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Introduction of Hog Cholera Virus Gene into Potato Plants by *Agrobacterium*-mediated Transformation and the Analysis of Its Expression

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Abstract

The HCV gene was expressed in potato plants under the control of the constitutive CaMV 35S promoter or tuber-specific patatin promoter. *Solanum tuberosum* plants carrying a plant expression vector harboring the encoding region of HCV gene were generated by *Agrobacterium tumefaciens*-mediated in vitro transformation methods. The presence of HCV gene in the plant genome was detected by PCR and DNA hybridization experiments. We obtained the 5 lines of transgenic potato with the pMBPHCV construct and 4 lines of transgenic potato with the pATHCV construct. The HCV transgene stably integrated into the potato genome, as well as their transcription. HCV mRNA was identified in leaf and tuber tissues of transgenic plants by Northern blot analysis. The transgenic potato plants produced the expected transcript, and the corresponding HCV protein accumulated in individual transgenic plants.

Introduction

The potential of molecular biology to increase the value of plants to agriculture, industry and health has led to the application of expensive resources to further develop this technology. Recent advances in the understanding of transgene expression and recombinant protein accumulation, stability, and processing in plants have allowed the development of novel strategies such as using edible plants for delivery of antigens for active immunization. The concept of producing a subunit vaccine in transgenic plants was first described in 1992 by Mason and co-workers who integrated the DNA coding for the hepatitis B virus major surface antigen into tobacco plants by *Agrobacterium*-mediated transformation. After that, the use of transgenic vegetables and fruits for the expression and delivery of recombinant protein antigens as edible vaccines has become an attractive topic for plant molecular biologists. Hepatitis B surface antigen (Thanavala et al., 1995), *E.coli* heat-labile enterotoxin antigen (Haq et al., 1995; Tacket et al., 1998), porcine transmissible gastroenteritis virus (Gomez et al., 1998; Tuboly et al., 2000) chorela toxin B subunit (Arakawa et al., 1998; Arakawa et al., 2001) were the vaccine antigens expressed in transgenic plants and tested for the immune response elicited in immunized animals. Additionally, rabies virus glycoprotein was expressed in transgenic tomatoes, but the immune response induced by administration of these plants to animals was not tested (McGarvey et al., 1995).

Hog Cholera (HC) is a highly contagious viral disease characterized by symptoms of haemorrhagic fever and immune depression, usually leading to substantial economic losses. HCV isolates and strains differ in virulence and antigenic properties, giving rise to four clinical forma of the disease; peracute, acute, subacute and chronic (Xinglong et al., 2001; de Smit et al., 2001). Highly virulent and moderately virulent strains provoke a fatal disease in pigs of all ages. Traditional immunization approaches using needle injection of various vaccines preparations have provided some degree of protection. However, needle injection requires the herding and restraint of the animals, inducing additional stress as well as incurring a sub-

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stantial labor cost. As an alternative, researchers propose to develop a noninvasive means of delivery of the vaccine via the oral route by using transgenic plants expressing recombinant immunogens. In the present study, we carried out the integration of the DNA coding for the hog chorela virus antigen into potato plants by *Agrobacterium*-mediated transformation. Also, we investigated the feasibility of expressing the hog cholera virus gene in transgenic plants through genomic DNA, mRNA and protein analysis.

Materials and Methods

Construction of plant expression vector

A 1023 bp DNA fragment containing the gene encoding Hog Cholera Virus was amplified with specific primers and pAcGP67B as template (provided by Dr. Cheoljung Kim, Chungnam Nat'l. Univ.). The specific primers containing ATG start codon of HCV were synthesized in the Genotech Co. (Daejeon, Korea) with *Bam*HI restriction endonuclease recognition site. After amplification (Perkin Elmer Gene Amp PCR System 7200), 1050 bp PCR product was cut with *Bam*HI and then *Bam*HI fragment was ligated into the binary plant vector pMBP1 to create pMBPHCV (Figure 1).

pATHCV was constructed as follows. The amplified HCV fragment was subcloned into pBluescriptKS at the *Bam*HI site. One clone (pKSHCV) was digested with *Kpn*I and *Sac*I; the resulting fragment was ligated with pPAT1.0 at *Kpn*I/*Sac*I. This gave pATHCV, a binary vector for expression of HCV in plants using the patatin promoter to drive transcription and the nopaline synthase terminator (Figure 1). The 1.0 kb patatin promoter from potato was cleaved from pUCPAT plasmid and ligated into the binary plant vector pBI121. The CaMV 35S promoter was removed and replaced by patatin promoter to give pPAT1.0. At preliminary examination, we also checked the efficiency of the 1.0 kb patatin promoter compared to CaMV35S promoter by GUS analysis (data not shown).

Plant transformation

Plasmids pMBPHCV and pATHCV were mobilized into *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method, using plasmids cloned in *E. coli* DH5. The structure of the plasmids was verified by restriction digestion. Potato leaf explants (*Solanum tuberosum* L. cv Desiree) excised from sterilized plants were transformed by *A. tumefaciens* mediated transformation methods. Leaf explants were infected by *A. tumefaciens*, harbouring the construct pMBPHCV and pATHCV, and cultured on selective medium. Shoots were generated from transformed callus selected on medium containing plant growth hormone (0.01 mg/1 NAA, 0.1 mg/1 GA_3, 2.0 mg/1 zeatin) and antibiotics (100 mg/1 of kanamycin and 1000 mg/1 of carbenecillin). Kanamycin resistant transformants were screened and investigated by Southern analysis, RNA hybridization and western blotting analysis.

Isolation of plant genomic DNA and Southern hybridization

Genomic DNA was isolated from transformed potato leaf tissues. Total cellular DNA was extracted using a cetyltrimethylammonium bromide (CTAB) based method (Doyle and Doyle, 1990). The presence of the HCV gene was determined by PCR analysis using the oligonucleotide primers specific for the T-DNA sequence. Transformed plant genomic DNA was used as a template to detect the HCV gene under the following PCR conditions; 94℃ for 1', 55℃ for 1' and 72℃ for 1' for a total 35 cycles.

The genomic DNA was digested with *EcoR*I to confirm the copy number of inserted T-DNA for pMBPHCV and pATHCV derived transformants. Digested DNA was electrophoresed on a 1.0% agarose gel and then transferred to nylon membrane, positively charged (Roche co., Germany) using Turboblotter system (Schleicher and Schuell). Dig-labeled probes were generated by PCR Dig Labeling Mix (Roche co., Germany) with the specific primer set for HCV gene. DNA gel blots were hybridized at 50℃ in DIG easy hybridization buffer (Roche co., Germany). After hybridization overnight at 50℃, blots were washed in SSC buffer series and then detected by Dig Detection Kit and manufacturer's instructions (Roche co., Germany).

RNA extraction and hybridization

Total RNA from the leaves and tubers of transformed plants was isolated using the RNAgents Total RNA isolation system (Promega co., USA) according to the manufacturer's instructions. Total RNAs from 30 μg of samples were denatured with formaldehyde and formamide and fractionated in a 1.0% agarose gel with 3-(N-morpholino) propanesulfonic acid (MOPS) buffer. The RNA was blotted to nylon membrane, positively charged (Roche co., Germany) and fixed by ultraviolet irradiation. The membranes were hybridized with Dig-labelled probes using template containing most of the coding region for HCV. After hybridization overnight at 50℃, blots were washed in SSC buffer series and then detected by Dig Detection Kit and manufacturer's instructions (Roche co., Germany).

Immunoblot detection of HCV protein in transformed potato tissues

Transgenic potato tissues were evaluated for the presence of HCV protein by western blotting analysis using an ECL plus kit (Amersham pharmacia biotech). Leaf tissues (~1g fresh weight) were homogenized by grinding in a mortar and pestle on ice in 1.0 mL of extraction buffer (200 mM Tris-Cl, pH8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsufonylfluoride, 0.05% Tween-20). The tissue homogenate was centrifuged twice at 17,000×g in Beckman TLX-120 centrifuge for 15min at 4℃ to remove insoluble cell debris. An aliquot of 10 μl lof supernatant, containing 30-50μg of total soluble protein, as determined by Bradford protein assay, was separated by 10% SDS-PAGE at 100V for 1hr in Tris-glycine buffer (25mM Tris base, 192 mM glycine, pH8.3, 0.1% SDS). Samples of the leaf homogenate were boiled for 3min prior to electrophoresis and loaded on the gel. The separated protein bands were transferred from the gel to PVDF membranes by electroblotting on a tank transfer for 1hr at 6v/cm. After completely dry of membrane, nonspecific antibody reactions were blocked by incubation of membranes in 25 mL of 5% skim milk in TBS buffer. The blocked membrane was incubated for 1h in TBS solution containing 1:5,000 dilution of HCV specific monoclonal antibody (provided by Dr. Cheoljung Kim, Chungnam Nat'l. Univ.). The anti-HCV primary antibody/HCV antigen complex was conjugated with secondary antibody by incubation of the membrane in a 1:5,000 TBS dilution of an anti-mouse Ig horseradish peroxidase. Following binding of the secondary antibody, the membrane was washed three times in TBS solution to remove unbound secondary antibody. Chemiluminescence detection of the recombinant protein was accomplished by The ECL Plus ™ Western blotting detection reagents and manufacturer's instruction.

Results and Discussions

Generation of transgenic potato plants

The HCV gene was placed under the transcriptional control of either the constitutively expressed CaMV 35S promoter (Benfey et al., 1989) or the tuber specific patatin promoter (Rocha-Sosa et al., 1989) in gene constructs pMBPHCV and pATHCV (Figure 1). The CaMV 35S promoter from cauliflower mosaic virus is a widely used example of a constitutive promoter and has been used to demonstrate the efficacy the introduction of foreign gene into the various plants. Patatin is a group of potato storage proteins that are represented by two different classes of proteins. Patatin is encoded by a multigene family and patatin genes are classified based on DNA sequence conservation into class Ⅰ and class Ⅱ genes (Bevans et al., 1986; Mignery et al., 1988). Class Ⅰ promoters have become an important system for gene expression studies due to both their tuber-specific expression and their ectopic expression by sucrose, a condition that mimics that of a sink tissue (Willmitzer et al., 1990). The right and left borders flank the expression cassettes and delineate the transferred DNA, which is stably integrated into nuclear chromosomal DNA at random sites and mediated by *Agrobacterium* infection. Two constructs contain the neomycin phospho-

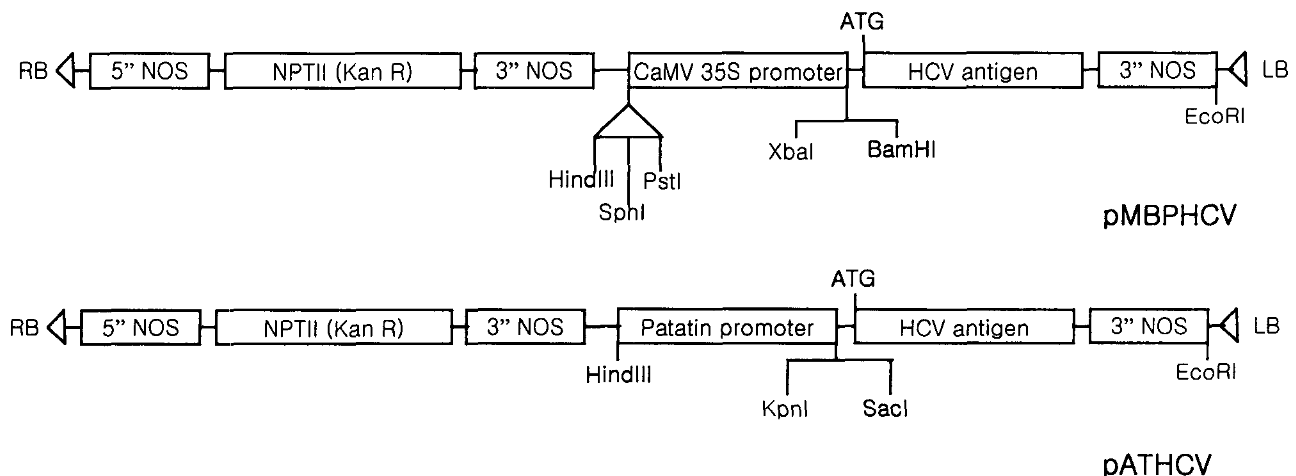

Figure 1. Schematic of gene constructs transferred to plants in binary vectors. 5'NOS: NOS promoter. NPTⅡ: plant-selectable kanamycin marker. 3'NOS: NOS terminator. The constructs carry the left and right borders (LB, RB) of the transferred DNA that demarcates the sequences that are incorporated into the plant genomic DNA via *Agrobacterium*-mediated transformation. pMBPHCV uses the cauliflower mosaic virus 35S promoter to drive transcription, while pATHCV uses the patatin promoter for tuber-specific expression.

transferase II (NPTII) gene for selection of transformed plants on kanamycin-containing growth medium. Through *Agrobacterium*-mediated transformation, 20 kanamycin-resistant putative transformants were screened with each construct and further analyses of HCV gene expressions were carried out on transgenic potato plants (Figure 2). PCR analysis was carried out as the first method to confirm the transgenic nature of the regenerated plants. The primers were derived from the 5′ and 3′ ends of the HCV coding sequence. The 1023-bp HCV fragment was amplified from genomic DNA for all transgenic plants tested (Figure 3-A). No amplification was observed for nontransgenic plants. The same fragment was amplified using plasmid pMBPHCV and pATHCV as a template (Figure 3-A, lanes 11 and 12).

Detection of the HCV gene in transformed potato

In order to confirm the insertion of HCV gene in potato plants, total DNA was isolated from transformed potato plants and analysed by DNA-gel blot. The total DNA was digested with *Eco*RI/*Hin*dIII completely and separated on a 1% agarose gel (Figure 3-B, b). After transfer the DNA onto a membrane, the paper was hybridized with digoxigenin labeled HCV encoding fragment. Positive bands were detected from each transgenic plant of pMBPHCV7 and pATHCV10, but not from a non-transgenic plant. The positions of bands are identical with that of the full size of T-DNA containing of HCV coding sequences. This result indicates that the HCV gene was inserted into some transgenic potato plants and suggests that the transgenic plants pMBPHCV7 and pATHCV10 were successfully transformed with HCV gene.

We performed the DNA-gel blot analysis to determine the number of T-DNA inserts of the selected transformant lines (Figure 3-B, a). Gel-blot of genomic DNA digested with *Eco*RI, which cuts in the T-DNA on one side of the HCV fragment. One copy of T-DNA was detected in the transgenic plants expressing pMBPHCV (No. 2 and No. 7), while two copies were detected in the transgenic plant expressing pATHCV (No. 10). There is no T-DNA was present in the non-transformed line (Figure 3-B, lane

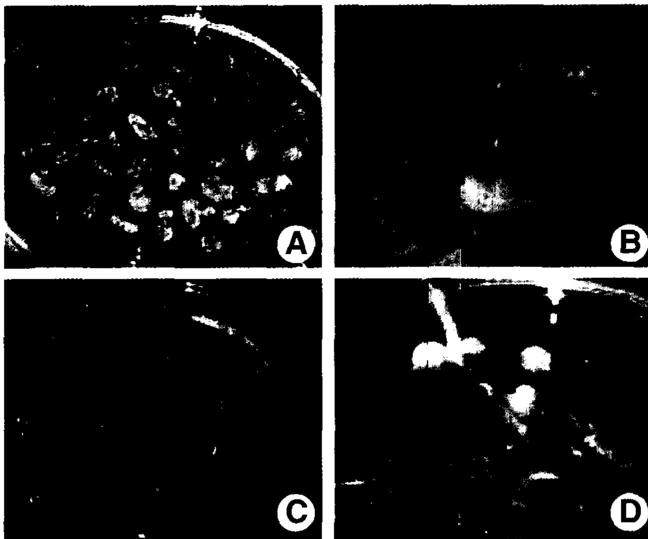

Figure 2. Formation of transgenic plants and microtuber in *in vitro*. (A) Culture of leaf discs after cocultivation with *Agrobacterium* carrying pATHCV and pMBPHCV plasmid DNA. (B) Callus formation and shoot induction at 2 weeks after transformation. (C) Culture of independent transgenic plant (D) Microtuber formation from independent transgenic plant.

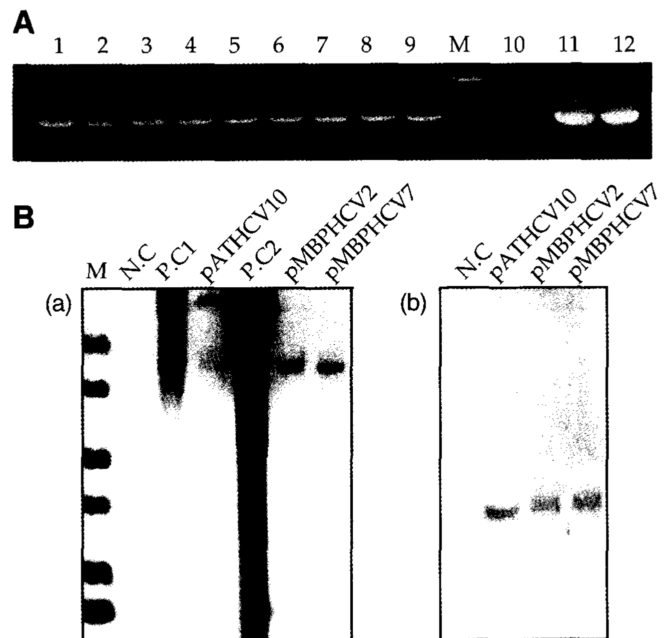

Figure 3. (A) HCV gene detection in genomic DNA of transformed potato leaves. Lane M is a 1 kb DNA ladder (MBI fermentas). DNA templates used for PCR amplification reaction of the HCV gene were as follows; Lanes 1-4, transformed potato plant genomic DNA from plants pATHCV No 2, No 7, No 10 and No 11. Lanes 5-9, transformed potato plant genomic DNA from plants pMBPHCV No 1, No 2, No 5, No 7 and No 10. Lane 10, untransformed potato plant genomic DNA. Lanes 11 and 12, pATHCV plasmid DNA (500 ng) and pMBPHCV plasmid DNA (500 ng). (B) Southern blot analysis of transgenic potato genomic DNA. Genomic DNA was isolated from leaves of potato. DNA (30 μg) was digested with *Eco*RI, which cuts in the T-DNA on one side of the HCV fragment (a), and *Eco*RI/*Hin*dIII, which cut in the T-DNA fragment containing promoter and HCV coding region (b), and electrophoresed on a 1.0% agarose gel. DNA transferred on a nylon membrane was hybridized with DIG-labeled HCV. M; Dig-labeled 1kb DNA ladder, N.C; non-transformed potato plant, P.C 1; pATHCV plasmid DNA (1 μg) digested with *Eco*RI/*Hind*III, P.C 2; pMBPHCV plasmid DNA (1 μg) digested with *Eco*RI/*Hin*dIII.

1). The T-DNA insertion patterns seen in this study are similar when compared to the simple insertion patterns commonly seen using *Agrobacterium* transformation. Olhodf and Somers (2001) reported all of the T_0 plants analyzed exhibited numerous transgene-hybridizing fragments with multiple copies. They believed that the multiple integrations of the T-DNA are due to the nature of the binary plasmid. Usually, it is known there is the correlation between transcripts level and T-DNA copy number in the transgenic plant. Arakawa et al. (2001) reported that highest level of PCR amplification of CTB fusion gene suggests the possibility of increased T-DNA copy number in the transformant.

Detection of the HCV transcripts and protein

The transformants were analyzed by hybridizing RNA samples with a labeled probe encompassing the coding region of the HCV gene. Figure 4 shows the results of an experiment where selected transformants harboring the pMBPHCV, pATHCV constructs and a wild-type control were probed. Those mRNAs were extracted from leaves of pMBPHCV lines 1, 2, 7 and 10 and pATHCV lines 2 and 10. Similar levels of HCV-specific RNA among the pMBPHCV independent transformants were apparent, but the expression bands of HCV-specific RNA in pATHCV independent transformants were slightly weaker than that in pMBPHCV transformants. The nontransformed control leaf and tuber RNA showed no detectable signal at this stringency of hybridization. We also compared HCV expression in leaves and tubers of transgenic plants. Figure 4(b) shows HCV-specific RNA accumulation in tubers of pATHCV line 10 higher than in leaves of pMBPHCV lines, respectively. The preferentially tuber-expressed patatin promoter that is activated at the onset of tuberization in potato and the constitutively expressed CaMV35S promoter were used to drive HCV gene expression. The patatin promoter was an effective in tuber as the CaMV 35S promoter, and transgenic plants containing the patatin promoter produced higher levels of HCV-transcript than the CaMV 35S promoter. These are very similar to the accumulation of HbsAg in tubers of transgenic plants transformed with constructs driven by the patatin promoter reported by Richter et al. (2000), and expression of Norwalk virus capsid protein in transgenic potato reported by Mason et al. (1996). In other case, Sandhu et al. (2000) reported that the average levels of RSV-F antigen in fruits of transgenic plants transformed with constructs with driven by the fruit-specific E8 and CaMV 35S promoter were similar.

The five selected transformed plants through genomic DNA analysis and Northern blotting were analysed for the presence of HCV protein by immunoblot analysis. No HCV-specific protein band was observed in untrans-

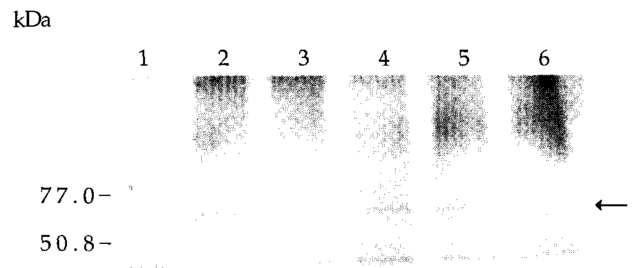

Figure 5. Immunodetection of HCV protein from tuber extract of the transgenic potato plants. Total soluble protein extract (50 μg) was fractionated by SDS-PAGE, blotted onto a PVDF membrane, and probed with a mouse anti-HCV monoclonal antibody. Molecular masses of the prestained SDS-PAGE standard are indicated at the left. Lane 1; protein extract from tuber tissue of untransformed plant. Lanes 2-4; protein extracts from tuber of transgenic plants pMBPHCV No. 2, 7 and 10, respectively. Lanes 5-6; protein extracts from tuber of transgenic plants pATHCV No. 2 and 10.

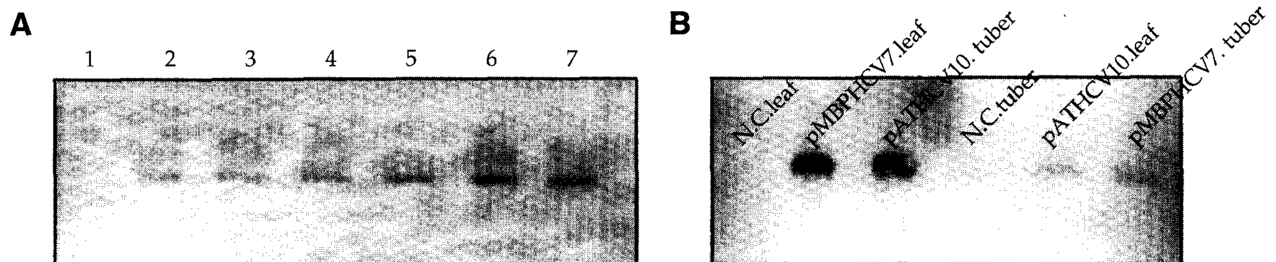

Figure 4. Untransformed and six independent transgenic potato lines carrying either the pMBPHCV or the pATHCV construct were analyzed for HCV-specific mRNA by RNA blot hybridization. (A) Total RNA (20 μg) was fractionated, blotted, and hybridized with HCV-specific probe. Lane 1; wild-type untransformed potato, Lanes 2-3; transgenic plants pATHCV No. 2 and No. 10, Lanes 4-7; transgenic plants pMBPHCV No. 1, No. 2, No. 7 and No. 10. (B) Expression of HCV mRNA extracted from tuber and leaf tissue (total RNA 30 μg) of transgenic lines pATHCV10 and pMBPHCV7. N.C; mRNA from leaf and tuber of wild-type untransformed potato.

formed plant homogenates (Figure 5, lane 1). Transgenic plants containing HCV expressing constructs showed detectable level of HCV-specific protein in the tuber (Figure 5). Thus, expression of a protein of the expected molecular mass (about 55kDa) in the potato plants suggested their possible use as edible vaccines. Through the future studies, mice test are planned to elicit the immune response in immunized mice, we are going to suggest the possibility of transgenic plants expressing HCV gene may be used as a source of recombinant antigen for vaccine production. Proteins involved in protective immune response can be produced at a low cost and easily purified from plant extracts for parental inoculation. The presence of specific antigens into plants, even at low levels, can rise by the oral route immune reactions comparable to those raised by conventional vaccines (Haq et al., 1995; Mason et al., 1996). At present, transformed plants synthesize relatively small amounts (approximately 0.001 0.3%) of foreign proteins. This factor significantly limits the application of edible plants for the production and delivery of therapeutic proteins. Since information on the increased production of foreign proteins in transformed plants is not abundant, alternative methods to enhance the effectiveness of the available therapeutic proteins must be found. Moreover, consistent expression levels of recombinant proteins in plants may be essential for production of edible vaccines in plants. In the investigation reported here, we successfully introduced HCV antigen into potato plants and confirmed the expression of HCV gene in transgenic potatoes. In conclusion, we view this as a successful first step in a long-term project to develop the edible vaccine for protection against the hog cholera disease.

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