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Expression of Human Papillomavirus Type 16 L1 Protein in Transgenic Tobacco Plants

Hong-Li LIU[1,2], Wen-Sheng LI[1], Ting LEI[1], Jing ZHENG[1], Zheng ZHANG[3], Xiao-Fei YAN[1], Zhe-Zhi WANG[3], Yi-Li WANG[1], and Lü-Sheng SI[1]*

[1]*Institute of Cancer Research, School of Life Science & Technology, Xi'an Jiaotong University, Xi'an 710061, China;*
[2]*First Hospital of Xi'an Jiaotong University, Xi'an 710061, China;*
[3]*Shaanxi Normal University, Xi'an 710062, China*

Abstract To develop a plant expression system for the production of the human papillomavirus type 16 (HPV16) vaccine, we investigated whether the HPV16 L1 protein can be expressed in tobacco plants and whether it can be used as the cheapest form of edible vaccine. The HPV16 L1 coding sequence was amplified by PCR using specific primers from the plasmid pGEM-T-HPV16 containing the template sequence, and subcloned into the intermediate vector pUCmT and binary vector pBI121 consecutively to obtain the plant expression plasmid pBI-L1. The T-DNA regions of the pBI-L1 binary vector contained the constitutive Cauliflower mosaic virus (CaMV) 35S promoter and the neomycin phosphotransferase *npt II* gene, which allowed the selection of transformed plants using kanamycin. The tobacco plants were transformed by co-cultivating them, using the leaf disc method, with *Agrobacterium tumefaciens* LBA4404, which harbored the plant expression plasmid. The regenerated transgenic tobacco plants were selected using kanamycin, and confirmed by PCR. The results of the Southern blot assay also showed that the HPV16 *L1* gene was integrated stably into the genome of the transformed tobacco plants. The Western blot analysis showed that the transformed tobacco leaves could express the HPV16 L1 protein. Furthermore, it was demonstrated by ELISA assay that the expressed protein accounted for 0.034%–0.076% of the total soluble leaf protein, was able to form 55 nm virus-like particles compatible with HPV virus-like particle (VLP), and induced mouse erythrocyte hemagglutination *in vitro*. The present results indicate that the HPV16 L1 protein can be expressed in transgenic tobacco plants and the expressed protein possesses the natural features of the HPV16 L1 protein, implying that the HPV16 *L1* transgenic plants can be potentially used as an edible vaccine.

Key words human papillomavirus (HPV); virus-like particle; transgenic tobacco; plant vaccine; *Agrobacterium tumefaciens*; L1 major capsid protein

Human papillomavirus (HPV) infection, the most common and widespread sexually transmitted disease that is found worldwide, has increased rapidly in recent years, especially in developing countries. High-risk HPVs, especially HPV type 16 (HPV16), are well known as the initiators of cervical cancer [1–3], which accounts for a quarter of the cancer cases in women from developing countries. Therefore, a lot of research has been carried out in an effort to develop cheap and effective prophylactic vaccines against the oncogenic types of HPV.

HPV, a species of animal papillomavirus (PV), has a variety of features in common with animal PVs in terms of its genome, proteosome, structure and pathogenicity. The success in using animal PV vaccines to prevent the corresponding viral infection has generated great enthusiasm for the development of the HPV prophylactic vaccine. However, because of the strict species-specificity and dependence on the maturity of squamous epithelia, HPV can not grow in animals or *in vitro*, which has hindered the development of the HPV vaccine. With the significant advances in molecular virology and genetic engineering,

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*Corresponding author: Tel, 86-29-82655191; Fax, 86-29-82655499; E-mail, slusheng@yahoo.com

researchers have made much progress in this field. They have tried to develop several different types of HPV16 prophylactic vaccines, including DNA vaccines, protein subunit vaccines, virus-like particle (VLP) vaccines and recombinant live vaccines [2,3].

It has been proven that the recombinant HPV16 L1 protein, a major capsid protein of HPV, can self-assemble into VLPs, which are capable of inducing strong antibody responses, and it has been widely acknowledged as one of the best candidates for the HPV16 prophylactic vaccine [4]. At present, the HPV16 L1 protein has been produced mainly in yeast expression systems and recombinant baculovirus-insect cell expression systems [5], but all of these expression systems are too expensive to be used in manufacturing affordable prophylactic vaccines for developing countries.

Transgenic plants are increasingly being used for expressing relevant proteins or antigens in the pharmaceutical field. Compared with conventional expression systems, transgenic plants offer many potential advantages for the production of recombinant therapeutics and subunit vaccines, such as ease of use, simplicity, convenience and low cost. Transgenic plants are an inexpensive source of antigens that can be parenterally administered or used as edible vaccines [6], which is especially important for HPV infections, as the majority of HPV infections are sexually transmitted diseases (STDs), and only mucosal immunity route administration can invoke an effective vagina mucosal immunity.

In this study, we try to investigate whether the *Agrobacterium*-transformation method can be used to efficiently express the HPV16 *L1* transgene in *the Nicotiana tabacum* L. cultivar Xanthi plants conformably, perfectly and functionally.

Materials and Methods

Materials

The restriction enzymes were obtained from TaKaRa (Dalian, China) and Promega Company (Madison, WI, USA). The plasmid pGEM-T-HPV16, containing the full-length HPV16 *L1* gene cDNA, was constructed in our laboratory [7]. The plant binary vector pBI121 was from Clontech (California, USA). The polyvinylidene difluoride (PVDF) membranes and nitrocellulose membranes were from Invitrogen (California, USA) and Amersham Biosciences Corperation (Centennial Avenue, USA), respectively. The digoxigenin (DIG)-labeling kit and DIG nucleic acid detection kit were supplied by Boehringer Mannheim Company (Mannheim, Germany). The antibodies, anti-HPV16 L1 monoclonal antibody and anti-mouse IgG, were from Neomarkers (Fremont, CA, USA) and DaKo (DaKo A/S, Denmark), respectively. Tobacco plants (*Nicotiana tabacum* L. cultivar Xanthi) were kindly provided by Prof. Zhe-Zhi WANG (Shaanxi Normal University, Xi'an, China).

Plasmid construction

The HPV16 *L1* gene cDNA (5637–7154 nt) was amplified by PCR using two specific primers, 5′-GA*CTCTAGA*-ATGTCTCTTTGGCTGCCT-3′ (forward primer) and 5′-CCA*CCCGGG*TTACAGCTTACGTTTTTTG-3′ (reverse primer), with *Xba*I/*Sma*I sites (in italic) at the 5′ and 3′ ends, and the plasmid pGEM-T-HPV16 was used as the template. After PCR, the amplified fragment was cloned into pUCmT (Promaga, Madison, WI, USA), generating the recombinant pUC-L1, which was identified by restriction enzyme digestion, PCR amplification and sequencing. The entire coding region of L1 was obtained from pUC-L1 by *Xba*I/*Sma*I digestion and inserted into the pBI121 binary vector, and the resulting recombinant pBI-L1 was characterized by endonuclease digestion and sequencing.

Agrobacterium transformation

Agrobacterium tumefaciens strain LBA4404 cells were transformed with the chimeric vector pBI-L1 by electroporation so as to prepare the recombinant *Agrobacterium* LBA4404/pBI-L1. The transformed cells were selected on YEB (each liter containing 1 g yeast extract, 5 g beef extract, 5 g peptone, 5 g sucrose, 2 M $MgSO_4 \cdot 7H_2O_2$, pH 7.0) plates containing kanamycin (50 μg/ml) and streptomycin (100 μg/ml) at 28 °C and screened by PCR.

Plant transformation and regeneration

Nicotiana tabacum L. cultivar Xanthi leaf discs were used for the transformation, as described by Horsch *et al.* [8]. Excised leaf discs (0.5 cm×0.5 cm) were infected with *Agrobacterium* LBA4404/pBI-L1 (A_{600} = 0.3–0.4) and grown on a co-cultivation medium [containing 1 μg/ml 6-benzyladenine (6-BA), 0.1 μg/ml naphthaleneacetic acid (NAA) and 400 μM acetosyringone (AS)] for 3 days in darkness, then placed on regeneration plates containing 100 μg/ml kanamycin and 200 μg/ml cefotaxime for callus formation. Petriplates were incubated at 4000 lx for 16 h per day at 25 °C. About 3–4 weeks later, the shoots regenerating from the callus were isolated and transferred to the selection medium. Thereafter, healthy shoots of 4–6 cm in height were transferred to the Murashige

and Skoog (MS) medium with antibiotic to initiate root growth. Following root induction, the plantlets were transferred onto soil and grown to maturity.

Screening of the transformed tentative plants

The transformed *Nicotiana tabacum* plants were initially selected by using kanamycin. Later, the transformed plants were screened and confirmed for the presence of the *L1* gene by PCR analysis. To extract the template DNA, the leaves of tentative plants were harvested, frozen, ground to powder in liquid nitrogen, and then the total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) technique.

Southern blot analysis

A Southern blot analysis was performed on the plant genomic DNA according to the instructions of Sambrook *et al.* [9]. The plant genomic DNA (10 μg) was extracted from transformed as well as untransformed leaves, digested with the restriction enzyme *Xba*I, separated on a 0.8% agarose gel and transferred onto nylon membrane. The membrane was hybridized with the digoxigenin-labeled *L1* gene probe at 42 °C overnight, and detected using the DIG nucleic acid detection kit.

Western blot analysis

The transgenic tobacco leaves were homogenized in 1:2 (*W*/*V*) extraction buffer [phosphate-buffered saline (PBS) containing 0.1% Triton X-100, 10 mM EDTA-Na_2, 0.02% NaN_3, 1 mM phenyl methyl sulfonyl fluoride (PMSF), pH 7.4] at 4 °C. Samples were centrifuged at 13,500 *g* for 20 min, and the supernatant was frozen dried and redissolved in 1/10 initial volume of distilled water. After heat denaturing, 20 μl protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. After blotting, the membrane was incubated with the anti-HPV16 L1 monoclonal antibody, anti-mouse IgG conjugated to horseradish peroxidase (HRP) and diaminobenzidine (DAB) solution consecutively.

ELISA quantification of HPV16 L1 protein expression

The total soluble leaf protein was quantified by the Bradford method. The amount of L1 protein in transgenic tobacco leaves was determined by using ELISA. Polystyrene microtiter plates were coated with 100 μl of plant protein extract or a certain quantity of purified baculovirus-produced HPV16 VLP diluted serially in bicarbonate-buffered saline (pH 9.6) and kept at 4 °C overnight. After blocking, the plates were incubated with the anti-HPV16 L1 monoclonal antibody, HRP-conjugated anti-mouse IgG and finally with 1,3,6′,8′-tetramethyl-dianthrone (TMD) substrate solution. When the reactions were complete, the absorbance at 450 nm was recorded by using an automated plate reader.

Electron microscopic analysis of transgenic plant leaf extracts

The transgenic plant leaf extract, in which the particles have been immunotrapped by the anti-HPV16 L1 monoclonal antibody or not, was dropped onto the carbon copper-coated grids. The grids were then negatively stained with phosphotungstic acid and viewed using the H-600 transmission electron microscope.

Hemagglutination and hemagglutination inhibition (HAI) assay

An erythrocyte suspension was prepared from the fresh blood of C57BL/6 mice. Then, 100 μl of plant extract was plated on a 96-well U-bottomed plate, and mixed with an equal volume of 1% (*V*/*V*) erythrocyte suspension. After incubation, visualization and photography were done. The HPV16 VLP produced by baculovirus-insect cells was used as a positive control, and the untransformed plant extract was used as a negative control.

The anti-HPV16 L1 monoclonal antibody is reported to inhibit HPV VLP-induced hemagglutination of mice [10]. So, an HAI assay was carried out according to basically the same procedure as the murine hemagglutination assay, except that the murine erythrocytes were pre-treated with the anti-HPV16 L1 monoclonal antibody.

Results

Construction of binary vector containing HPV16 *L1* gene

PCR-amplified HPV16 *L1* gene cDNA (1518 bp) was cloned into pUCmT, and the generated recombinant pUC-L1 was identified by *Xba*I/*Sma*I restriction digestion, PCR and sequencing (**Fig. 1**). The HPV16 *L1* fragment excised from pUC-L1 was cloned into the pre-digested pBI121 binary vector, and the recombinant vector was confirmed by sequencing and named pBI-L1, in which the HPV16 *L1* fragement was located between the Cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator. The neomycin phosphotransferase *npt II* gene on the T-DNA of the binary vector can be used as an

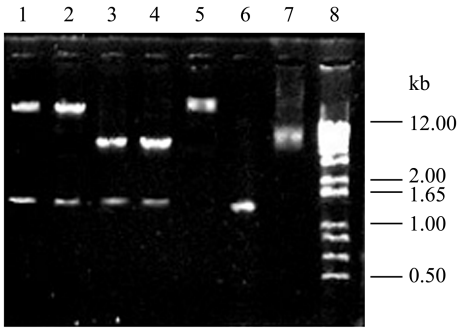

Fig. 1 Identification of the recombinant plasmids pUC-L1 and pBI-L1 by endonuclease digestion

1 and 2, pBI-L1 digested with *Xba*I/*Sma*I; 3 and 4, pUC-L1 digested with *Xba*I/*Sma*I; 5, plasmid pBI-L1; 6, pUC-L1 PCR production; 7, plasmid pUC-L1; 8, 1 kb DNA marker (Gibco BRL, USA).

selection marker of transformed plants (**Fig. 2**).

Genetic analysis of transformed tobacco plants

Twenty-five plants that were successfully transformed were identified by PCR screening (**Fig. 3**). The genomic DNA of all PCR-positive plants was shown to contain the integrated HPV16 *L1* cDNA as shown by Southern blot analysis. The results of Southern hybridization showed intense signs indicating the presence of the HPV16 *L1* cDNA in the transformed plants, and there were 1–2 copies of the HPV16 *L1* cDNA in the transgenic tobacco genome, but the untransformed plants did not show any positive results (**Fig. 4**).

Protein analysis

The extract from the transformed plants was further analyzed for the recombinant HPV16 L1 protein expression. The SDS-PAGE result showed a novel extra protein band of 58 kDa in accordance with the expected molecular weight of the HPV16 L1 protein, which reacted

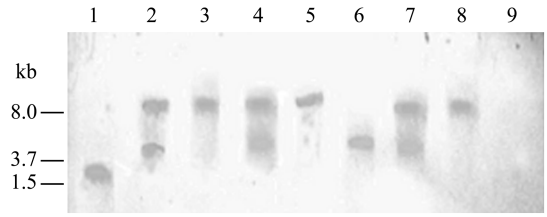

Fig. 4 Southern blot analysis of HPV16 *L1* transgenic tobacco plants

1, positive control of pBI-L1 (digested with *Xba*I/*Sma*I); 2–8, genomic DNA of transformed tobacco plants (digested with *Xba*I); 9, genomic DNA of untransformed tobacco plants (digested with *Xba*I).

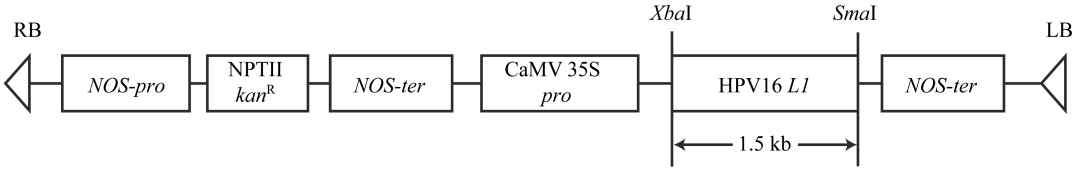

Fig. 2 Structure of the T-DNA region of the pBI-L1 binary vector

RB and LB, the right and left borders; NPTII, *npt II* gene for kanamycin resistance in plants; CaMV 35S, Cauliflower mosaic virus 35S promoter; HPV16 *L1*, HPV16 *L1* gene cDNA.

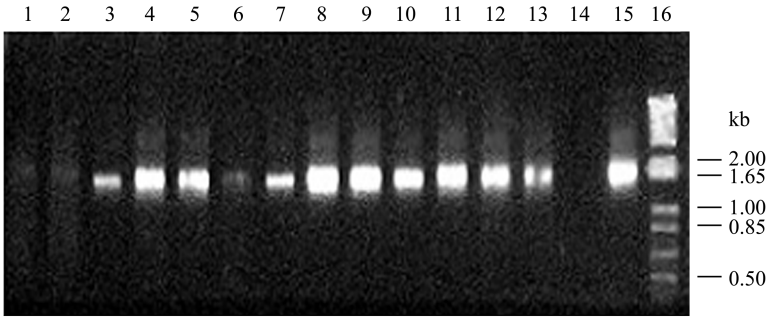

Fig. 3 PCR analysis of transgenic tobacco plants for the HPV16 *L1* gene

1–13, transformed tobacco plants; 14, untransformed tobacco plants; 15, positive control of pUC-L1; 16, 1 kb DNA marker (Gibco).

specifically with the anti-HPV16 L1 monoclonal antibody as shown by Western blot analysis (**Fig. 5**). The expression of HPV16 L1 was quantified by ELISA assay, and the yield accounted for 0.034%–0.076% of the total soluble protein of the transformed plants.

Electron microscopic analysis

Hollow spherical particles of 55 nm in diameter were seen in the transgenic plant samples using the H-600 transmission electron microscope, implying that the recombinant HPV16 L1 protein is able to self-assemble into VLPs (data not shown).

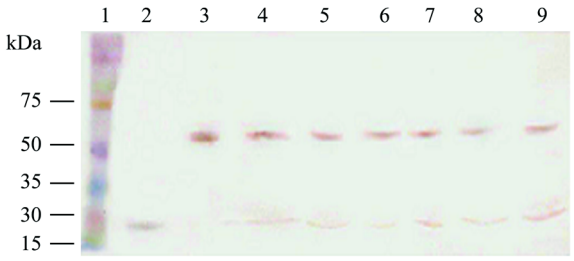

Fig. 5 Western blot analysis of HPV16 L1 expression in transgenic tobacco plants

1, RPN800 marker (yellow, 75 kDa; purple, 50 kDa; blue, 35 kDa; orange, 30 kDa); 2, leaf protein of untransformed plants; 3, HPV16 L1 protein expressed by Sf9 cells; 4–9, leaf protein of transformed plants.

Biological activity of recombinant HPV16 L1 protein

The HPV16 L1 protein expressed in transgenic plants can cause murine erythrocyte agglutination and its hemagglutination function can be specifically abolished by the anti-HPV16 L1 monoclonal antibody (**Fig. 6**).

Discussion

In recent years, significant progress has been made in the development of a high-risk-HPV recombinant prophylactic vaccine, and the HPV16 VLP vaccine expressed in Sf9 cells has been proven to be effective in clinical trials [11]. However, the VLP vaccine is expensive, which makes it unaffordable for common people, especially those who are living in developing countries.

Transgenic plants are being developed as an alternative expression system to produce various foreign pathogenic proteins, which might be used as edible vaccines without the need for a tedious purification procedure [5,12]. The advantages of the transgenic plant vaccine have made it popular, and a number of different viral proteins have already been generated in transgenic plants. The virus capsid proteins expressed, such as HBsAg, Norwalk virus and HPV11 [13–15], have been proven to be able to self-assemble into VLPs, but what is of concern is that the oral administration of vaccines might lead to tolerance rather

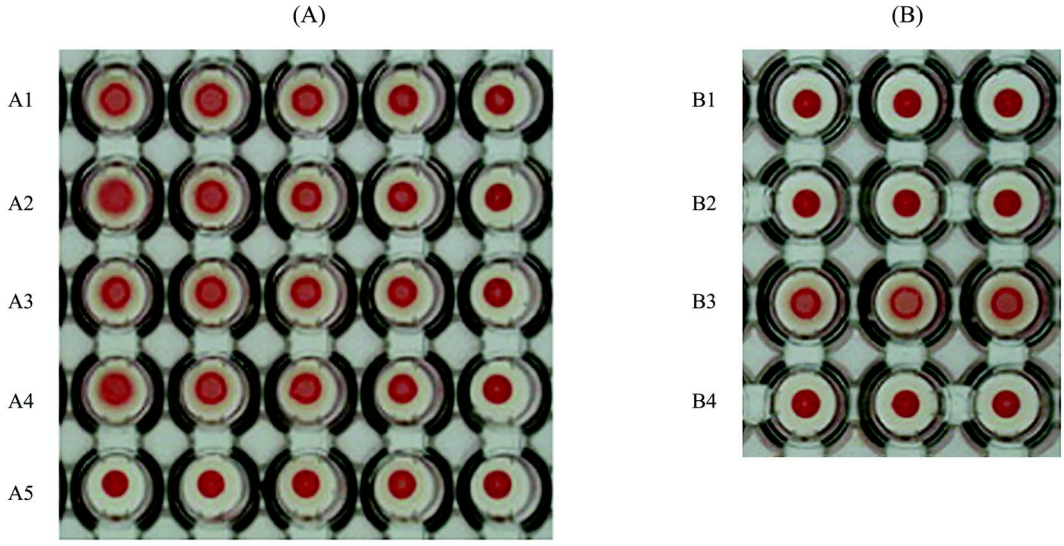

Fig. 6 Hemagglutination assay

(A) Hemagglutination assay. A1, HPV16 VLP positive control; A2–A4, leaf tissue protein of transformed plants; A5, leaf tissue protein of untransformed plants. (B) Hemagglutination inhibition assay. B1 and B2, leaf tissue protein of transformed plants; B3, leaf tissue protein of untransformed plants; B4, HPV16 VLP positive control.

than immunity. However, Warzecha *et al*. [15] and Rocha-Zavaleta *et al*. [16] have proven that the oral delivery of HPV VLP leads to the induction of protective immune responses. For example, in our study, we found that LT-B, a well-known mucosal adjuvant, can strengthen the genital tract mucosal immunoresponse when co-administered orally with the HPV16 VLP vaccine [17] (data not shown). So transgenic plants are the bioreactors for HPV16 VLP production and can be used as edible vaccines.

In the present study, we constructed the HPV16 L1 plant binary vector containing the CaMV 35S promoter, nopaline synthase terminator and *npt II* gene in the T-DNA, and used it to perform *Agrobacterium tumefaciens*-mediated transformation of tobacco plants. We obtained 25 strains of transformed tobacco plants, in some of which the HPV16 *L1* cDNA was integrated in the transformed plant genome. Among them, a novel extra protein band at 58 kDa, thought to be the HPV16 L1 protein, was identified by SDS-PAGE and confirmed by Western blotting.

In general, the expression levels of foreign proteins in transgenic plants are variable, ranging from 0.001% to 0.37% of the total soluble protein [11]. Various approaches have been suggested to increase the target protein expression level in transgenic plants, including codon optimization, modification of plant promoters for transcription of the genes and introduction of a 5′-untranslated *Tobacco mosaic virus* sequence or other translational enhancers [5]. Nonetheless, our data on the expression of HPV16 L1 in transgenic plants suggest that this system can potentially be used for vaccine production.

Furthermore, the HPV16 L1 protein expressed by transgenic tobacco can self-assemble into VLPs and maintain the natural conformation, meaning that the recombinant protein possesses the fundamental characteristics of HPV16 L1. The present results are important for the development of HPV plant-based vaccines that will contribute effectively to the prevention of HPV16 infections.

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Zu-Xun GONG