Expression of Human papillomavirus type 52 L1 capsid gene in Oryza sativa involved in cytoprotective activities

Kuan-Hung LIN¹, Shwu-Fen PAN², Chiu-Chen CHEN², Wen-Shian LI², Chih-Ming CHIANG²*  

¹Chinese Culture University, Department of Horticulture and Biotechnology, Taipei 11114, Taiwan; rlin@faculty.pccu.edu.tw  
²Ming Chuan University, Department of Biotechnology, Taoyuan 333, Taiwan; sfpan@mail.mcu.edu.tw; cmchiang@mail.mcu.edu.tw (*corresponding author)

Abstract

Female cervical cancer is largely formed by Human papillomavirus (HPV), the second leading cause of cancer deaths in women worldwide. HPV-52 is a regionally common high-risk type of cervical cancer found mostly in Asia and reveals geographical variations, in order of importance, as types HPV-16 and -18. However, the differing propensities of HPV types in progressing to cancer, focusing on HPV-52 vaccines, are limited. Several plant-based vaccines against cancer have been developed, and the production of candidate HPV therapeutic vaccines using plant-derived expression platforms is also proven. The objectives of this study were to assess the HPV-52L1 Capsid gene by transferring HPV-52L1 Capsid cDNA into rice (Oryza sativa L.) via an Agrobacterium-mediated transformation, and accumulating HPV-52L1 Capsid proteins in a plant-based expression system to maintain and improve antigenicity. Crude protein extracts containing 5~20 μg from OsHP-52L1 transgenic lines induced cell death and significantly reduced cell proliferation in HPV-positive HeLa cervical cancer cells compared with those non-transformant (NT) rice plants. However, no significant cytotoxicity of induced human breast MDA-MB-231 cell proliferation (as negative control) was observed at any dose compared with NT groups. HeLa cells ameliorated the effects of OsHPV crude protein extracts on cell viability as the extract concentration increased, and treatment with 20 μg of the extract from OsHPV-3 significantly reduced cell viability in HeLa cells (26%) compared with the control group (57%). Our results can be used for exploring the potential of plants for increasing the immunogenicity of OsHPV-52L1 Capsid DNA vaccines, and support the development of cost-effective HPV vaccines, which is highly desirable for resource-poor countries.

Keywords: cervical cancer; Human papillomavirus; Oryza sativa; plant vaccine; type 52 L1 capsid protein

Abbreviations: HPV, Human papillomavirus; MTT, 3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide; NT, non-transformant; OsHPV, Oryza sativa Human papillomavirus; VLPs, virus-like particles
Introduction

Human papillomavirus (HPV) related cancers account for 5% of all human cancers (Chabeda et al., 2018). At least 170 HPV genotypes have been identified, and more than 15 of them are considered to have carcinogenic potential in organs such as the cervix, vulva, vagina, penis, and anus, and can also cause cancer in subsets of oropharyngeal carcinogenesis or tumors of the base of the tongue and tonsils (Cardona and García-Perdomo, 2018). HPV infects mucosal and cutaneous basal epithelial cells after tissue microtrauma (Kines et al., 2009). HPV is a small non-enveloped double-stranded DNA virus with a genome size of approximately 8 kb encoding for six early (E) regulatory proteins and two late (L) structural proteins (Conway and Meyers, 2009). The virus capsid consists of major and minor proteins, L1 and L2, respectively, and L1 assembles into virus-like particles (VLPs) in the L2 minor capsid protein (de Villiers et al., 2004). VLPs retain the immunological properties of native papillomaviruses and produce high titers of neutralizing antibodies when used as a vaccine (Karanam et al., 2009; Schellenbacher et al., 2017). Currently available preventive vaccines against HPV are based on VLPs prepared by the recombinant expression and assembly of L1, and different types of prophylactic vaccines based on the immune-dominant L protein are currently on the market and are effective in preventing cervical disease (Naud et al., 2014; Huh et al., 2017). However, the cost of HPV vaccines remains expensive due to the systems of manufacture, meaning that even if a vaccine were commercialized, its cost would render it less accessible to populations in developing countries where the burden of cervical cancer is highest (Roden and Stern, 2018; Hefferon, 2017). Chen et al. (2018) indicates that the insertion or substitution of several peptides into several L1 surface loops does not affect chimeric VLPs assembly, with both anti-L1 and anti-L2 responses observed. Although HPV infection is preventable through very efficient recombinant vaccines developed against variously incident oncogenic genotypes in yeast and insect cells, and despite cervical cytology and DNA testing, HPV-related preinvasive and invasive diseases remain critical public health problems. Furthermore, currently available treatments against HPV-related disease are only moderately successful, with radiotherapy, chemotherapy, and surgery being very poorly efficient against high-grade lesions (Cordeiro et al., 2018).

Plants provide a convenient protein production platform for potentially reducing the cost of vaccine production compared to traditional microbial fermentation or mammalian/insect cell expression systems. This approach is especially advantageous in the field of prevention and treatment of infections and cancer (Loh et al., 2017). The expression of therapeutic proteins using in vitro plant systems under contained conditions represents a profitable manufacturing approach in terms of uniform cultivation conditions, product quality, and downstream purification processing (Santos et al., 2016; Massa et al., 2018). Several plant-made vaccines against cancer have been developed using viral vectors and peptide/protein-based strategies, and it is thought that these have the potential for reaching the market (Wong-Arce et al., 2017). Among protein-based formulations, the production of candidate HPV therapeutic/prophylactic vaccines using plant-derived expression platforms is also proven. Different plant-based expression systems were considered, from whole plant approaches for transient expression to stably transformed green microalgae, using single HPV antigens or fusion to peptides to improve accumulation yield, to intracellular targeting strategies (Chabeda et al., 2018). HPV are categorized into two groups: low-risk types, including HPV-6/11/40/42/43/44/54/61 and -72, and high-risk types including HPV-16/18/31/35/39/45/51/52/56/58/66 and -68, which is responsible for 99.7% of all cervical cancer cases (Parkin and Bray, 2006). HPV VLPs have been successfully produced in plants via transient expression to stably transformed green microalgae, using single HPV antigens or fusion to peptides to improve accumulation yield, to intracellular targeting strategies (Chabeda et al., 2018). HPVs are categorized into two groups: low-risk types, including HPV-6/11/40/42/43/44/54/61 and -72, and high-risk types including HPV-16/18/31/35/39/45/51/52/56/58/66 and -68, which is responsible for 99.7% of all cervical cancer cases (Parkin and Bray, 2006). HPV VLPs have been successfully produced in plants via transient expression to stably transformed green microalgae, using single HPV antigens or fusion to peptides to improve accumulation yield, to intracellular targeting strategies (Chabeda et al., 2018). HPV are categorized into two groups: low-risk types, including HPV-6/11/40/42/43/44/54/61 and -72, and high-risk types including HPV-16/18/31/35/39/45/51/52/56/58/66 and -68, which is responsible for 99.7% of all cervical cancer cases (Parkin and Bray, 2006). HPV VLPs have been successfully produced in plants via transient expression to stably transformed green microalgae, using single HPV antigens or fusion to peptides to improve accumulation yield, to intracellular targeting strategies (Chabeda et al., 2018).
intraperitoneally (Kohl et al., 2007). Higher yields of HPV-16L1 and VLPs were observed via agroinfiltration-mediated transient expression or via chloroplast expression (Fernandez-San Millan et al., 2008). Heterologous DNA prime-protein followed by recombinant protein boost regimens can be used as a tool for envisaging new therapeutic options in HPV-associated infection and cancer (Peng et al., 2016). Plant DNA vaccines deliver genes encoding protein antigens into host cells, enabling their production in vivo. Vici et al. (2016) reported that genetic immunotherapy has become a pharmacological tool and therapeutic option against cervical disease, with HPV DNA vaccines reaching encouraging results in phase II clinical trials. Moreover, genes encoding tumor-associated antigens and viral coat proteins of HPV can be expressed in plants that not only retain their native immunological activity but also receive adjuvant activity from the plant extract itself (Chabedaa et al., 2018). Safety, efficacy, and potential immunogenicity are also features of plant DNA vaccines targeting HPV (Franconi et al., 2010).

HPV-52 is a regionally common high-risk type of cervical cancer found mostly in the Asia-Pacific region, and reveals geographical variations as, in order of importance, HPV-16 and -18 (Parkin et al., 2008). Nevertheless, the differing propensity of HPV types for progressing to cancer (focusing on HPV-52) is limited. A pre-screening system for the production of plant-based HPV-52L1 vaccines for cervical cancer needs to be established. Therefore, the objectives of this study were to assess the HPV-52L1 Capsid gene by transferring HPV-52L1 Capsid cDNA into rice (Oryza sativa L.) via an Agrobacterium-mediated transformation, and accumulating HPV-52L1 Capsid proteins in a plant-based expression system for maintaining and improving antigenicity. An MTT assay was used to analyze cell growth in HPV-positive HeLa cervical cancer cells treated with OsHPV crude protein extracts, and the effects of these extracts on the cytoprotective activities and cell death of HPV-positive cervical cancer cells were determined. The medical potential for a transgenic plant protein as the cytotoxic component of an immuno-toxin provided further stimulus toward the development of a candidate HPV therapeutic vaccine, thus expanding the nature of the possible immune-enhancers of HPV-52L1. The characterization and functional analysis of HPV-52L1 genes should facilitate our understanding of the cytotoxic-response mechanism in transgenic rice plants for the bioproduction of a candidate therapeutic vaccine endowed with a specific cell-mediated response associated with anticancer activity against HPV in a HeLa cell model.

Materials and Methods

Plant materials

Japonica rice ’Tainung’ (TN) 67 is one of the most widely grown rice cultivars in Taiwan. ’TN 67’ seeds were sterilized with 1.5% (v/v) sodium hypochlorite, rinsed with distilled-deionized (dd) H2O, sown into 3.6-inch plastic pots containing a commercial potting soil mixture, and grown in a growth chamber under 450 μmol m−2 s−1 light with a 16 h photoperiod and 28/20 °C (16 h day/8 h night) temperatures at a relative humidity of 80%. Plants were watered three times a week, and an optimal amount of compound fertilizer solution (N-P2O5-K2O, 20-20-20) was applied once a week.

Reverse transcription (RT)-polymerase chain reaction (PCR) analysis of HPV52L1 Capsid gene expression and amplification of cDNA

Total RNA was isolated from 0.1 g of ’TNG67’ leaves with a Qiagen RNeasy Plant Mini Kit (Valencia, CA, USA) and then poly (A)+ mRNA was extracted from total RNA with a Qiagen Oligotex Mini Kit according to vendor instructions. Paired primer sequences HPV52L1-5F (ACCGTCGACATGCTACAGATTTTATTCTTA) and HPV52L1-3R (GCGCAGCTCTACCTTTAACCCTTTTCT) were used for amplification. Original HPV-52L1
coding sequences with adapted codon usage for expression in yeast can self-assemble into VLPs and used in a VLP-based vaccine (Bryan et al., 2010). The PCR was carried out in an Eppendorf Mastercycler Gradient Thermal Cycler (Hamburg, Germany) with the following thermal program: initial denaturation at 94 °C for 5 min, followed by 25 cycles at 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The products were electrophoretically separated on 1.5% agarose gel, the predicted size of 1.59 Kb of the HPV 52L1 gene (accession no. FJ615303.1) was verified with a 1 Kb DNA ladder marker, and the sequences were checked. Contamination of RNA samples by genomic DNA was excluded by an additional PCR reaction without preceding reverse transcription.

**Plasmid construction and gene cloning**

*Escherichia coli* strain TOP 10 (Invitrogen, Carlsbad, CA, USA) and the destination vector pPZP200/Ubi-HA-ccdB-Nos-35S-hpt-tml (Chiang et al., 2017) were respectively used for gene construction and transformation. Briefly, HPV52L1 was constructed by the Invitrogen pENTR/D-TOPO Expression Vector System for plant transformation. HPV-52L1 was amplified with the above-described paired primers (HPV52L1-5F and -3R) from the RT-PCR using high-fidelity DNA polymerase (New England Biolabs, Beverly, MA, USA). HPV52L1 fragments were amplified to 1.59 kb, in which water was used as a negative control. The PCR product was purified using the SNAP Gel Purification Kit (Invitrogen), ligated to the pENTR TOPO vector, and transformed into TOP10. After pENTR cloning, the constructed plasmid, named pENTR-OsHPV52L1 (Supplemental Figure S1A), was cloned into the destination vector, pPZP200/Ubi-HA-ccdB-Nos-35S-hpt-tml. After Spectinomycin screening of the colony, plasmid insertion was confirmed by PCR, and DNA sequences of the pPZP200/Ubi-HA-HPV52L1-Nos (pPZP200-OsHPV52L1) clones were confirmed (Supplemental Figure S1B).

**Agrobacterium transformation and genomic PCR analysis**

A freeze-thaw method was used to transform plasmids in *Agrobacterium tumefaciens* strain EHA105, and *Agrobacterium*-competent cells were prepared for transformation according to Jyothishwaran et al. (2007). Briefly, agrobacterium-containing plasmid pPZP200/OsHPV52L1 was used for rice transformation. Transgenic seedlings were selected on MS medium with 50 mg/l of hygromycin. Leaves of T1 transgenic rice were ground to a fine powder with a mortar and pestle in liquid nitrogen. Genomic DNA was prepared essentially as described by Doyle and Doyle (1990). A PCR analysis was performed using the specific primer hygromycin phosphotransferase (HPT)-5F (GCTGGGGCGTGTTTCC) and HPT-3R (CACACCGGACGTCTGTC) to amplify the hpt gene, and the annealing temperature was 55 °C. PCR was carried out in an Eppendorf Mastercycler Gradient Thermal Cycler with a thermal program consisting of an initial denaturation at 94 °C for 5 min, followed by 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s, with a final extension at 72 °C for 10 min. PCR products were electrophoretically separated on a 1.5% agarose gel, and the predicted size of 987 bp hpt was verified with a 100 bp DNA ladder of a DNA marker. Positive transgenic plants were chosen for further biological and physiological analysis.

**Western blot analysis**

The protocol for protein extraction was modified from Wang et al. (2006). In brief, total soluble proteins were prepared from 300 mg of fresh seedling leaves from both transgenic and NT plants and quantified with a Bradford Protein Assay Kit (BioRad, Hercules, CA, USA). Forty micrograms of total protein in each sample were electrophoresed on a 4% stacking gel and 12% resolving gel (sodium dodecylsulfate polyacrylamide gel electrophoresis; SDS-PAGE) on Mini PROTEAN III equipment (Bio-Rad). Following electrophoresis, proteins were transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA, USA). The membrane was incubated at 4 °C overnight with a mouse anti-HA antibody (Sigma H9658). Bands were detected with anti-mouse immunoglobulin G (IgG) peroxidase-conjugated secondary antibody for 1 h. The
3,3',5,5' tetramethyl benzidine (TMB) substrate from the TMB Kit (BioLegend, San Diego, CA, USA) was used for staining, as it is catalyzed by peroxidase to produce a pale-blue color.

**Crude protein extracts from OsHPV transgenic plants**

Surface-sterilized NT and T3 transgenic seeds were placed in MS agar media without and with 50 mg/L hygromycin and 2 mg/L 2,4-D, respectively, induction callus for 3 weeks at 28/20 °C, 16 h light/8 h dark cycle. Fifty milligrams of callus from both transgenic and NT plants were extracted with 450 µL of CCLR buffer containing 100 mM K-phosphate pH 7.8, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and 7 mM 2-mercaptoethanol (Liao et al., 2016). The crude protein was centrifugated and the supernatant collected. The amount of crude protein concentration was determined in an optical density of 595 nm with a Protein assay Dye Reagent Concentrate (BioRad 500-0006) using a spectrophotometer (U-2000, Hitachi, Tokyo, Japan). Commercial bovine serum albumin (Sigma A9418) was used as a standard.

**Cell culture and a 3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity test**

HeLa cervical cancer cells and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum and a 1% penicillin/streptomycin mix (Invitrogen, Camarillo, CA, USA) in 10 cm Petri dishes at 37 °C in a 5% CO₂ environment as previously described (Cory et al., 1991). Cells were seeded in a 96-well plate at a density of 1500-2000 cells per well and incubated at 37 °C. After overnight incubation, cells were treated with different concentrations (5, 10, 15, and 20 µg/mL) of crude protein extracted from OsHPV T3 transgenic plants dissolved in DMEM (containing 0.3% dimethyl sulfoxide, DMSO) and the final volume of each well was adjusted to 50 µl with growth media. The cytotoxic effects of the OsHPV crude protein extracts against HeLa or MDA-MB-231 cells were determined. After 24 h incubation, 50 µL of tetrazolium/formazan (MTT, 0.5 mg/mL, Sigma-Aldrich, Merck KGaA, USA) was added into a subset of wells. After 4 h incubation, cells were washed with phosphate-buffered saline (PBS) and dissolved in 120 µL of DMSO, and absorbance at 560 nm (A₅₆₀) was measured in an ELISA Reader (SpectraMax 190, San Jose, CA, USA) for cell viability. Cell viability (%) = [(A₅₆₀ of the treated sample - A₆₅₀ of the treated sample) / (A₅₆₀ of the blank - A₆₅₀ of the blank)] x 100%. The blanks were cells with no added H₂O₂ or sample extract.

HeLa cells were treated with 5, 10, 15, and 20 µg of T3 OsHPV crude protein extracts for 24 h. Pictures of five different fields were captured at 0 h and 24 h under an inverted microscope (Axiovert 100M, Carl Zeiss, Champaign, IL, USA) at 100x magnification (Schug et al., 2014).

**Statistical analysis**

Statistical analyses of ELISA data were performed using the one-way analysis of variance (ANOVA) with the least significant difference (LSD) test at p < 0.05 using the SAS program ver. 9 (SAS Institute, Cary, NC, USA).

**Results**

**Identification and analysis of transgenic plants by genomic PCR, RT-PCR, and Western blotting**

After transformation, seeds were collected and germinated on MS medium containing 50 mg/l of hygromycin for 7 days. Transgenic seedlings were then transplanted into soil for continued growth. Transgenic seeds were then collected, oven-dried, and stored at 4 °C until use. Transformation efficiency of hygromycin-
resistant OsHPV transgenic plants was 1% (Table S1). Five healthy, robust, independent hyg-resistant T1 transgenic lines of rice were generated and designated OsHPV-1 to -5, and used for the production of T2 and T3 progenies. T3 transgenic lines were detected by genomic DNA PCR amplification and electrophoresis, and all transgenic lines displayed the expected size (987 bp) of the hygromycin gene (Figure S2)

To investigate whether the OsHPV gene was overexpressed in transformed rice, an RT-PCR analysis was performed with extracted RNA from 7-day-old NT and transgenic plants. Figure 1 shows that all transgenic T3 lines except OsHPV-5 presented different expression levels, and OsHPV transcript was not detected in NT plants. Therefore, transgenic line OsHPV-5 was not used for the below-described Western analysis. Tubulin (TUB), a housekeeping gene consistently expressed in plants (212 bp), was used as an internal control.

**Figure 1.** Transcription reverse transcriptase (RT)-PCR analysis of rice human papillomavirus (OsHPV) lines 1~5 compared to a non-transgenic (NT) rice plant (upper panel). The tubulin gene (TUB) was used as an internal control and transcripts were exhibited in all T3 transgenic and NT plants (lower panel). The expected sizes of OsHPV and TUB at 1.59 kb and 212 bp, respectively, are indicated by an arrowhead. Total RNA in all tested plants was extracted from 7-day-old plants.

**Figure 2.** Western blot analysis of the OsHPV lines 1~5 and NT plants (anti-HA antibody). Forty micrograms of total protein were used for each sample. M, Molecular Weight Standard Mixture (Sigma) used during SDS-PAGE separation, and the molecular weight of T3 OsHPV at 59 kDa is indicated by an arrowhead.
To estimate the relative amounts of OsHPV protein accumulating in transgenic lines, a Western blot analysis was conducted followed by using antiserum anti-HA to identify OsHPV expression. OsHPV transcripts responded differently in the various transgenic lines (Figure 2). A clear band (59 kDa) appeared in all transgenic lines (OsHPV-1, -2, -3, and -4), but this protein was not detected in NT plants. Thus, these four lines presenting high expression levels were selected for further work, although OsHPV-2, -3, and -4 showed higher accumulations of the OsHPV protein than OsHPV-1.

**OsHPV crude protein extracts reduce cell proliferation in HeLa and MDA-MB-231 cells**

The effects of the OsHPV1~4 crude protein extracts at four concentrations (5, 10, 15, and 20 μg) on the viability of HeLa and MDA-MB-231 are shown in Figure 3, and the extracts exerted different cytotoxicity effects on the proliferation of HeLa and MDA-MB-231 cells. Treatments with OsHPV crude protein extracts in any concentration significantly reduced the proliferation of HeLa cells compared with the NT group (Figure 3A); however, no significant cytotoxicity of induced MDA-MB-231 cell proliferation was observed at any dose compared with the NT group (Figure 3B). It was also revealed that crude protein extracts from OsHPV transgenic plants inhibited the proliferation of HeLa cells in a dose-dependent manner, and HeLa cells ameliorated the effects of OsHPV crude protein extracts on cell viability as the extract concentration increased. A lower cell viability value indicates higher cytoprotective activity by the OsHPV crude protein extracts. In particular, treatment with 20 μg of the extract from OsHPV-3 tremendously reduced the viability of HeLa cells (26%) compared with the control group (57%).

![Figure 3](image.jpg)
Immune responses of the OsHPV crude protein extracts (5, 10, 15, and 20 μg) against cell metabolic activity in HeLa cells by microscopy are given in Figure 4, and in general, OsHPV crude protein extracts stimulated a HPV52L1-specific cytotoxic T cell immune response. Live HeLa cells of about 40~50 μm in diameter were anchored on the plate; however, dead cells became floating black spots about 20~30 μm in diameter. The concentrations with 5~20 μg of the protein extracts seemed to affect the viability of HeLa cells in a dose-dependent manner. HeLa cells treated with 20 μg of the extract (Figure 4E) had higher cell death counts and were reduced in size compared to those treated with 5~15 μg of the extracts (Figure 4 B-C). Compared with NT (Fig. 4A), OsHPV crude protein extracts showed significant effects on HeLa cells.

Figure 4. HeLa cells following treatments with 5, 10, 15, and 20 μg of NT and T3 OsHPV crude protein extracts for 24 h, followed by photographs at 100x magnification. Arrows indicate the putative dead HeLa cells observed. (A) No treated crude protein extracts HeLa cells as the negative control, (B) five microgram of crude protein extracts from NT and OsHPV1~4, (C) ten microgram of crude protein extracts from NT and OsHPV1-4, (C) fifteen microgram of crude protein extracts from NT and OsHPV1-4, (D) twenty microgram of crude protein extracts from NT and OsHPV1-4, and (E) twenty microgram of crude protein extracts from NT and OsHPV1-4. Scale bar indicates 50 μm

Discussion

In an attempt to produce a valid immunotherapy at low cost for HPV-associated lesion/cancer, our study focused on the development of low-cost platforms such as DNA and plant biotechnologies. Plants can
be utilized as bio-factories of immune stimulators to produce tailor-made and potentiated formulations. OsHPV cDNA was over-expressed in rice plants under the control of the ubiquitin constitutive promoter. Studying gene expression at the level of RNA abundance can give a reliable estimate of gene activation. Different transgenic lines present different expression levels, and the RNA expression levels of OsHPV-1, -2, -3, and -4 lines were up-regulated compared to NT plants. However, the OsHPV gene transcript was not detected in the OsHPV-5 transgenic line, and this could be due to gene silencing. Hsing et al. (2007) reported that after Agrobacterium transformation, > 75% of transgenic plants contained one or two copies inserted and had a lower chance of inhibiting the expression of the transgene due to RNA-induced gene silencing. The highly expressed RNA levels also represented high accumulations of the OsHPV protein in OsHPV-2, -3, and -4 transgenic lines, whereas the relatively lower level of protein expressed was detected in the transgenic OsHPV-1 line. Observed variations in relative RNA abundances may have resulted from changes in random T-DNA tagging from Agrobacterium-mediated transformation (Kim et al., 2007) or RNA lifetimes (transcript-specific stabilization or degradation), resulting in each line unequally protecting plants against the tested cell’s cytotoxic activity. Transformants that possessed a T-DNA in the proximity of an endogenous matrix attachment region (MAR) sequence (e.g., OsHPV-2~4) differed in their expression profiles from those that did not (e.g., OsHPV-1). Therefore, it is not possible to conclude whether integration in close proximity to endogenous MAR sequences is necessary for high transgene expression (De Buck et al., 2004).

Plant expression systems have a significant advantage compared to other methods of recombinant protein production, since plants are much cheaper and easier to cultivate than mammalian cell cultures and they provide an optimal system for the expression of recombinant proteins free of contamination by bacterial toxins or animal pathogens. They also offer an eukaryotic protein modification machinery, allowing subcellular targeting, proper folding, and posttranslational modifications. In this work, both NT and T3 homozygote transgenic seeds were placed on 3M filter papers wetted with ddH2O, and germinated at 28/20 °C under 16/8 h (light/dark) conditions watered with ddH2O for 7 days. The seed germination rates of all transgenic lines and NT plants were > 95%. The germinated seedlings were then transplanted into 3.6-inch plastic pots containing commercial potting soil and kept in a growth chamber for 21 days under 450 μmol m⁻² s⁻¹ light with a 16 h photoperiod and 28/20 °C (day/night) temperatures at a relative humidity of 80%. No differences were observed between NT and transgenic plant (including seedling) growth as evaluated by plant height in a growth chamber (Figure S3) despite the over-expression of OsHPV. T1 transgenic OsHPV plants were not obviously different from T2 and T3 plants in regards to germination rates and plant heights under a growth chamber conditions (data not shown). These results further support the usefulness of this gene in the genetic engineering improvement in other model plants such as Arabidopsis, tobacco, and tomato.

To analyze the impact of OsHPV crude protein extracts on the growth of HeLa and MDA-MB-231 cells, both cells were treated with 5, 10, 25, and 50 μg of the extracts for the measurement of cell viability by MTT assay. It is a standard and rapid colorimetric method to determine the effects of potential agents or compounds on the proliferation of cells. OsHPV proteins were effectively expressed in the rice transgenic lines and OsHPV crude protein extracts promoted cell death in HPV-52L1 cervical cancer cells. OsHPV in transgenic plants was involved in the immune response and thus helped to overcome the cytotoxic effects induced by HeLa cells, whereas MDA-MB-231 breast cells immunized with VLP did not present significant cytoprotective activity. The immune response of the OsHPV crude protein extracts against cell metabolic activity in MDA-MB-231 breast cells showed a similar effect as NT treatment (photos not shown), as Figure 3B reveals no significant cytotoxicity of induced MDA-MB-231 cell proliferation at any dose compared with the NT group. These results suggest that immunization with OsHPV crude protein extracts elicit the T cell immune responses of HeLa cells specific to epitopes from type 52L1 protein (Lamprecht et al., 2016). Yields of OsHPV crude proteins harvested from the T3 callus were calculated to be between 0.05 ~ 0.1% of total soluble protein using an ELISA assay. Crude protein levels in T3 plants were similar to T2 (data not shown), indicating that the expression of the transgene is stable through generations. Our results also suggest the
possibility of devising a different plant-based platform for stable transformation to achieve better protein yield and purification in native conditions. The accumulated expressed product could be used directly in vaccination, and its concentration might be further increased by purification or freeze drying for applications where higher levels of an immunogen might be desirable.

The regional importance of HPV-52 in Eastern Asia can be seen in high-grade squamous intraepithelial lesions and normal cytology, but large differences in HPV type distribution in normal cytology tends to disappear with increasing severity of lesions leading to cancer (Parkin et al., 2008). This highlights the differing propensity of HPV types to progress to cancer, and hence the need to focus on cervical cancer to define priorities for HPV types in future vaccines. The major accomplishments in plant-produced VLPs prove that HPV-L1 can self-assemble in transgenic plants (Warzecha et al., 2003; Biemelt et al., 2003), and the derived VLP induces immune responses in rabbit models (Kohl et al., 2006). In our study, we show that it is possible to obtain a recombinant OsHPV from transgenic rice lines with immunological and anticancer activity against HPV experimental tumors, especially in combination with a DNA vaccine based on the same sequences. These results pave the way for more studies on the production of vaccines in plant-based expression systems and their combination with other treatment modalities for the development of effective and more specific therapeutic intervention against HPV infection and related cancers. Specific immune responses were induced in mice by tobacco plant extracts containing the HPV-16E7 protein, and the higher level of E7 within tobacco extracts increased immunological responses and therapeutic vaccine effectiveness in the mouse model (Franconi et al., 2006; Venuti et al., 2015). In addition, an HPV-16L1 produced in tomato and tobacco plants were able to elicit humoral and cytotoxic T-cell epitope activity in mice (De la Rosa et al., 2009; Šmídková et al., 2010). The expression of HPV-16L1 capsomeres with glutathione-S-transferase as a fusion protein in tobacco chloroplasts has been reported to generate elevated immune responses against HPV (Hassan et al., 2014). The production of human β-glucocerebrosidase in Nicotiana benthamiana for the replacement therapy of rare diseases is also reported (Naphatsamon et al., 2018). Recently, Massa et al. (2019) illustrated that tomato hairy root cultures were used to express the HPV-16E7 protein fused to the saporin protein from Saponaria officinalis to improve E7-specific cell-mediated responses as a therapeutic fusion DNA vaccine. The potential of plants to manufacture engineered compounds from small to complex protein molecules for pharmaceutical purposes allows the expression of HPV antigens, and possibly also the regulation of immune functions, to develop very specific therapies to reinforce available nonspecific therapies and preventive vaccination in developed countries.

A high level of cytotoxic activity from the overexpression of OsHPV-1~4 lines could modulate the genes involved in inflammatory/defense response and cytokine and cell cycle pathways, which eventually lead to the restoration of cellular homeostasis and detoxification of toxins (García-Piñeres et al., 2009). OsHPV crude protein extracts trigger ROS generation and activate caspase-3 mediated apoptosis in part by modulating gene expression, which eventually leads to the restoration of cellular homeostasis and detoxification of toxins (Gansukh et al., 2019). Immune responses, including T cell proliferative (CD4 and CD8) and cytokine responses, increased with an increasing concentration of OsHPV crude protein extracts to a certain extent. Furthermore, OsHPV crude protein extracts might play diverse roles in resistance to HeLa cells and possibly also in mediating signal transduction involved in activating naïve CD4+ T helper cells and trigger the CD8+-mediated cellular immune response or B cell-mediated humoral immune response (Kaliamurthi et al., 2018). The overexpression of OsHPV in transgenic plants obviously decreased cell viability compared to NT plants, exhibiting unique abilities and specificities through OsHPV crude protein extract content, indicating that OsHPV plants might use HPV-dependent mechanisms to cope with HeLa cells. OsHPV crude protein extracts inhibited cell proliferation and increased cell death rate in HeLa cells with an increasing extract concentration. These transgenic OsHPV seeds can be further used for direct seeding into soils, and use the expression of the HPV gene in transgenic rice plants for the bioproduction of a candidate therapeutic vaccine endowed with a
specific cell-mediated response associated with anticancer activity against HPV in a human HeLa cell model as utilized for immune therapies with genetic or plant-derived therapeutic vaccines. Nevertheless, the efficient expression of OsHPV crude protein extracts in animal models like the Syrian hamster (Wang et al., 2019) remain to be established. Platforms for plant molecular farming are different and may involve the use of whole plants or plant cell/organ cultures subjected to a transient or stable expression, and may be intended for purification or administration as a crude extract or whole plant tissues. All these aspects emphasize the advantages of plant-based systems for the expression of pharmaceutical proteins and support the development of cost-effective HPV vaccines, which is highly desirable for resource-poor countries.

Conclusions

Plant expression systems have successfully been used to produce biologically relevant products and offer platforms to manufacture cheaper vaccines. Crude protein extracts from OsHPV-52L1 transgenic lines with 5, 10, 15, and 20 µg concentrations effectively inhibited human HeLa cell proliferation. To date, no therapeutic vaccine has been approved for commercial use in the treatment of HPV52 infections and related malignancies. Current vaccines have no therapeutic effects and thus there is a need for therapeutic HPV52 vaccines to reduce the burden of cervical cancer. OsHPV-52L1 crude protein extracts have potential as novel drugs for the treatment of cervical cancer in future clinical practice.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Bryan JT, Brownlow MK, Schultz LD, Jansen KU (2010). U.S. Patent No. 7,700,103. Washington, DC: U.S. Patent and Trademark Office.
Cardona CE, García-Perdomo HA (2018). Incidence of penile cancer worldwide: systematic review and meta-analysis. Revista Panamericana de Salud Publica 41:e117.
Chabeda A, Yanez R, Lamprecht R, Meyers A, Rybicki E, Hitzeroth I (2018). Therapeutic vaccines for high-risk HPV-associated diseases. Papillomavirus Research 5:46-58.
Chiang CM, Chen CC, Chen SP, Lin, KH, Chen LR, Su YH, Yen HC (2017). Overexpression of the ascorbate peroxidase gene from eggplant and sponge gourd enhances flood tolerance in transgenic Arabidopsis. Journal of Plant Research 130(2):373-386.
Chen X, Zhang T, Liu H, Hao Y, Liao G, Xu X (2018). Displaying 31RG-I peptide on the surface of HPV16 L1 by use of a human papillomavirus chimeric virus-like particle induces cross-neutralizing antibody responses in mice. Human Vaccines and Immunotherapeutics 14(8):2025-2033.
Conway M, Meyers C (2009). Replication and assembly of human papillomaviruses. Journal of Dental Research 88:307-317.
Cordeiro MN, De Lima RC, Paolini F, Melo AR, Campos AP, Venuti A (2018). Current research into novel therapeutic vaccines against cervical cancer. Expert Review of Anticancer Therapy 18:365-376.
De la Rosa GP, A Monroy-García, ML Mora-García, CG Peña, J Hernández-Montes, B Weiss-Steider, MA Lim (2009). An HPV 16 L1-based chimeric human papilloma virus-like particle containing a string of epitopes produced in plants is able to elicit humoral and cytotoxic T-cell activity in mice. Virology Journal 6:2.
De Buck S, Windel P, De Loose M, Depicker A (2004). Single copy T-DNAs integrated at different positions in the Arabidopsis genome display uniform and comparable β-glucuronidase accumulation levels. Cellular and Molecular Life Sciences 61(19-20):2632-2645.
de Villiers EM, Fauquet, C, Broker, TR, Bernard, H.U, Zur HH (2004). Classification of papillomaviruses. Virology 324:17-27.
Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.
Fernandez-San MA, Ortigosa SM, Hervas-Stubbbs S (2008). Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. Plant Biotechnology Journal 6:427-441.
Franconi R, Massa S, Illiano E (2006). Exploiting the plant secretory pathway to improve the anticancer activity of a plant derived HPV16 E7 vaccine. International Journal of Immunopathology and Pharmacology 19:187-197.
Franconi R, Demurtas OC, Massa S (2010). Plant-derived vaccines and other therapeutics produced in contained systems. Expert Review of Vaccines 9(8):877-892.
García-Piñeres AJ, Hildesheim A, Dodd L, Kemp TJ, Yang J, Fullmer B, Pinto LA (2009). Gene expression patterns induced by HPV-16 L1 virus-like particles in leukocytes from vaccine recipients. The Journal of Immunology 182:1706-1729.
Gansukh E, Mya KK, Jung M, Keum YS, Kim DH, Saini RK (2019). Lutein derived from marigold (Tagetes erecta) petals triggers ROS generation and activates Bax and caspase-3 mediated apoptosis of human cervical carcinoma (HeLa) cells. Food and Chemical Toxicology 127:11-18.
Hefferon K (2017). Plant virus expression vectors: a powerhouse for global health. Biomedicines 5:44.
Hassan SW, MT Waheed, M Muller, JL Clarke, ZK Shinwari, AG Lossl (2014). Expression of HPV-16 L1 capsomeres with glutathione-S-transferase as a fusion protein in tobacco plastids: An approach for a capsomerebased HPV vaccine. Human Vaccines and Immunotherapeutics 10(10):2975-2982.
Hsing YI, Chern CG, Fan MJ, Lu PC, Chen KT, Lo SF, ... Lee KW (2007). A rice gene activation/knockout mutant resource for high throughput functional genomics. Plant Molecular Biology 63(3):351-364.
Huh WK, Joura EA, Giuliano AR, Iversen, OE, de Andrade RP, Ault KA (2017). Final efficacy, immunogenicity, and safety analyses of a nine-valent human papillomavirus vaccine in women aged 16–26 years: A randomised, double-blind trial. Lancet 390:2143-2159.
Kaliamurthi S, Selvaraj G, Kaushik AC, Gu KR, Wei DQ (2018). Designing of CD8+ and CD8+-overlapped CD4+ epitope vaccine by targeting late and early proteins of human papillomavirus. Biologics Targets Therapy 12:107.
Kim SI, Veena SB, Gelvin SB (2007). Genome-wide analysis of Agrobacterium T-DNA integration sites in the Arabidopsis genome generated under non-selective conditions. The Plant Journal 51:779-791.
Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM (2009). The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proceedings of the National Academy of Science 106:20458-20463.
Kohl T, Hitzeroth I, Stewart D (2006). Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. Clinical Vaccine and Immunology 13:845-853.
Kohl T, Hitzeroth I, Christensen N, Rybicki E (2007). Expression of HPV-11 L1 protein in transgenic Arabidopsis thaliana and Nicotiana tabacum, BMC Biotechnology 7(1):56.
Karanam B, Subhashini J, WK Huh, Roden RB (2009). Developing vaccines against minor capsid antigen L2 to prevent papillomavirus infection. Immunology and Cell Biology 87:287-299.
Lamprecht RL, Kennedy P., Huddy SM, Bethke S, Hendrikse M., Hitzeroth II, Rybicki EP (2016). Production of Human papillomavirus pseudovirions in plants and their use in pseudovirion-based neutralisation assays in mammalian cells. Scientific Reports 6:20431.
Liao YD, Lin KH, Chen CC, Chiang CM (2016). Oryza sativa protein phosphatase 1a (OsPP1a) involved in salt stress tolerance in transgenic rice. Molecular Breeding 36:22.
Loh HS, Green, BJ, Yussov V (2017). Using transgenic plants and modified plant viruses for the development of treatments for human diseases. Current Opinion in Virology 26:81-89.
Massa S, Presenti O, Benvenuto E (2018). Engineering plants for the future: farming with value-added harvest. In: Progress in Botany 80:65-108.
Massa S, Paolini F, Marino C, Franconi R, Venuti A (2019). Bioproduction of a therapeutic vaccine against human papillomavirus in tomato hairy root cultures. Frontiers in Plant Science 10:452.
Naphatsamon U, Ohashi T, Misaki R, Fujiyama K (2018). The production of human β-glucocerebrosidase in Nicotiana benthamiana root culture. International Journal of Molecular Sciences 19(7):E1972.
Naud PS, Roteli-Martins CM, De Carvalho N, Teixeira JC, de Borba PC, Sanchez N (2014). Sustained efficacy, immunogenicity, and safety of the HPV-16/18 AS04-adjuvanted vaccine: final analysis of a long-term follow-up study up to 9.4 years post-vaccination. Human Vaccines and Immunotherapeutics 10:2147-2162.

Parkin DM, Bray F (2006). The burden of HPV-related cancers. Vaccine 24:S1-S25.

Parkin D, Maxwell S, Louie S, Clifford G (2008). Burden and trends of type-specific human papillomavirus infections and related diseases in the Asia Pacific Region. Vaccine 26:L1-L15.

Peng S, Qiu J, Yang A, Yang B, Wang JW (2016). Optimization of heterologous DNA-prime, protein boost regimens and site of vaccination to enhance therapeutic immunity against human papillomavirus-associated disease. Cell and Bioscience 6(1):16.

Pineo CB, Hitzeroth I, Rybicki EP (2013). Immunogenic assessment of plant-produced human papillomavirus type 16 L1/L2 chimaeras. Plant Biotechnology Journal 11:964-975.

Roden RB, Stern PL (2018). Opportunities and challenges for human papillomavirus vaccination in cancer. Nature Reviews Cancer 18:240-254.

Santos RB, Abranches R, Fischer R, Sack M, Holland T (2016). Putting the spotlight back on plant suspension cultures. Frontiers in Plant Science 7:60-72.

Schug Z, Peck B, Jones D, Zhang Q, Alam I, Witney T, Aboagye E (2014). Acetyl-coA synthetase 2 promotes acetate utilization and maintains cell growth under metabolic stress. Cancer Cell 27(1):57-71.

Schellenbacher C, Richard R, and R Kirnbauer (2017). Developments in L2-based Human Papillomavirus (HPV) Vaccines. Virus Research 231:166-175.

Šmídková MM, Müller N, Thönes K, Piuko P, Angelisová J, Velemínský KJ (2010). Angelis. Transient expression of human papillomavirus type 16 virus-like particles in tobacco and tomato using a tobacco rattle virus expression vector. Biologia Planarum 54:451-460.

Suhandono S, Unga DAK, Kristianti T, Sahiratmadja E, Susanto H (2014) Cloning, expression and bioinformatic analysis of human papillomavirus type 52 L1 capsid gene from Indonesian patient. Microbiology Indonesia 8:2.

Venuti A, G Curzio, L Mariani, F Paolini (2015). Immunotherapy of HPV-associated cancer: DNA/plant-derived vaccines and new orthotopic mouse models. Cancer Immunology and Immunotherapy 64:1329-1338.

Vici P, Pizzuti L, Mariani L, Zampa G, Santini D, Di Lauro L (2016). Targeting immune response with therapeutic vaccines in premalignant lesions and cervical cancer: Hope or reality from clinical studies. Expert Review of Vaccines 15:1327-1336.

Wang Y, Miao J, Chard L, Wang Z (2019). Syrian hamster as an animal model for the study on infectious diseases. Frontiers in Immunology 10:2329.

Wang W, Vignani R, Scali M, Cresti M (2006). An universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Electrophoresis 27:2782-2786.

Wong-Arce A, González-Ortega O, Rosales-Mendoza S (2017). Plant-made vaccines in the fight against cancer. Trends in Biotechnology 35:241-256.

Warzecha H, Mason HS, Lane C (2003). Oral immunogenicity of human papillomavirus-like particles expressed in potato. Journal of Virology 77:8702-8711.
Supplemental Figure S1. Vector maps of pENTR-OsHPV52L1 (A) and pPZP200-OsHPV52L1 (B)

Supplemental Figure S2. Analysis of transgenic rice (*Oryza sativa* L.) by a genomic PCR for the hygromycin gene (*HPT*). The expected size of *HPT* gene fragments (indicated by an arrowhead) was 987 bp. +, positive control (using the pPZP200-Ubi-HA-HPV52L1-NOS plasmid as a template); NT, non-transgenic plants; lanes 3-7, transgenic rice human papillomavirus (OsHPV) 1~5. Lanes 3~7, OsHPV-1, OsHPV-2, OsHPV-3, OsHPV-4, and OsHPV-5, respectively.
Supplemental Figure S3. Morphology of T3 transgenic plants carrying transgene HPV-52L1 showing healthy phenotypes. The seedling and plant growth of all transgenic lines were similar to NT plants, and no differences were observed between NT and transgenic plants as evaluated by plant height in a growth chamber.

| Plasmids of transformation | Total number of transfected callus (A) | Hygromycin (50 mg/l) resistant rice explants (B) | Transformation efficiency % (B/A) |
|----------------------------|----------------------------------------|---------------------------------------------|----------------------------------|
| pPZP200-OsHPV-52L1        | 500                                    | 5                                           | 1                                |