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Plant-Produced Cottontail Rabbit Papillomavirus L1 Protein Protects against Tumor Challenge: a Proof-of-Concept Study

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The native cottontail rabbit papillomavirus (CRPV) L1 capsid protein gene was expressed transgenically via *Agrobacterium tumefaciens* transformation and transiently via a tobacco mosaic virus (TMV) vector in *Nicotiana* spp. L1 protein was detected in concentrated plant extracts at concentrations up to 1.0 mg/kg in transgenic plants and up to 0.4 mg/kg in TMV-infected plants. The protein did not detectably assemble into viruslike particles; however, immunoelectron microscopy showed presumptive pentamer aggregates, and extracted protein reacted with conformation-specific and neutralizing monoclonal antibodies. Rabbits were injected with concentrated protein extract with Freund's incomplete adjuvant. All sera reacted with baculovirus-produced CRPV L1; however, they did not detectably neutralize infectivity in an in vitro assay. Vaccinated rabbits were, however, protected against wart development on subsequent challenge with live virus. This is the first evidence that a plant-derived papillomavirus vaccine is protective in an animal model and is a proof of concept for human papillomavirus vaccines produced in plants.

Papillomaviruses (PVs) are small double-stranded DNA viruses (family *Papillomaviridae*) that infect many different vertebrate species (12). Human papillomaviruses (HPV) are known to cause warts and have also been associated with certain cancers in humans (46). Specific high-risk types of HPV are causally associated with cervical cancer (43); this is the second most prevalent cancer in women worldwide and the most common cancer in South African women (30). Vaccination against papillomaviral disease should result in reduced disease burden, which has resulted in a worldwide effort to develop prophylactic vaccines against HPV.

The efforts to develop candidate HPV vaccines have been made more difficult by the fact that the protective efficacy of vaccines cannot be evaluated in animals. In contrast, however, development of animal PV vaccines allows evaluation by immunization of the respective hosts, followed by an experimental challenge with live virus. Cottontail rabbit papillomavirus (CRPV) in rabbits provides a robust model to study viral interaction with the host and progression to cancer and for viral vaccine studies. Naive domestic rabbits can be protected from experimental challenge with live CRPV after vaccination with a nondenatured L1 and/or whole L2 protein or peptides derived from the L2 protein (2, 5, 8, 9, 24, 27, 28).

The most successful HPV prophylactic vaccine candidates to date are based on L1 viruslike particles (VLPs) produced by recombinant baculovirus and yeast: these VLPs are almost indistinguishable from native virions in morphology and induce effectively identical immune responses (for a review see reference 30). In animal and human studies, VLP vaccines have been well tolerated and have induced high titers of neutralizing antibodies as well as protecting against papillomaviral infection and especially disease (3, 22). However, these vaccines will be expensive and not affordable in developing countries where they are needed most (37, 39).

Plants have been investigated extensively recently as alternative and cheaper production vehicles for vaccines and other high-value pharmaceuticals: proteins can be expressed in plants which have been stably transformed or transiently transformed by use of plant viral vectors or by infiltration with *Agrobacterium tumefaciens* (15, 16). In particular, it is thought that gene expression can be significantly increased over the transgenic case by utilizing recombinant tobacco mosaic virus (TMV) vectors (20). We and others have previously reported the transgenic expression of HPV-16 L1 in tobacco and potatoes (1, 29, 40, 44), and we have recently reported the use of a TMV-based plant virus vector to express HPV-16 L1 transiently in *Nicotiana benthamiana* (41). However, in all cases except for a human codon usage-optimized HPV-16 L1 gene in tobacco (1), expression levels were very low and the immunogenicity of the products, whether administered orally or by injection, was uniformly low. Moreover, it has not yet been shown in these five published reports of plant expression of HPV L1 proteins that these proteins elicit either neutralizing antibodies or protection from disease.

Here we focus on the evaluation of a plant-produced CRPV vaccine, which was produced in plants by expressing the native

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L1 gene either transiently using a TMV-based vector or in *Agrobacterium tumefaciens*-transformed transgenic plants. We investigated whether a plant-derived PV vaccine was indeed capable of eliciting an immune response similar or identical to that achieved by vaccination of animals with recombinant-baculovirus-produced VLPs. We further investigated whether protective immunity was established after vaccination of New Zealand White rabbits with plant-derived CRPV VLPs in vivo by challenging animals with live virus.

MATERIALS AND METHODS

Cloning of the CRPV L1 gene. The CRPV *L1* full-length gene was amplified by PCR using the following primer pair: Forward, 5′-TTAATTAA**ATG**GCAG TGTGGCTGTCTACG-3′ (PacI site is underlined; start codon is in boldface); Reverse, 5′-CTCGAG**TTA**AGTTCTCTTGCGTTTAGATGATTTC-3′ (XhoI site is underlined; stop codon is in boldface) from a full-length CRPV virus clone (N. D. Christensen). The PCR product was cloned into the pGEM-T Easy vector (Promega), and the sequence was verified.

The CRPV *L1* gene was directionally subcloned using PacI and XhoI into the Geneware vector pBSG1057 (Large Scale Biology Corporation, Vacaville, Calif.), thereby replacing the 30B-GFPC3 gene. It was also cloned into the *Agrobacterium* vector pART7 using EcoRI. The cassette containing the CRPV *L1* gene, the CaMV35S promoter, and the octopine synthase gene terminator (*ocs* 3′) was excised by NotI digestion and subcloned into the binary vector pART27 (19).

Transformation of *Nicotiana tabacum* cv. Xanthi. *N. tabacum* leaf disks were transformed with *A. tumefaciens* carrying the pART27 CRPV L1 binary vector, and transgenic plants were regenerated according to a standard protocol (23). Flowering regenerated plants (R_0 generation) were self-pollinated and the seeds collected. Dry seeds were screened on plant tissue culture media containing kanamycin (250 μg/ml), and putative transgenic seedlings were transferred to soil, once the fourth leaves had grown to maturity.

Screening of transformed *N. tabacum* plants for the CRPV L1 gene. Plant genomic DNA was extracted in extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM NaCl, pH 8.0) from putative transgenic and wild-type *N. tabacum* leaves using the Dellaporta method (11). DNA from transformed *N. tabacum* plants was screened by PCR for the CRPV L1 gene using the same primer pair and conditions as those used for the initial amplification of the gene.

Inoculation of *Nicotiana benthamiana* plants with CRPV RNA. Transcripts were synthesized in vitro using the T7 RNA polymerase (RiboMAX large-scale RNA production system T7; Promega) and capped by addition of the RNA cap structure analogue m7G(5)ppp(5)G (New England Biolabs).

Inoculation of 3-week-old *N. benthamiana* plants and subsequent monitoring of infection were done as described previously (41).

Analysis of total plant RNA extracts. Total RNA was extracted from fresh leaf material from transgenic plants and from individual leaves from TMV-infected plants at 14 days postinoculation using the TRIzol reagent (Life Technologies). Total RNA samples were predigested with RNase-free DNase (Promega) at 37°C for 30 min. DNase stop solution was added to the samples, and the DNase was heat inactivated at 65°C for 10 min. CRPV L1 RNA was amplified by reverse transcription-PCR (RT-PCR) using the Access RT-PCR system (Promega). For CRPV, forward primer 5′-AAAGCATGGCGTTCGACC-3′ and reverse primer 5′-GCACACAGATGCAGGGAGAG-3′ were used to amplify an internal, 421-base-pair fragment situated between nucleotides 433 and 854. TMV coat protein mRNA was amplified from TMV-infected plants using forward primer 5′-CA TTAGCGCTGCGGCCGCCCTTATACAATCAACTCTCCG-3′ and reverse primer 5′-ATAAGAATGCGGCGGCTCGCGAAGTAGCCGGAGTTGTTGT C-3′, which gives a 474-bp product.

Processing and concentration of plant material. Leaf material was harvested and homogenized in 1:2 (wt/vol) cold high-salt phosphate-buffered saline (PBS; 1.47 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 500 mM NaCl, pH 7.4). Homogenate was filtered through cheesecloth and centrifuged at 6,000 × *g* for 10 min to remove plant debris. To precipitate TMV particles in the TMV-infected plants, 4% polyethylene glycol (PEG; molecular weight, 8,000) was added to the supernatant and it was centrifuged at 6,000 × *g* for 20 min. Six percent PEG was added to the subsequent supernatant. Transgenic plant material was treated directly with 10% PEG and centrifuged at 6,000 × *g* for 20 min. The resulting pellet was resuspended in 1/10 starting volume of PBS, the suspension was centrifuged for 20 min at 6,000 × *g*, and the supernatant was then centrifuged at 100,000 × *g* for 2 h to pellet high-molecular-weight aggregates. Pellets were resuspended in 1/10 starting volume and further analyzed by enzyme-linked immunosorbent assay (ELISA) and electron microscopy.

Monoclonal antibody characterization of plant-derived protein. CRPV L1 protein-containing extracts derived from *N. tabacum* and *N. benthamiana* together with nontransgenic plant protein extracts were characterized by direct ELISA using two monoclonal antibodies (MAbs) against CRPV. Monoclonal antibody CRPV:5A is a conformation-specific neutralizing antibody, and CRPV:10B recognizes a linear surface epitope (7). ELISA plates were coated with protein extracts for 1 h and then blocked in 2% nonfat milk in PBS (1.47 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) for 1 h. MAbs diluted at 1:1,000 were incubated with the plant-derived antigen for 1 h. Anti-mouse immunoglobulin G-alkaline phosphatase-conjugated secondary antibody (diluted 1:5,000; Sigma) was allowed to bind the primary antibody for 1 h at 37°C. The secondary antibody was detected using *p*-nitrophenylphosphate (Sigma), and the absorbance was measured using a Titrex ELISA plate reader at 405 nm. All samples were analyzed in triplicate to determine the mean absorbance and calculate the respective standard deviation.

Expression of the CRPV L1 gene in Sf21 cells via recombinant baculovirus. The CRPV L1 gene was directionally cloned into the pFastBac1 vector (Invitrogen) using the EcoRI restriction enzyme sites. This DNA was used for the transfection of maximum-efficiency DH10Bac-competent *Escherichia coli* cells for the preparation of bacmid clones. Recombinant bacmid DNA was isolated and used for transfection of *Spodoptera frugiperda* (Sf21) cells (Invitrogen) in the presence of Cellfectin (Invitrogen) according to the manufacturer's Bac-to-Bac protocol.

To purify CRPV L1 VLPs, Sf21 cells were pelleted, resuspended in PBS containing 0.4 g/ml CsCl and complete protease inhibitor (Roche), and sonicated. The sonicated suspension was centrifuged at 100,000 × *g* at 10°C for 24 h. Two distinct bands were observed on the CsCl gradient: the top band was extracted by puncturing the tubes and dialyzed against PBS (1.47 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 500 mM NaCl, pH 7.4) for 48 h. Dialyzed protein was divided into 100-μl aliquots and frozen at −70°C for further use.

Electron microscopy of plant-derived CRPV L1 protein. Protein extracts from transgenic and infected plants and insect cell-derived CRPV L1 VLPs were viewed under the electron microscope after absorption of the respective preparations onto carbon-coated copper grids for direct viewing or L1 protein detection by immunogold labeling.

Basic procedures were done as described previously (40). Protein samples were directly adsorbed onto copper grids for 30 min and then stained in 2% uranyl acetate for 2 min. Immunotrapping of plant- and insect cell-derived L1 protein was done with rabbit anti-CRPV L1 antiserum raised against insect cell-derived CRPV L1 VLPs diluted 1:50 in PBS. Grids were incubated with plant protein extract for 30 min, washed, and stained in 2% uranyl acetate for 2 min.

For immunogold labeling, immunotrapping of transgenic, transiently expressing, and nontransgenic plant protein extracts was done as described above. Grids were then washed and probed with CRPV L1 MAbs CRPV:5A and CRPV:10B (diluted 1:1,000 in 1% bovine serum albumin-PBS) for 60 min at room temperature. Another 2-min washing step preceded the 60-min incubation of grids in the gold-labeled anti-mouse immunoglobulin G-conjugated secondary antibody (30-nm gold particles) diluted 1:100 in PBS. Grids were washed one final time in sterile distilled water (twice for 2 min each) before being stained with 2% uranyl acetate for 2 min.

Immunization of rabbits and evaluation of sera. Two groups of three New Zealand White rabbits each were inoculated with concentrated extracts of transgenic *N. tabacum* or recombinant-TMV-infected *N. benthamiana*. The first inoculum (1 ml of extract) was administered by subcutaneous (three sites) and intramuscular (one site) injection. An additional two booster inoculations consisting of 500 μl protein extract mixed in a ratio of 1:1 with Freund's incomplete adjuvant were administered as described above on days 23 and 41. Serum was collected on days 1, 23, 41, and 51 and analyzed by direct ELISA and Western blotting (1:20 dilution) against CRPV L1 insect cell-derived VLPs at a concentration of 1.2 μg/ml.

Preimmune sera all displayed high reactivity to normal plant proteins and to baculovirus and insect cell proteins, probably because of exposure of rabbits to these proteins via feed. Consequently, all sera were preabsorbed with nontransgenic plant extract and nonrecombinant baculovirus insect cell debris by incubating serum dilutions with nitrocellulose membrane pieces (~100 cm^2) which had been coated with the respective antigens as raw homogenates and then washed and blocked as for Western blots (36).

Challenge of rabbits with infectious CRPV. Challenge of immunized rabbits with infectious CRPV virus stock was performed according to a method used by the Christensen group (6, 9). The rabbits were in four groups: group 1 was three

animals immunized with transgenic plant-derived CRPV L1 protein, group 2 was three animals immunized with TMV-derived CRPV L1 protein, group 3 was a control group of four animals immunized with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) expressing an irrelevant rotavirus protein, and group 4 was a positive control group of five animals immunized with insect cell-derived CRPV VLPs. The two control groups received three immunizations at 2-week intervals as described previously (21). At 10 weeks after the final immunization, the two groups of animals immunized with plant-produced CRPV were challenged, while the two control groups were challenged 6 weeks after last inoculation. It has previously been shown by our group that the timing of challenge does not play a significant role in determining response within the parameters used in these experiments (21). All rabbits were challenged with infectious CRPV stock: each rabbit was inoculated at two sites for each dilution of the infectious virus stock (10^{-2} and 10^{-3}), and a 10^{-3} dilution of the virus stock produces papillomas at 50% of challenged sites (9). Papilloma size was measured as length by width by height in millimeters starting 14 days postchallenge. The geometric mean diameter (GMD) was calculated for each papilloma. The means and standard deviations for the GMDs in each treatment group were plotted against time after challenge with virus.

Western blot analysis of sera. CRPV L1 expressed in insect cells was denatured at 100°C for 10 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) gel loading buffer with reducing agents. The denatured protein extracts were resolved on a 12% SDS-PAGE gel and electrophoretically blotted (Trans-Blot Semi-Dry, Transfer Cell; Bio-Rad) onto nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in PBS for 1 h and incubated in prebleed sera or day 51 sera from rabbits inoculated with plant-produced CRPV L1 protein. The blot was probed with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma) at a dilution of 1:5,000, and binding was detected with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and 4-nitroblue tetrazolium chloride in substrate buffer (Roche).

Virus neutralization experiments. CRPV L1 pseudovirions were generated according to the protocol described by Pastrana et al. (33), with plasmids obtained from John Schiller (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Md.). Sera from inoculated rabbits collected on the last day of the experiment (day 51) as well as the preinoculation sera (day 1) were evaluated for their capability to neutralize the CRPV pseudovirions in vitro as described previously (33). Threefold dilutions of all pre- and postinoculation sera ranging from 1:25 to 1:12,150 were prepared, sera were incubated with CRPV pseudovirions, and the resulting secreted alkaline phosphatase (SEAP) content was determined by application of the Great ESCAPE SEAP chemiluminescence kit (BD Clontech) according to the manufacturer's instructions.

RESULTS

Genetic analysis of transgenic plants. Wild-type *N. tabacum* leaf disks were successfully transformed with *A. tumefaciens* carrying the pART27 CRPV L1 binary vector, and putative transgenic plants were regenerated and screened for the CRPV L1 gene by PCR. Integration of the L1 gene was confirmed in 5 out of 15 regenerated *N. tabacum* lines. R_0 generation plants were grown until sexual maturity and self-pollinated under controlled conditions, and the T_1 generation seeds were harvested. These were germinated on kanamycin-containing tissue culture media. Total genomic DNA was extracted from T_1 generation plantlets of the respective transgenic lines and again screened by PCR. A 1.5-kb amplicon was generated by PCR for all five transgenic lines, indicating the integration and confirming the inheritance of the CRPV L1 gene (data not shown).

Total RNA was extracted from individual transgenic plants and analyzed for CRPV L1 gene transcription. RT-PCR products resulting from the amplification of an internal gene fragment indicated that the integrated gene is transcribed in all confirmed transgenic plants (Fig. 1A).

Expression of CRPV L1 in TMV-infected plants. Plants were mechanically inoculated with synthesized transcripts of the respective recombinant TMV vectors and closely monitored for a period of 14 days. During the observation period, control plants infected with the pBSG1057 vector expressing the green fluorescent protein (GFP) were closely monitored using a UV lamp. GFP expression throughout all leaves of control plants was synonymous with the appearance of leaf curling and mosaic patterns.

On the day prior to harvesting the infected *N. benthamiana* plants (day 13), leaf disk samples were taken from each leaf, from the inoculated leaf to the last leaf on the apex, and total RNA was extracted. RT-PCR analysis was performed to confirm the presence of CRPV L1 mRNAs (Fig. 1B).

Low levels of expression of the CRPV L1 gene could be detected in the inoculated leaf through to the fifth leaf, after which it was not possible to detect the 421-bp amplicon (Fig. 1B). Concurrent RT-PCR analysis detected the 474-bp TMV coat protein mRNA in all leaves of infected plants, confirming the systemic spread of TMV from the inoculated leaf to the apical leaf (see Fig. 2B). This indicates deletion of the transgene in the vector; this has been seen previously with TMV expressing HPV-16 L1 (41).

Concentration of plant-derived CRPV L1 protein. Plant material was harvested, processed, concentrated, and characterized by direct ELISA using conformation-specific and neutralizing MAb CRPV:5A and surface linear epitope-specific MAb CRPV:10B. The TMV-infected plants were harvested 14 days postinoculation once expression of the CRPV L1 genes was confirmed by RT-PCR. The conformation-specific MAb CRPV:5A bound strongly to both plant extracts (optical density at 405 nm [OD_{405}] = 1 and 0.6 for transgenic and transient material, respectively), and MAb CRPV:10B slightly less so (OD_{405} = 0.5 and 0.2 for transgenic and transient material, respectively). The strong binding of the conformation-specific MAb CRPV:5A with the plant extracts suggests that most of the plant-derived protein is assembling into higher-order structures displaying appropriate conformational epitopes. Furthermore, the surface-linear-epitope-specific MAb CRPV:10B also bound strongly, indicating the presence of L1.

The amount of plant-derived CRPV L1 protein was measured by comparison to a standard curve of the OD_{405} values plotted against known concentrations of insect cell-derived CRPV L1 protein in the colorimetric ELISA (data not shown). Nontransgenic plant protein extract was spiked with insect cell-derived CRPV L1 VLPs, resulting in a known concentration of 0.12 μg per well (100 μl). The amount of CRPV L1 produced in transgenic plants ranged from 0.4 to 1 mg/kg, and CRPV L1 protein from infected *N. benthamiana* plants ranged from 0.15 to 0.6 mg/kg of total leaf mass.

Electron microscopy of plant-derived protein extracts. Preliminary experiments showed that the immunotrapping and decorating protocol efficiently trapped and labeled intact insect cell-derived VLPs and capsomeres (data not shown). Results from transgenic extracts (Fig. 2) show that both gold-labeled MAbs bound presumptive CRPV L1 protein, with no evidence of any VLP-like structures, but evidence of capsomeres or smaller aggregates (compare Fig. 2B and D with C and E).

Analyses of the TMV-derived CRPV L1 protein extract were qualitatively identical, with no distinct higher-order structures seen after trapping with the CRPV:5A MAb. Parallel analyses of proteins extracted from a noninfected *N. benthamiana* plant showed no MAb binding at all (Fig. 2C and E).

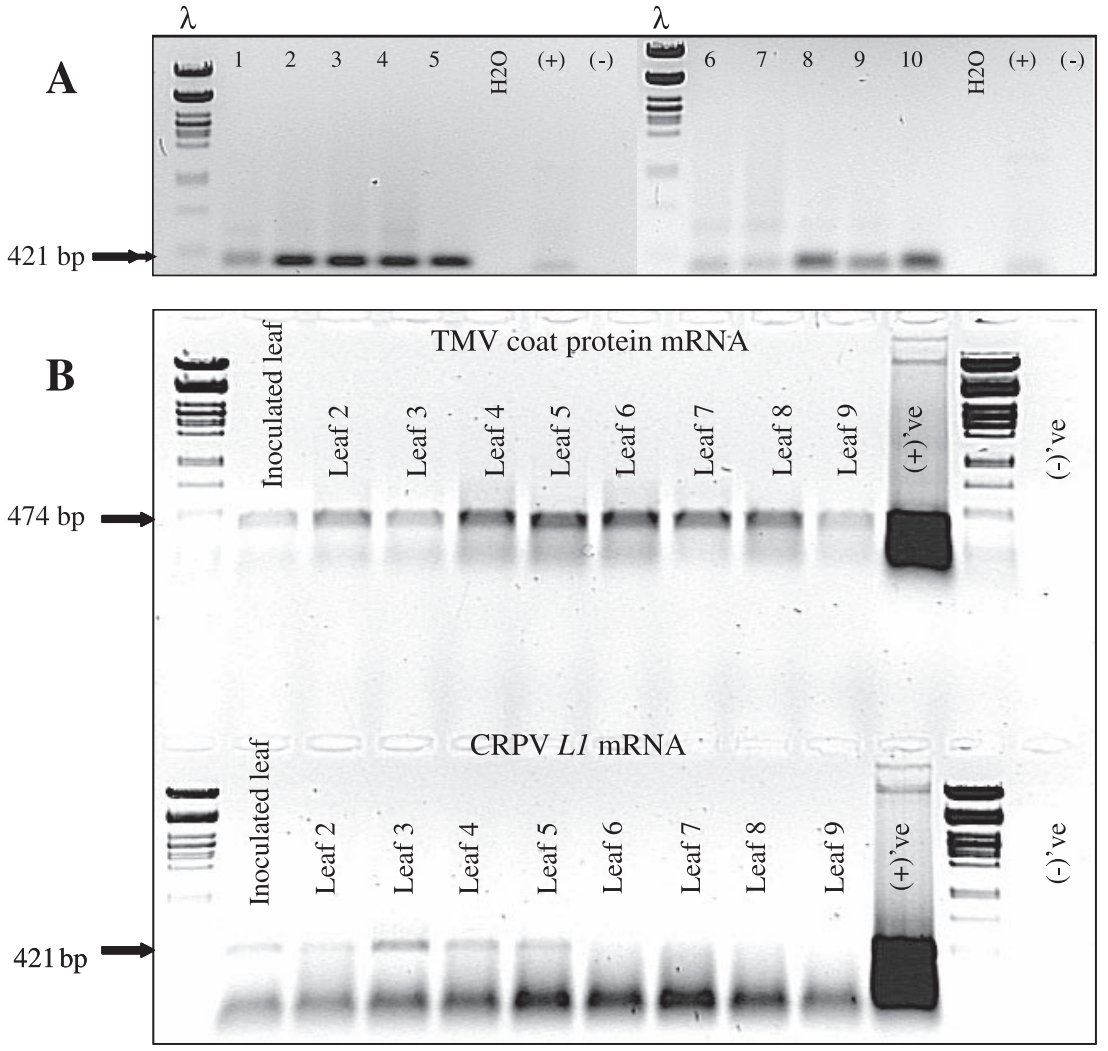

FIG. 1. RT-PCR RNA analysis of (A) PCR-positive transgenic *N. tabacum* plants from the T_1 generation and (B) *N. benthamiana* plants 13 days after inoculation with TMV-CRPV *L1* synthesized transcripts. In panel B total RNA samples were also analyzed for the TMV coat protein mRNA. The amplification of a 421-bp product by RT-PCR indicates the presence of the CRPV *L1* transcript within total plant RNA. Total RNA extracted from a noninfected/nontransgenic tobacco plant is the negative control; positive controls are synthesized transcripts prepared from pBSG-CRPV *L1* plasmid DNA.

Analysis of the immune response of animals. Two groups of three rabbits were immunized with transgenic-plant-derived (rabbits 901, 902, and 903) or transiently expressed (rabbits 904, 905, and 924) CRPV L1 protein extract. The initial 1-ml inoculum contained approximately 22 μg to 50 μg or 2 to 8 μg CRPV L1 from transgenic or recombinant-TMV-infected plant extract, respectively. Serum was evaluated by direct ELISA (1:20 dilution) against insect cell-derived CRPV L1 VLPs at a concentration of 0.12 μg/well (Fig. 3A and B).

These results show that immunization of the rabbits with transgenic-plant-derived CRPV L1 protein elicits a type-specific antibody response (Fig. 3A). Rabbit 902 shows the weakest response of the three animals on day 51. Rabbits 902 and 903 show increasingly elevated antibody responses to CRPV L1 VLPs over the course of 51 days in comparison to their respective antiserum reactivity on day 1. Analysis of the sera collected from rabbits inoculated with TMV-derived CRPV L1 protein is shown in Fig. 3B. Rabbit 905 showed the strongest reactivity for antisera collected on day 51. Although not to the same magnitude, a similar trend in increasing reaction levels was observed for the sera collected from rabbits 904 and 924.

To confirm the results presented in Fig. 4A and B, the preabsorbed prebleed (day 1) and last-bleed (day 51) sera collected from all six rabbits were tested by Western blotting against denatured insect cell-derived CRPV L1 protein. All the last-bleed sera collected from all rabbits injected with L1-containing protein extracts were capable of specifically binding denatured CRPV L1 protein (data not shown).

Rabbit challenge and CRPV pseudovirus neutralization assay. The data presented in Fig. 4 indicate that vaccination of rabbits with plant-derived CRPV L1 protein does protect from challenge with the higher dosage (10^{-2} dilution) of infectious virus. Over the course of 63 days, vaccination did not entirely prevent the growth of papillomas in one animal (903) but

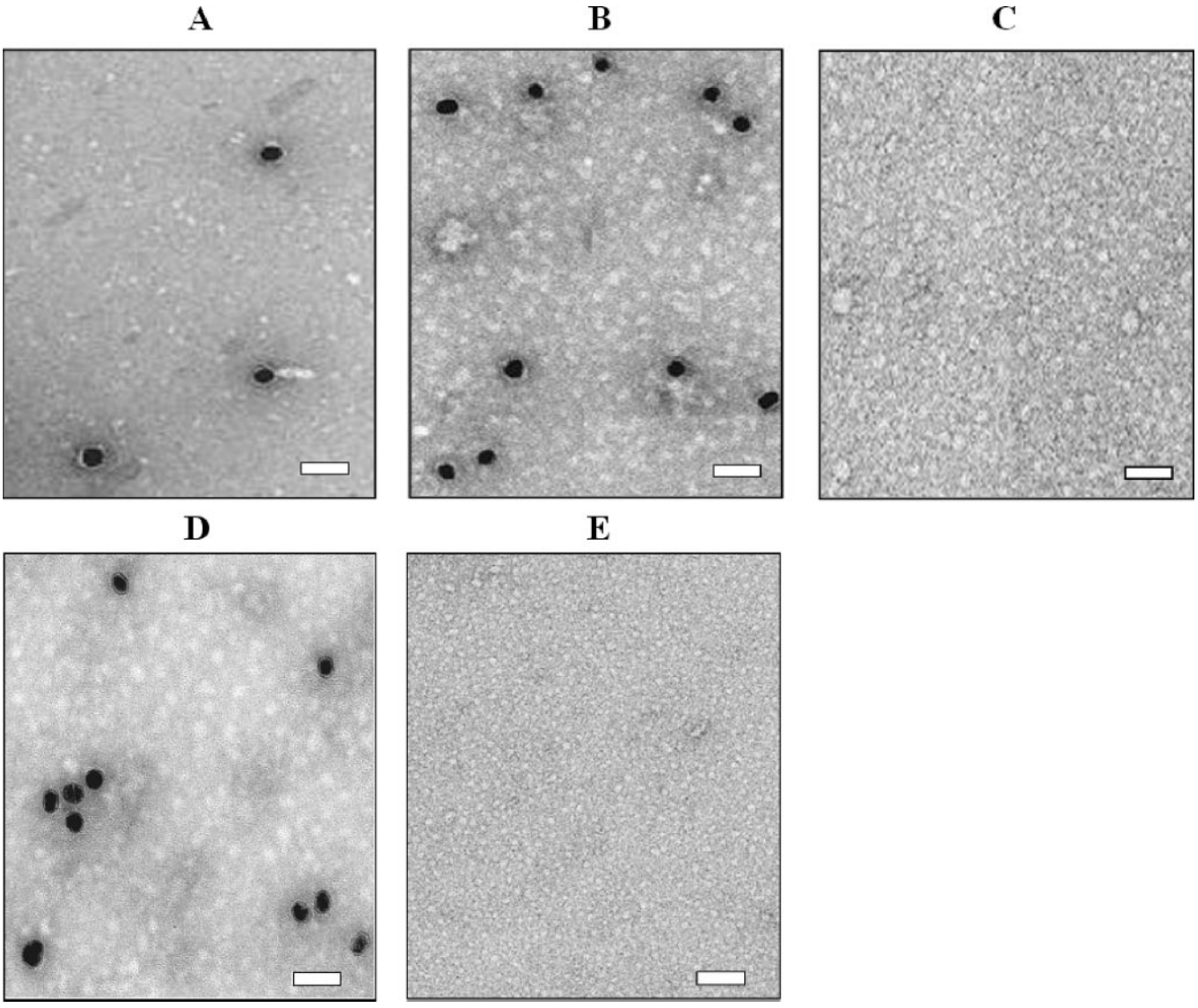

FIG. 2. Electron micrograph images of immunogold-labeled CRPV L1 protein extracted from transgenic *N. tabacum* (A and B). Plant protein extract was trapped onto carbon-coated copper grids using anti-CRPV L1 rabbit polyclonal antiserum, decorated with monoclonal antibodies CRPV:5A (A) and CRPV:10B (B), and detected with an anti-mouse gold-conjugated secondary antibody. Gold particles are 30 nm in diameter. (C) Nontransformed *N. tabacum* plant protein extract. (E) Noninfected *N. benthamiana* protein extract. (D) TMV-derived CRPV L1 protein extract immunogold labeled with CRPV MAb 5A. Scale bars = 100 nm.

drastically reduced the average size of papillomas. The other two rabbits (901 and 902) immunized with transgenic-plant extract formed no papillomas. Rabbit 905, immunized with TMV-derived extract, developed papillomas only on day 35 of this experiment. Measurements taken on day 35 showed papillomas of 0.5 mm in diameter. On the final day (day 63), this diameter had increased to approximately 1 mm. In contrast, the remaining two challenged rabbits (rabbit 904 and rabbit 924) had already developed papillomas on day 14. Measurements showed that the diameters of papillomas on the backs of rabbits 904 and 924 were around 0.8 mm and 1.45 mm, respectively. Over the period of 63 days these papillomas grew in size, resulting in measurements of 2 mm and 2.1 mm for rabbits 904 and 924, respectively. In comparison to the control group displaying an average papilloma size of 13 mm on day 63 (all four animals affected), the sizes of papillomas were reduced by approximately 6.5-fold in the group vaccinated with the transgenic- or TMV-infected-plant-derived CRPV L1 protein.

Serum collected from all animals was tested for the ability to neutralize CRPV pseudoviruses in vitro. Serum collected from naive rabbits before vaccination was also tested, and, as expected, the absence of type-specific antibodies in the serum resulted in the expression of alkaline phosphatase, thereby confirming the inability of the serum to neutralize the CRPV pseudovirus. However, no neutralization of the pseudovirus was observed in the postinoculation serum (day 51) harvested from all animals up to a dilution of 1/25. The included internal positive-control serum, collected from rabbits that were previously immunized with insect cell-derived CRPV VLPs, show neutralization of the virus up to and including the 1:450

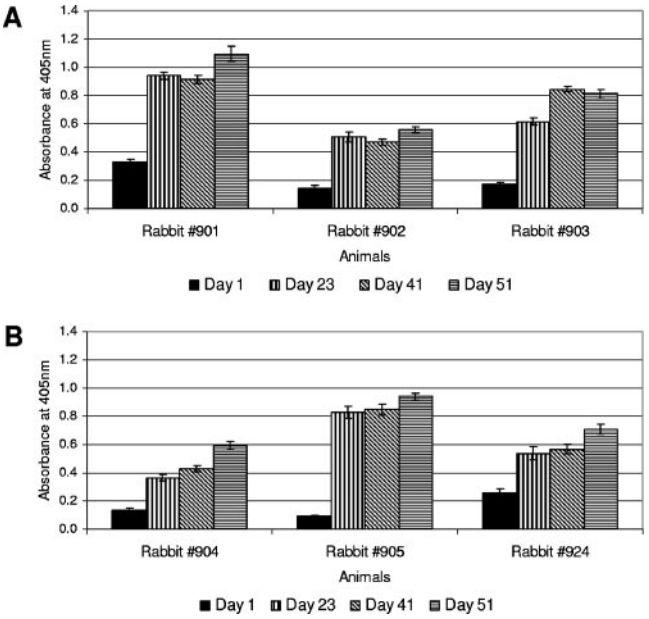

FIG. 3. Analysis of serum collected from New Zealand White rabbits inoculated with transgenic (A) or transiently derived (B) CRPV L1 protein extract. Serum collected over 51 days was tested against insect cell-derived CRPV L1 VLPs at a concentration of 0.12 μg/well.

dilution. The known CRPV conformation-specific and neutralizing MAb CRPV:5A, derived from a hybridoma, is capable of neutralizing the CRPV pseudovirus at a much higher dilution of 10^{-5}.

DISCUSSION

The aims of this study were to determine, first, whether or not wild-type CRPV L1 could be produced in plants; second, whether or not the plant-produced L1 protein was capable of eliciting a type-specific antibody immune response; and third, and most importantly, to see if this response could protect against challenge by the live virus. Here we report the first successful expression in plants of CRPV L1, both in transgenic *N. tabacum* and via transient expression in *N. benthamiana* using a TMV-based plant virus vector. While the protein did not appear to assemble into higher-order structures, it did react with conformation-specific and neutralizing MAbs and it did elicit CRPV L1-specific antibodies. While these were apparently not capable of neutralizing pseudovirion infectivity in the assay system, rabbits were protected against developing disease after virus challenge. This is therefore the first report of the efficacy of any plant-derived papillomavirus vaccine.

HPV L1s have been expressed in transgenic plants previously at a very wide range of concentrations: Biemelt et al. (1) achieved a yield of ~12 mg/kg of L1 from a human codon-optimized HPV-16 L1 gene in potatoes, Warzecha et al. (44) got ~20 μg/kg L1 VLPs from a plant codon-optimized HPV-11 L1 gene in potato tubers, Varsani et al. (40) got 4 μg/kg leaf tissue of HPV-16 L1 in tobacco from a native viral gene, and Liu et al. (29) got ~0.05% total soluble leaf protein (around 3 mg/kg in our hands) in tobacco from a presumably native gene. The expression level achieved for CRPV L1 in our transgenic tobacco (~1 mg/kg) was reasonable in light of these results, and especially so given that an unmodified viral gene was used. However, it is not up to the level (1% total soluble leaf protein)

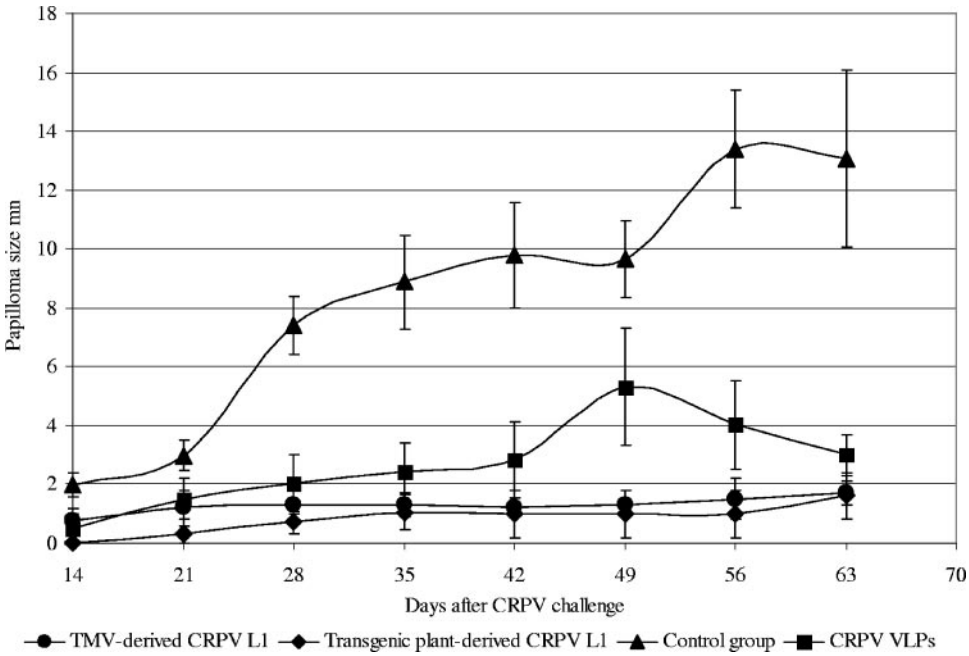

FIG. 4. Papilloma growth on the backs of rabbits following challenge with infectious CRPV. Papilloma sizes were measured weekly beginning at day 14 and the GMDs calculated. The mean GMDs and standard errors of the means of papillomas were plotted against time for the sites challenged with the 10^{-2} dilution of infectious CRPV. The control group was immunized with BCG expressing irrelevant rotavirus antigen. CRPV VLPs, rabbits immunized with purified CRPV L1 VLPs produced via baculovirus in insect cells. Data from rabbits 901 and 902, which showed no papilloma growth, are included.

considered as being the lower limit for commercial exploitation (15) and as justifying industrial purification. Increasing this expression level is not a trivial matter and is not apparently amenable to prediction; for example, Warzecha et al. (44) achieved only very low expression levels of a plant codon-optimized HPV-11 L1 gene in transgenic potatoes, while our group has achieved much higher levels in transgenic tobacco using a native viral HPV-11 L1 gene (25). Biemelt et al. (1) also found a human codon-optimized HPV-16 L1 expressed far better than a native or a plant-optimized version.

Given that various virus-based transient expression systems are known to allow for the synthesis of large amounts of antigen within a short period of time (15, 16), we investigated the TMV-based transient expression system to determine if we could obtain higher yields of protein. Our group has also expressed HPV-16 L1 transiently in *Nicotiana benthamiana* via the same TMV vector as used here; this allowed a 10-fold increase in production over the transgenic case (41). However, yields of CRPV L1 were lower than those obtained with transgenic plants (~0.4 mg/kg versus ~1 mg/kg); this was unexpected, but virus-based expression systems do have the disadvantage that the gene needs to be stably expressed, which is not always guaranteed. Our results showed that the mRNA for CRPV L1 was expressed only in the first five leaves, while the TMV coat protein mRNA was expressed in leaves 1 (inoculated) through 9. This suggests that the CRPV L1 gene must have been excised and that the recombinant virus had reverted back to wild type. Rabindran and Dawson (34) have previously shown, using the same vector, that sequences were deleted from the recombinant virus on passage from plant to plant, resulting in loss of GFP expression; this also happens with the wild-type HPV-16 L1 gene in recombinant TMV (41). Donson et al. (13) found evidence that the size of the inserted gene was the direct cause of vector instability, and the L1 gene is at the upper limit of sequences to have been expressed successfully in this vector (K. E. Palmer, Large Scale Biology Corp., personal communication).

While the expression levels of the L1 protein in plants in this work were too low for commercial exploitation, both the expression systems used here can be significantly improved: our group has shown in other work that it is possible to achieve yield increases in transient and transgenic expression of HPV-16 L1 of several orders of magnitude, by an optimization procedure involving a combination of vector choice, subcellular localization, and codon optimization (J. Maclean, M. Koekemoer, A. J. Olivier, D. Stewart, I. Hitzeroth, T. Rademacher, R. Fischer, A.-L. Williamson, and E. P. Rybicki, unpublished results).

The transgenic-plant- and TMV-derived CRPV L1 protein apparently does not assemble into VLPs, but apparently only into capsomeres and possibly other aggregates (Fig. 3A); this is in contrast to the reported assembly of the HPV L1 proteins into 55-nm VLPs in plants and the proven assembly of the CRPV protein expressed from the same gene in insect cells in our hands. This is most probably due to low protein concentrations that are not capable of driving the equilibrium towards the assembly of VLPs; this has been postulated as the reason for the predominance of lower-order structures in HPV L1 transgenic plants (1, 40, 41). However, in this case it appears as though the assembly of CRPV capsids requires a significantly higher L1 concentration than do either HPV-11 or HPV-16 VLPs, given that VLPs could be seen in tobacco expressing only 4 μg/kg of HPV-16 L1 (40). While the electron microscope study detected no obvious higher-order structures bigger than pentamers (~10 nm in diameter), the trapping of these and immunogold labeling of protein by the conformation-specific and neutralizing MAb CRPV:5A are proof that the protein assembled into an antigenically appropriate form. We note that, while papillomavirus VLPs are the "gold standard" in current vaccine development, capsomere-only vaccines have also been shown to be effective in eliciting both neutralizing antibodies (35) and cytotoxic T lymphocytes capable of causing tumor regression (32).

A relatively strong CRPV L1-specific immune response was elicited by the plant-derived protein in all rabbits immunized. It was noteworthy that, in the case of the TMV-derived protein, immunization with only 2 to 8 μg of L1 protein per dose elicited an immune response equivalent to the ~30 μg of L1 derived from transgenic plant material. While Zhang et al. (45) have demonstrated that HPV-6b VLPs are potent immunogens in the absence of adjuvant, our immunogen was not in this form. However, Franconi et al. (17, 18) have found that crude extracts of *N. benthamiana* sap containing HPV-16 E7 protein expressed via a potato virus X vector have a potent natural adjuvant activity, which could potentially be exploited to the advantage of plant-expressed vaccines. The same activity probably contributed to the response in our rabbits.

We further determined whether the immune response in rabbits resulting from inoculation with plant-derived CRPV L1 protein was capable of protecting the animals from wart development upon live-virus challenge. Results were obtained which compared very favorably with the baculovirus-produced CRPV L1 VLP positive-control model used both here and in a parallel study (21), with all immunized animals being effectively protected from disease. This compares well with other studies of the efficacy in animals of plant-derived vaccine antigens, such as the protection of rabbits from lethal challenge with rabbit hemorrhagic disease virus by vaccination with VP60 protein produced in *N. benthamiana* via potato virus X (14) and the protection of beagles from infection by canine parvovirus (CPV) by administration of a UV-inactivated recombinant cowpea mosaic virus displaying a CPV VP2 capsid protein epitope (17 amino acids) fused to the coat protein and displayed on the surface of the virus particle (26). Moreover, ours is the first successful test of efficacy in an animal model system of a plant-produced vaccine equivalent to vaccines of proven efficacy for a major human disease pathogen (22, 42) and as such represents an important proof of concept.

Protective immunity against HPV in particular, but also against all PVs tested so far, is assumed to be due to the presence of virus-neutralizing antibodies (30). Various animal immunization and subsequent challenge experiments performed using PV subunit vaccines on rabbits, cattle, and dogs have been found to protect their respective hosts from challenge with live virus and have resulted in the induction of virus-neutralizing antibodies (4, 24). However, in this study we were unable, using a relatively new PV pseudovirion neutralization assay, to demonstrate the presence of infectivity-neutralizing antibodies in the sera of immunized rabbits that were subsequently shown to be protected against CRPV infection,

albeit in antiserum diluted ≥25-fold. In our hands it has proved difficult to prove neutralizing activity in anti-PV L1 sera diluted <50-fold, and it may be that the older live-virus infectivity neutralization tests are more sensitive (D. Stewart, unpublished results). We note that Suzich et al. (38) found only a low titer of neutralizing antibodies in dogs protected by L1 vaccination against canine oral papillomavirus infection and cautioned that the levels of virus-neutralizing antibodies might be underestimated. Indeed, others in our research group have previously demonstrated protection of rabbits against CRPV infection by vaccination with BCG-vectored CRPV L1, with only very weak demonstration of neutralizing antibodies by the same assay as used here (21). It is also possible that protection from virus challenge in our case could be by stimulation of the cell-mediated immune response, as this has been argued to be an underappreciated component of PV immunity and clearance of warts and other lesions or tumors (10, 31, 32); however, this was not explicitly investigated.

In conclusion, this is the first report that shows that the plant-produced papillomavirus L1 protein can be used as a vaccine capable of protecting against challenge by live virus. This is a valuable first step towards the production of an HPV vaccine by a system that is cheap enough to be used in and for developing countries.

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