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Research Article

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

Kuan-Hung LIN[1], Shwu-Fen PAN[2], Chiu-Chen CHEN[2], Wen-Shian LI[2], Chih-Ming CHIANG[2]*

[1]*Chinese Culture University, Department of Horticulture and Biotechnology, Taipei 11114, Taiwan; rlin@faculty.pccu.edu.tw*
[2]*Ming 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

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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). 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 assemble into higher order structures, and elicit anti-L1 and anti-L2 antibody responses that neutralize HPV-16 and HPV-52 pseudovirions (Pineo *et al*., 2013; Suhandono *et al*., 2014). Plant virus particles are easily purified, stable at high temperatures, and functionally indistinguishable from VLPs produced in the conventional system, and they have stronger CD8+ T cell immune responses (Lamprecht *et al*., 2016). Furthermore, plant-produced VLPs are immunogenic when administered in mice either orally or

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 (Chabeda *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) H_2O, 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-P_2O_5-K_2O, 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 (ACGCGTCGACATGGTACAGATTTTATTTTA) and HPV52L1-3R (GCCGAGCTCTTACCTTTTAACCTTTTTCT) 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 (GCTGGGGCGTCGGTTTCC) and HPT-3R (CACACCGCGACGTCTGTC) to amplify the hyg 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 hyg 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% CO_2 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 (A_{560}) was measured in an ELISA Reader (SpetraMax 190, San Jose, CA, USA) for cell viability. Cell viability (%) = [(A_{560} of the treated sample - A_{650} of the treated sample) / (A_{560} of the blank - A_{650} of the blank)] x 100%. The blanks were cells with no added H_2O_2 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.

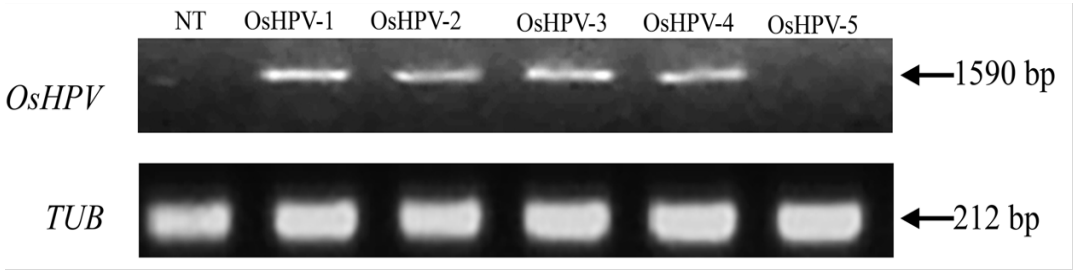

Figure 1. Transcription reverstase (RT)-PCR analysis of rice human papillomavirus (OsHPV) lines 1~5 compared to a non-transgenic (NT) rice plant (upper panel). The tubulin gene (TUB) was used as an internal control and transcripts were exhibited in all T3 transgenic and NT plants (lower panel). The expected 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

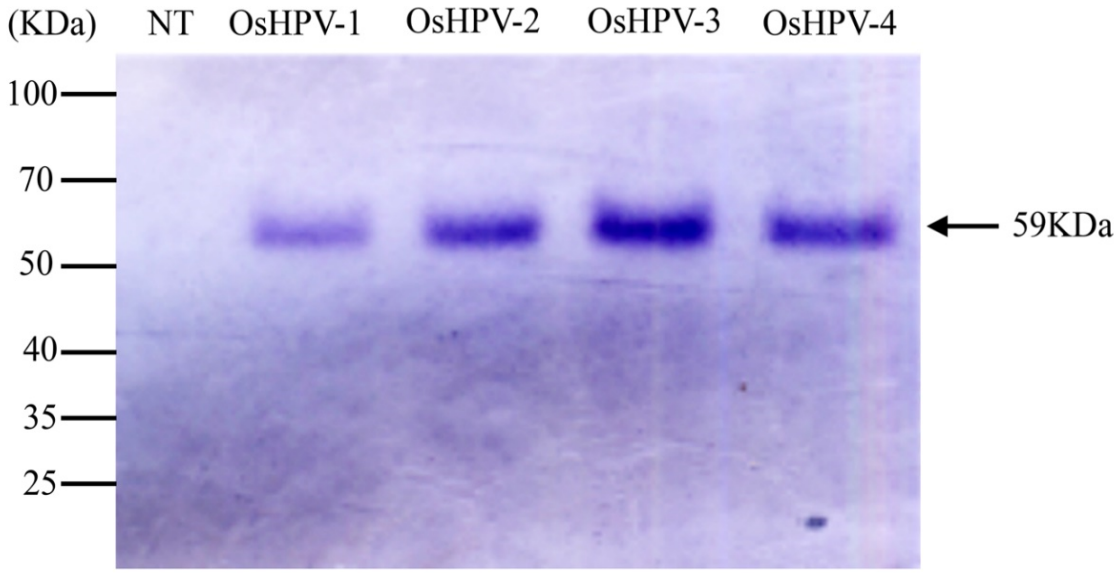

Figure 2. Western blot analysis of the OsHPV lines 1~5 and NT plants (anti-HA antibody). Forty micrograms of total protein were used for each sample. M, Molecular Weight Standard Mixture (Sigma) used during SDS-PAGE separation, and the molecular weight of T3 OsHPV at 59 kDa is indicated by an arrowhead

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 ug of the extract from OsHPV-3 tremendously reduced the viability of HeLa cells (26%) compared with the control group (57%).

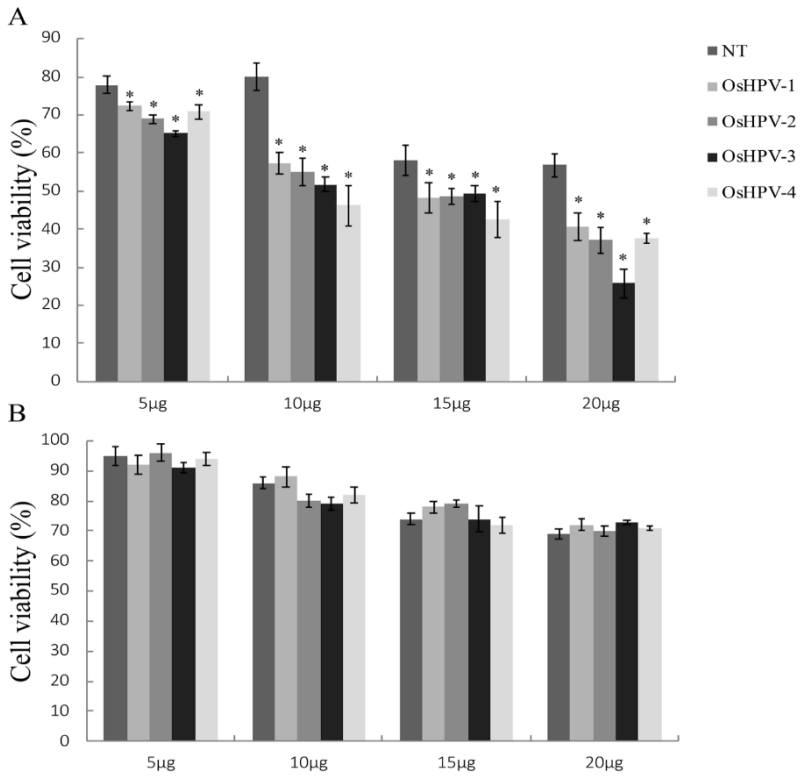

Figure 3. Cell toxicity effects of crude protein extracts from T3 OsHPV-1~4 transgenic lines using different extract concentrations (5, 10, 15, and 20 μg) on the viability of HeLa cells (A) and MDA-MB-231 cells (B). Among each concentration, four OsHPV transgenic lines were compared to NT plants; an asterisk indicates a significance level of $p \leq 0.05$. Cell viability (%) = [(A_{560} of the treated sample - A_{650} of the treated sample) / (A_{560} of the blank - A_{650} of the blank)] x 100%. The blank was cells with no added H_2O_2 or sample extract. Vertical bars indicate standard deviations (n = 3)

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 um 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.

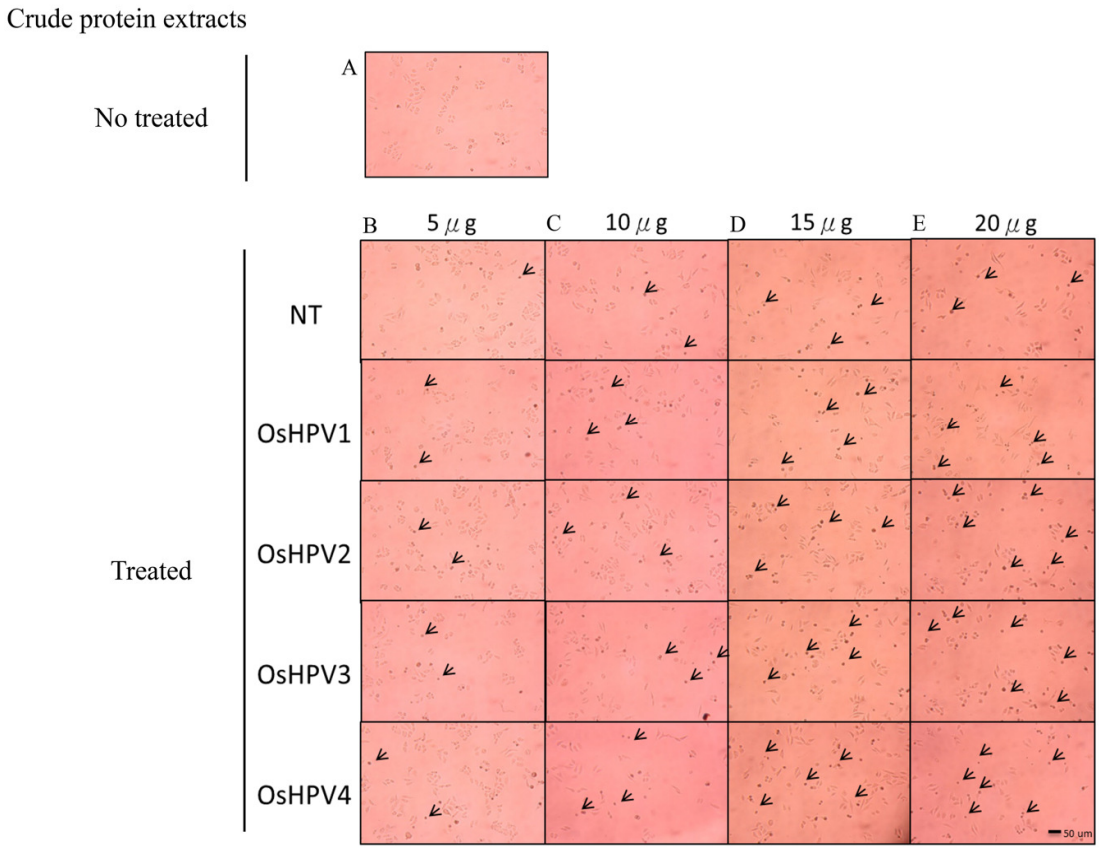

Figure 4. HeLa cells following treatments with 5, 10, 15, and 20 µg of NT and T3 OsHPV crude protein extracts for 24 h, followed by photographs at 100x magnification. Arrows indicate the putative dead HeLa cells observed. (A) No treated crude protein extracts HeLa cells as the negative control, (B) five 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 ddH_2O, and germinated at 28/20 °C under 16/8 h (light/dark) conditions watered with ddH_2O 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-231cell 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 been 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 naive $CD4^+$ 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.

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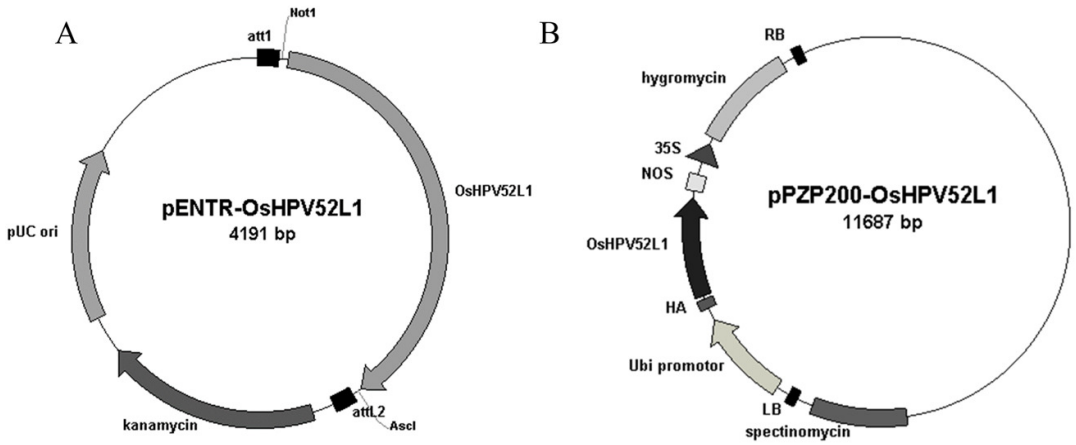
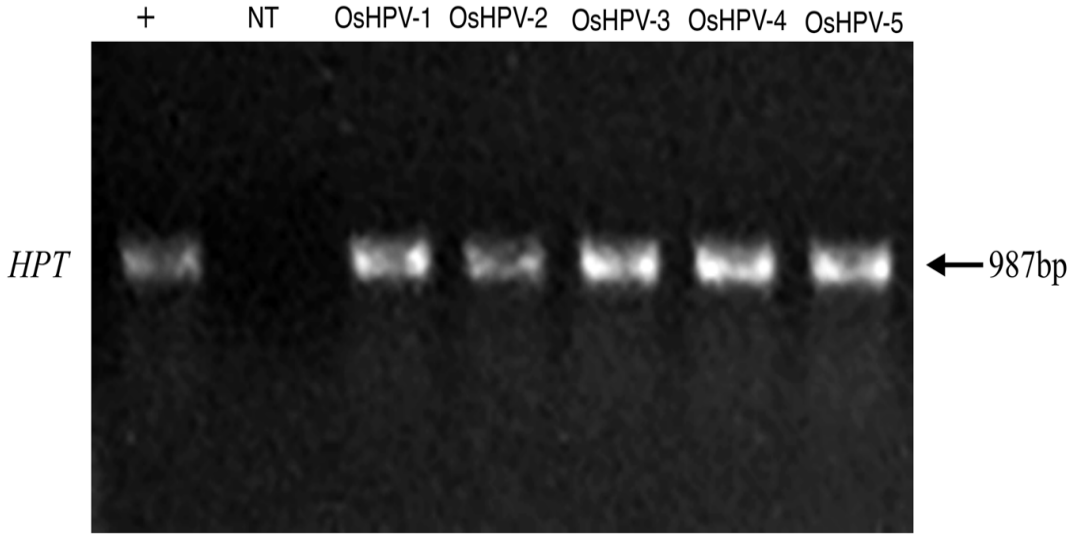
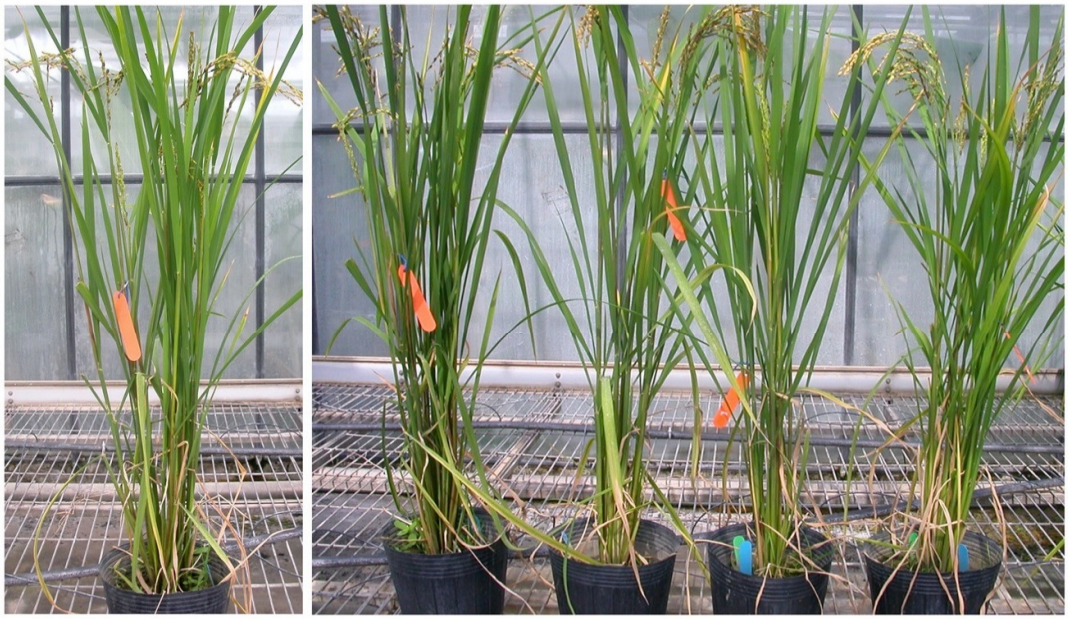


Supplemental Figure S1. Vector maps of pENTR-OsHPV52L1 (A) and pPZP200-OsHPV52L1 (B)



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

Table S1. Efficiency of plasmid transformation in rice

Plasmids of transformation	Total number of transfected callus (A)	Hygromycin (50 mg/l) resistant rice explants (B)	Transformation efficiency % (B/A)
pPZP200-OsHPV52L1	500	5	1