 viral stock was obtained after 72 h of incubation at 27 °C. The baculovirus was amplified following the manufacturer's instructions until a titre of 2.5×10^9 plaque-forming units/mL was reached. For the production of VLPs, Sf21 insect cells were grown to a density of $(1–1.2) \times 10^6$ cells/mL on BD BaculoGold™ TNM-FH Insect Medium (BD Biosciences) supplemented with 50 μg/mL gentamycin, 50 units/mL penicillin and 50 μg/mL streptomycin in 75-cm^2 flasks. Cells were then infected at a multiplicity of infection of 1–5 and, after 72 h of incubation, infected cells were pelleted by centrifugation at 1000 ***g*** for 5 min.

Purification of VLPs

Insect cells

Pellets of infected insect cells were resuspended in 16 mL of PBS (0.5 M NaCl)/g cells and sonicated for 1 min. Extracts were layered on to a 40% sucrose cushion and centrifuged in a swinging bucket rotor (Kontron TST4114, Kontron, Milano, Italy) for 2 h at 140 000 ***g*** at 4 °C. The resulting pellets were resuspended in 10 mL of 27% (w/v) CsCl in PBS (0.5 M NaCl) solution and centrifuged for 20 h at 260 000 ***g*** in a swinging bucket rotor at 10 °C. Five hundred microlitre fractions were collected and their densities were measured with a refractometer. The desired fractions were dialysed overnight against PBS (0.5 M NaCl).

Plant VLPs

For the extraction of plant VLPs, 4 g of mature tobacco leaves were ground in liquid nitrogen, homogenized with 26 mL of PBS (0.5 M

NaCl) and sonicated for 5 min. After centrifugation at 20 000 ***g*** for 5 min at 4 °C, supernatants were filtered through a 0.2-μm mesh and layered on to a 40% sucrose cushion. The rest of the protocol was similar to that described for insect cell VLP extraction.

Electron microscopy and immunogold labelling

For negative staining, 20-μL samples of the positive fractions in the CsCl gradient were dialysed against PBS (0.5 M NaCl) on floating filter pads (pore size, 0.2 μm; Millipore, Bedford, MA, USA). The samples were placed on to carbon-coated copper grids (400 mesh size) covered with Formvar membrane and stained with 1% uranyl acetate solution for 1 min. The samples were inspected under a Zeiss EM 910 transmission electron microscope (Zeiss, Oberkochen, Germany) operating at 60 and 80 kV.

For ultrastructural observations, leaf samples from glasshouse-grown plants were fixed in Karnovsky fixative (4% formaldehyde + 5% glutaraldehyde in 0.025 M cacodylate buffer, pH 6.7), postfixed in 2% OsO_4, dehydrated in an acetone series for 3 days and slowly embedded in Epon resin for 2 days. Epon blocks were polymerized at 60 °C for 2 days. Thin sections (~80 nm) were collected on 100-mesh copper grids, counterstained with uranyl acetate and lead citrate, and observed using a Philips CM10 transmission electron microscope (Philips, Hillsboro, OR, USA) operating at 100 kV.

For immunogold labelling, leaf samples were fixed in 4% formaldehyde in PBS and dehydrated in acetone by the 'Progressive Lowering of Temperature' (PLT) method in a Leica AFS automated system (Leica, Wetzlar, Germany). Then, samples were infiltrated in Lowicryl K4M resin and polymerized at –30 °C under ultraviolet light. For immunolabelling, Lowicryl sections (thickness, ~80 nm) were deposited on Formvar- and carbon-coated nickel grids. Immunogold labelling was performed as described previously (Segui-Simarro *et al*., 2003). Briefly, sections were hydrated, floated in PBS, blocked with 5% BSA in PBS and incubated with the anti-L1 Cam Vir-1 mAb diluted 1 : 200 in 1% BSA for 1 h at room temperature. Sections were then incubated with an anti-mouse IgG antibody conjugated to 10 nm gold particles, diluted 1 : 25 in 1% BSA for 45 min. Finally, sections were washed, air dried, counterstained with uranyl acetate and lead citrate, and observed in a Philips CM10 transmission electron microscope operating at 100 kV. Controls were performed excluding the anti-L1 antibody from the incubation buffer.

Trypsin digestion of plant-synthesized L1

Soluble extracts from transgenic plants were partially purified by a 2-h centrifugation step through a 40% sucrose cushion in a swinging bucket rotor at 140 000 ***g***. His-tagged L1 monomeric protein and insect cell-derived VLPs purified by CsCl gradients, as described above, were used as controls. Approximately 1 μg of protein was digested for 24 h with 0.5 μg of trypsin (Promega) at 37 °C. Controls without trypsin were also kept at 37 °C for 24 h. After trypsin inactivation by boiling for 5 min in loading buffer, samples were analysed by western blotting.

Analysis of L1 assembly by sucrose sedimentation

To identify different assembly forms of L1, soluble extracts from insect cells [200 mg/2 mL PBS (0.5 M NaCl)] and tobacco leaves [500 mg/2 mL PBS (0.5 M NaCl)] were prepared as described previously for western blotting. The samples were loaded on to linear 10%–65% (w/v) sucrose gradients. After 2 h of centrifugation at 150 000 ***g*** in a swinging bucket rotor, 20 fractions of 500 μL were collected, their densities were determined by refractometry, and they were analysed by ELISA with the H16.V5 conformational antibody. Sedimentation of L1 capsomers was calibrated with catalase from bovine liver (Sigma) as sedimentation marker (11.3S).

Analysis of the antigenicity of the chloroplast-derived L1 protein by ELISA

Microtitre plates (Maxisorp, Nunc, Rochester, NY, USA) were coated with plant extracts or VLPs purified from Sf21 insect cells. After overnight incubation at 4 °C and two washes with PBS-T, the wells were blocked with PBS supplemented with 1% fetal calf serum (FCS) for 1 h at 37 °C. The wells were then incubated with either H31.D24 or H16.V5 mAb (1 : 500 and 1 : 5000, respectively) in dilution buffer (PBS 5×, 1% Tween-20, 10% FCS) for 1 h at 37 °C. The plates were washed four times and probed with peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich; 1 : 5000 dilution) for 1 h at 37 °C. After four washes, 0.4 mg/mL *o*-phenylenediamine and H_2O_2 diluted in 25 mM sodium citrate and 50 mM Na_2HPO_4 were added. The reaction was stopped after 30 min by the addition of 2 M, and the optical densities were read at 492 nm.

Immunization of animals

Female Balb/c mice were purchased from Harlan Ibérica (Barcelona, Spain). Groups of 8-week-old mice ($n = 6$) were immunized by intraperitoneal injection (0.2 mL) with partially purified leaf extracts from L1-transformed plants in complete Freund's adjuvant (CFA, Sigma) or aluminium hydroxide gel (Sigma). The leaves were ground in liquid nitrogen and homogenized in PBS (0.5 M NaCl). The tissue homogenates were sonicated, centrifuged and filtered (0.45-μm mesh) to remove cellular debris. Partial purification of the leaf extracts was performed by 20% ammonium sulphate precipitation. The precipitated proteins were resuspended in PBS (0.5 M NaCl) and stored at –80 °C until use. Each mouse received 30 μg of L1 recombinant protein. The amount of TSP injected varied between 400 and 500 μg. As a negative control, a group of mice ($n = 6$) was intraperitoneally immunized with leaf protein extracts from wild-type plants (400–500 μg TSP/mouse). Animals were boosted at days 21 and 35 (using incomplete Freund's adjuvant instead of CFA). Blood was collected from the retro-orbital plexus at days 0, 21, 35 and 47. Pooled sera were titrated against purified L1 native or denatured VLPs produced in insect cells by an in-house ELISA test. Denaturation of VLPs was performed by boiling 100 ng aliquots for 10 min (Sasagawa *et al*., 2005). 96-Well microtitre plates were coated overnight at 4 °C with 50 μL of insect cell-derived VLPs (100 ng/well) diluted in PBS (pH 7.4). Subsequently, the wells were blocked for 1 h at room temperature with PBS-TM and then incubated for 1 h at 37 °C with serial dilutions of pooled sera in PBS-TM. Then, the plates were incubated for 1 h at 37 °C with a goat anti-mouse IgG antibody-horseradish peroxidase conjugate. Washes were performed between each step with PBS-T. The plates were developed by adding ABTS. The absorbance at 405 nm was measured in a microplate plate reader. Antibody titres were expressed as the highest serum dilution to yield twice the absorbance mean of pre-immune sera.

Detection of anti-HPV-16 neutralizing antibodies

Neutralization assays were performed by inhibition of infection in COS-7 cells (a human embryonic kidney cell line expressing SV40 T-antigen) with infectious pseudovirions containing a luciferase expression plasmid having the SV40 replication origin. In these assays, HPV-16 pseudovirions were pre-incubated with pooled mice sera, and the inhibition of gene transfer expression was measured in COS-7 cells.

HPV-16 pseudovirions were generated as described previously (Touze and Coursaget, 1998) with some modifications. Briefly, HPV-16 VLPs (100 μg), purified from Sf21 insect cells infected with a recombinant baculovirus encoding HPV-16 *L1* and *L2* genes, were incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM dithiothreitol (DTT) and 1 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) for 30 min at room temperature. At this stage, expression plasmids encoding luciferase (10 μg) were added to the disrupted VLPs. The preparation was then diluted with increasing concentrations of $CaCl_2$, up to a final concentration of 5 mM, in a buffer containing dimethylsulphoxide (DMSO) and $ZnCl_2$ (10 mM). Pseudovirions were then dialysed in PBS overnight and stored at 4 °C until use.

After 24 h of incubation at 37 °C, COS-7 cells were washed twice with Dulbecco's modified Eagle's medium (DMEM). Sera (dilutions 1 : 25–1 : 600) from mice immunized with leaf extracts from L1-transformed plants were incubated with diluted HPV-16 pseudovirion stocks. The quantity of HPV-16 pseudovirion was adjusted to give a luciferase activity of approximately 1500 counts per second (cps). Pseudovirions (100 μL) were added to 100 μL of mice sera diluted in incomplete DMEM. After 30 min of incubation at 37 °C, the mixture was added to the cells. After 3 h at 37 °C, the supernatant was removed and 200 μL of complete DMEM was added to the cells. The cells were then incubated for 48 h at 37 °C. Luciferase gene expression was measured by luminescence assay (luciferase reporter gene assay with constant light signal; Roche Molecular Biochemical, Meylan, France). The results were expressed as the percentage of inhibition of luciferase activity: 100% inhibition corresponded to the luciferase activity obtained with DNA alone and 0% inhibition corresponded to the luciferase activity obtained with pseudovirions. The cut-off value used in this assay was an inhibition of gene transfer greater than 80%, a value previously determined using the sera from 20 infants and 10 non-immunized mice (diluted 1 : 20) that demonstrated less than 80% inhibition of luciferase expression.

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