# *Expression of the Hepatitis A virus empty capsids in suspension cells and transgenic tobacco plants* (Nicotiana tabacum *L.)*

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RESEARCH

***ABSTRACT***

The unique open reading frame (ORF) encoding the Hepatitis A virus (HAV) polyprotein was expressed in tobacco NT1 suspension cells and transgenic tobacco plants. The full transgene was transcribed in both host systems, as confirmed by Reverse Transcriptase-PCR analysis. The formation of the HAV empty capsid was demonstrated by ELISA using the conformation-specific monoclonal antibody 7E7. Electron microscopy studies revealed the presence of 27 nm virus-like spherical structures similar to HAV particles. This is the first paper reporting the formation of HAV empty capsids in transgenic plant cells and supports the possibility of using transgenic plant cells to express complex viral particles for vaccine production.

*Keywords*: HAV, plant vaccine, VLP, transgenic, polyprotein

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***RESUMEN***

**Expresión de cápsidas vacías del virus de la hepatitis A en suspensiones celulares y plantas transgénicas de tabaco (*Nicotiana tabacum* L.).** El único marco de lectura abierto que codifica para la poliproteína del virus de la Hepatitis A (VHA) se expresó en suspensiones celulares de tabaco NT1 y en plantas transgénicas de tabaco. El transgén se transcribió en los dos sistemas como se confirmó por análisis de reacción en cadena de la polimerasa por Reverso transcriptasa. La formación de cápsides vacías de VHA se demostró por ELISA usando el anticuerpo monoclonal conformacional 7E7. Estudios de microscopía electrónica revelaron la presencia de partículas esféricas tipo virus de 27 nm semejantes a las partículas del VHA. Este es la primera publicación que reporta la formación de cápsidas de VHA en células de plantas transgénicas y sostiene la posibilidad del uso de las células vegetales para expresar partículas víricas complejas útiles en la producción de vacunas.

*Palabras clave*: VHA, vacunas en plantas, VLP, transgénicas, poliproteínas

## *I*ntroduction

Transgenic plant technology was initially used as a way to achieve resistance to pathogenic microorganisms [1], to insects [2] and herbicides [3]. But the evidence of vegetable cell capacity to correctly assembly foreign proteins of a high structural complexity rapidly indicated its potential value as a new strategy to improve the economical production of recombinant proteins of industrial and biopharmaceutical interest [4, 5].

The HAV is a 27 nm non-enveloped RNA virus belonging to the Hepatovirus genus [6] of the *Picornaviridae* family. The HAV genome is a single stranded positive polarity RNA with approximately 7.5 kb, coding for a 253 kDa polyprotein [7]. The HAV replication exclusively occurs within the hepatocyte cytoplasm [8]. The polyprotein suffers both translational and post-translational processes, creating mature structural (VP1, VP2, VP3, VP4 and 2A) and non structural (2B, 2C, 3A, 3B, 3C and 3D) proteins [9].

The HAV polyprotein is cleaved by the 3C protease present in its P3 domain [10]. Thus, a differential proteolytic process is needed for HAV replication and the proper formation of its capsid. The characteristic 27 nm viral capsid comes from the unification of the viral proteins. The assembly of capsid proteins into subviral or virion structures is necessary for the generation of efficient HAV-neutralizing epitopes.

Both, pentamers and viral particles, induce neutralizing antibodies and therefore they can be useful for vaccine development [11]. The ORF expression and capsid assembling was reported in two eukaryotic expression systems, baculovirus [12], and vaccinia virus [13]. However, at present, only inactivated or live-attenuated vaccines are available to prevent HAV infection. Baculovirus and vaccinia virus vectors have some difficulties such as high production cost and safety concerns.

Plant-based vaccines are relatively inexpensive to obtain and their production can be rapidly scaled up [14]. There is also the potential for oral delivery of these vaccines, which can dramatically reduce distribution and delivery costs.

An increasing body of evidences shows that these plant-produced antigens can induce immunogenic responses and confer protection when are delivered orally [15, 16]. Although plant technology is very promising for the expression of foreign proteins, epitopes or adopting tridimensional structures [17, 18]. However, the practical use of transgenic plants as a source of antigen for vaccine production would undoubtedly require, in most cases, the expression of more complex antigenic structures [19].

Here, we report for the first time the expression of the HAV empty capsids in the cytosol of tobacco cells.

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These results demonstrate the possibility of using transgenic plants to express complex antigenic structures for vaccine production.

## Materials and methods

### Construction of expression plasmids

The HAV cDNA was obtained from the M2 RNA strain, isolated and characterized in Cuba [20]. The total RNA was isolated using a purification kit (Promega Corp., Madison, WI) and the DNA fragment (6.7 kb) was amplified by means of the reverse transcription-polymerase chain reaction technology (RT-PCR). The RT was carried out using 1 µg of RNA, 30 pmol of specific primer (5'CTTAATCTAGAATGAA TATGTCCAA-3') complementary to the 3D protein gene (based on published HAV sequences), 200 U of Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), 40 U of RNasin (Promega Corp., Madison, WI), 250 µM each dNTP (Roche Molecular Biochemicals, Indianapolis, IN), 4 µL of 5X reaction buffer in a final volume of 20 µL at 42 ºC for 1 h, followed by incubation for 10 min at 65 ºC.

The PCR was then carried out in a 25-µL mixture containing 2.5 µL of cDNA, 5 units of 0.2 units of *Pfu* DNA Polymerase (Promega Corp., Madison, WI), 1X *Pfu* DNA Polymerase Reaction Buffer and 250 µM of each dNTP and 20 pmol each of sense and antisense primers. The oligonucleotide pairs employed to amplify this regions were 5'-GAAAGAA ATAAAGGTACCTCAG-3' and 5'-CTTAATCTAG AATGAATATGTCCAA-3', respectively. The sequence of the first oligonucleotide contained nucleotides corresponding to the beginning of the ORF sequence and included the initiation codon. The second oligonucleotide corresponded to the sequence complementary to the end of the ORF sequence (3D). The amplification program consisted of an initial denaturation at 94 °C for 6 min followed by 30 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min) and extension (72 °C for 10 min). A final extension of 8 minutes at 72 °C was performed.

The amplified band (6.7 kb) was cloned in Blue Script vector (KS+), previously digested with *Sma*I endonuclease to obtain the pMLA plasmid. It was sequenced in a DNA automatic sequencer (Wageningen University, The Netherlands).The pKMLA plasmid was obtained by cloning the ORF band (digested with *Sma*I) under the 2x 35S CaMV promoter and TEV leader sequence. As terminator, the one of 35S of Ca MV was used. For cloning of the band, the vector pKTPL was *Nco*I digested followed by blunting with the *Klenow* fragment of DNA polymerase I and finally digested with *Sma*I.

The binary pBMLA plasmid (Figure 1) was obtained by *Sph*I digestion of the pKMLA plasmid and subsequent treatment with *Mung Bean* nuclease, rendering a band of 7.5 kb that was cloned in the binary pDE1001 vector previously digested with *Sma*I.

The resultant pBMLA plasmid contains: the neomycin phosphotranferase II gene (*npt* II) which acts as selection marker conferring kanamycin resistance; the ORF gene which codes for the HAV polyprotein and the border sequences of T-DNA to facilitate its transference to the plant genome.

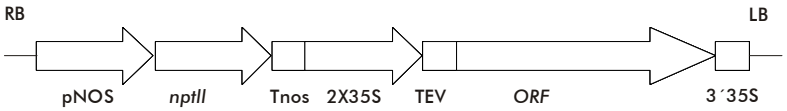


Figure 1. Schematic representation of the binary vector for Agrobacterium-mediated tobacco transformation. pNOS: nopaline synthase promoter; nptII: neomycin phosphotransferase gene; Tnos: nopaline synthase terminator; 2X 35S: CaMV 35S promoter with dual enhancer; TEV: tobacco etch virus enhancer sequence; ORF: HAV Open Reading Frame; 3' 35S: CaMV terminator; LB and RB: left and right T-DNA border.

### Transformation of cell suspensions

The *Agrobacterium tumefaciens* strain At 2260 [21] was transformed by the liquid nitrogen method [22] with the developed binary pBMLA plasmid and then used to transform *Nicotiana tabacum* cv. Bright Yellow 2 (suspension NT-1 line) cells. Transformants were obtained as described [23] and selected on the medium supplemented with 100 mg/L kanamycin (Sigma Chemical Co., St. Louis, Mo.). Chosen lines were maintained on NT-1 selective medium. The two highest-expression lines were placed into liquid suspension culture in 250 mL shaker flasks at 25 °C and 120 rpm and passed every 10 days to fresh medium after suspension establishment.

Suspension cultures were vacuum filtered through MiraCloth (Calbiochem, La Jolla, CA), rinsed once with distilled water, and refiltered. Fresh weight (fw) was detemined and cell samples were stored at -70 °C for subsequent analysis.

### PCR and RT-PCR analyses

The presence of the HAV nucleotide sequences in suspension cells and transgenic plants were detected by PCR. Genomic DNA was extracted from frozen tobacco cells using the Nucleon Phytopure extraction and purification kit (Amersham International plc, Buckinghamshire, England). PCR reactions were carried out using 500 ng of genomic DNA, 40 pmol each primer, 250 µM dNTPs (Roche Molecular Biochemicals, Indianapolis, IN), and 2.5 units of *Taq*-polymerase 4 °C (Promega, Madison, Wi, USA). After an initial denaturation step for 5 min, the PCR reactions were performed for 30 cycles including 1 min of denaturation at 95 °C, 1 min of annealing at 55 °C, and finally 1 min of polymerization at 72 °C. Forward primer, 5'-CCTCATGGTCTGTTAAATTGCAATATCA-3' and reverse primer, 5'-GTAAAAGTGATGGACCT GTA GGT-3' were used for amplification of the region between positions 504 and 902. The non transformed tobacco cells were used as negative control.

The transcription of the transgenes was analyzed by performing RT-PCR. Total RNA from suspension cell (1 g) and transgenic tobacco leaves (500 mg) was isolated using a purification kit according to the manufacturer's recommendation (Promega Corp., Madison, WI).

RT-PCR (Access RT-PCR Introductory System; Promega Corp, Madison, USA) was performed using 6 µg of total RNA according to manufacturer's recommendation in 50 µL reaction volumes. Primers were designed to amplify a 995 bp fragment of the HAV ORF (between positions 4262 and 5256). The forward primer was 5'-CTCAGGGAATTTCAGATGATGAC AATG-3', and the reverse primer was 5'-TACATTCA TTGAACATTGAGTAAATTC-3. Regenerated but

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non-transformed plants, pBMLA plasmid and reactions without RT were used as experimental controls.

The PCR parameters: 94 °C for 2 min one cycle, 94 °C for 1 min (denaturing) 55 °C for 1 min, (annealing) and 72 °C for 1 min (extension) for 30 cycles, with final extension 72 °C for 10 min.

### Protein analysis

For ELISA analysis, proteins were extracted from NT-1 cells and plants (25 g) by grinding with liquid nitrogen up to obtaining a very thin dust. 25 mL of cold extraction buffer (50 mM $Na_2HPO_4$, pH 7; 50 mM $NaH_2PO_4$, pH 7; 10 mM DTT, 0.1% Sarkosyl; 0.1% Triton X-100) were added to each tube. The insoluble material was removed by centrifugation at 9 000 *x g* for 15 min. Total soluble proteins were determined as previously described (Bradford *et al.*, 1979), using bovine serum albumin (BSA) as protein standard (Bio-Rad Laboratories, Hercules, CA).

### Sucrose precipitation

For particles purification, 20 mL of the protein extract was pelleting through 40 mL 20% sucrose cushion, and ultracentrifuged at 40 000 rpm for 2 h at 4 °C with a SW40 rotor in a Beckman ultracentrifuge. The pellet obtained was resuspended in 1 mL of PBS (100 mM NaCl, 80 mM $Na_2PO_4$, 20 mM $NaH_2PO_4$, pH 7.4).

### Sample concentration

The purified particles were concentrated by filtration through centrisart-C4 (100 000 NMWL cut-off) (Sartorius, Göttingen, Germany), at 7 000 *x g* centrifugation. Samples were stored at 4 °C and then analyzed by ELISA and electron microscopy. A non transgenic NT-1 cell extract was prepared in the same way as negative control. The total soluble proteins were measured by the BioRad Bradford method.

### Immunoenzymatic assay

The HAV particles were detected by a specific "sandwich type" ELISA using the neutralizing 7E7 monoclonal antibody (MAb) as capture and detection antibody [24]. The 96 well plate (Maxisorp, Nunc) was covered with 10 µg/mL of the 7E7 MAb (Mediagnost, Tübingen, Germany) in carbonate buffer ($Na_2CO_3$ 0.015 M, $NaHCO_3$ 0.028 M, pH 9.6) during 4 hours at 37 °C. The blocking step was carried out during 2 h at 37 °C, with 5% skim milk. Later 100 µL of the samples were added. The plate was incubated overnight at 4 °C. After washing with 0.1% Tween 20 in PBS, 100 µL of the 7E7 MAb, conjugated with alkaline phosphatase (Mediagnost, Tübingen, Germany) at 1/1000 dilution in PBS containing 0.5% skim milk, were added. The plate was incubated at 37 °C during 2 h. The reaction was developed by addition of 4-nitrophenylphosphate (substrate of the enzyme), prepared in 0.1% of diethanolamine buffer, pH 9.8. The color development was followed during a 60 min period. The absorbance was read in a spectrophotometer at 405 nm wavelength. The plate was washed three times with PBS-Tween at all stages of the ELISA.

### Transmission electron microscopy

The cell suspension samples (line 27 and non-transformed cell) were fixed for 1h at 4 °C in 1% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde, rinsed in 0.1 M sodium cacodylate (pH 7.4), post-fixed for 1 h at 4 °C in 1% $OsO_4$, and dehydrated in increasing concentrations of ethanol. The embedding process was done as previously described with minor modifications [25]. Briefly, ultra thin sections (400-500 Å) made with an ultramicrotome (NOVA, LKB) were placed on 400 mesh grids and stained with saturated uranyl acetate and lead citrate.

The purified preparation from broth samples were negatively stained with 2% uranyl acetate prior to analysis by transmission electron microscopy as previously described [26]. All samples were examined with a JEOL/JEM 2000 EX transmission electron microscope (JEOL, Japan).

## Results

### Production and genetic analysis of transformed tobacco cell lines and plants

The plasmid pMLA carrying the HAV ORF from M2 strain, isolated and characterized in Cuba, was sequenced and the sequences were deposited in GenBank with accession number AY974170.

In an attempt to express HAV particles in the cytosol of tobacco cells, NT-1 cells and plants were transformed with pBMLA plasmids (Figure 1). Two tobacco cell line suspensions and ten transgenic tobacco plants containing the ORF of HAV gene were initially produced. The presence of the HAV ORF genes was detected in all the transformed cell lines and plants by PCR amplification of the expected size (400 bp). On the other hand, this product was consistently absent in non-transformed cells and plants (Figure 2B).

We confirmed the transcription of the recombinant genes from the lines and transformed plants by RT-PCR; Amplification was not observed in non-transformed cell lines or plants. Controls without RT were used to confirm the absence of genomic DNA contamination (Figure 2C).

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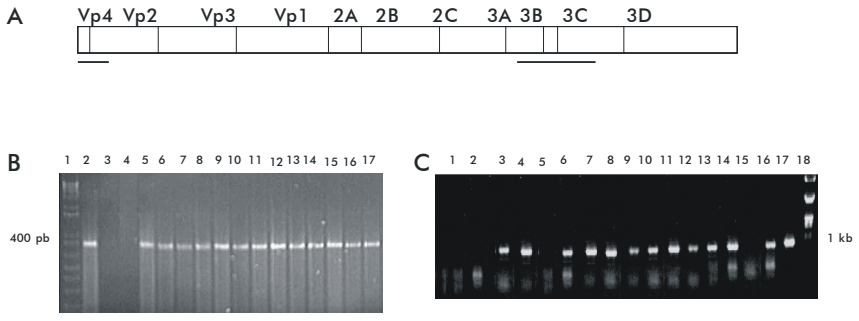


Figure 2. Analysis of transformed tobacco suspension cells and plants. A: Schematic showing HAV polyprotein. B: Detection of the fragment VP0 genes in transgenic cells by PCR. Tobacco DNA was isolated from cell extract, and PCR was performed with a pair of primers designed to amplify the region between positions 502 and 904 base of the VP0 genes. Lanes: 1, DNA market; 2, pBMLA plasmid; 3, not transformed NT1 cell; 4, not transformed tobacco plant; 5, transformed line 20; 6, transformed line 27; 7, transformed line 33; 8-17, transformed tobacco plants. C: Detection of the fragment 3ABC genes in transgenic plants by RT-PCR. Tobacco RNA was isolated, and RT-PCR was performed with a pair of primers designed to amplify the region between positions 4262 and 5256 base of the 3ABC gene (0.9 kb). Lanes: 1, transformed line 20 without RT-PCR; 2, not transformed NT1 cell; 3, transformed line 20; 4, transformed line 27; 5, not transformed tobacco plant; 6-14, transformed tobacco plants (clon 1-9); 17, transformed tobacco plant (clon 10) without RT; 14, transformed tobacco plants 10; 15, pBMLA plasmid; 16, λ *HindIII-Eco*RI DNA market.

### Detection of the presence of HAV empty capsids in the selected tobacco cell lines and plants

The presence of empty capsids in the cell suspensions (20 and 27 lines) or leaf extracts from ten plants were analyzed using the conformation-specific neutralizing 7E7 MAb, by ELISA assay. The HAV particles were detected in the suspension cells. The antigenicity of the concentrated extracts of the lines 20 and 27 were always higher than the untransformed (C-) plant cell culture and PBS buffers, designed as negative control for the ELISA (Figure 3A). A 1/20 dilutions of the purified HAV (0.302 ng/μL) were used as positive controls for all the assays. The expression in plants did not behave in similar way (Figure 3B). The concentration of the HAV particles expressed in the transgenic tobacco cells was estimated to be 0.0015% of the total soluble protein (TSP).

These results suggest that HAV proteins expressed in transgenic cells were able of correctly self-assembled into particles.

Tobacco cells (L 27) were examined for HAV particles using a transmission electron microscope. This analysis revealed vesicular bodies in transgenic cells which can contain particles of approximately 27 nm (indicated by arrows in Figure 4B). On purified proteins we observed numerous aggregates of particles with approximate diameter of 27 nm which were indistinguishable from HAV, both in size and shape (Figure 4D). Microscopic examination of non-transformed cells (Figure 4A) or non-transformed tobacco cell sample purified in the same way did not show evidence of particle formation (data not shown) or any subcellular vesicles containing particles

## *D*iscussion

The use of viral empty capsids or pentamers is essential to achieve an adequate protective immune response against HAV. A variety of potential expression systems have been investigated for the expression of HAV particles. However, only four inactivated hepatitis A vaccines are currently available [27].

The possibility to insert the HAV ORF into the plant genome and its constitutive expression rendering of empty capsids or pentamers, offers an alternative to produce vaccines against HAV. The electron microscopy confirmed the presence of VLP that are morphologically similar to HAV. The HBsAg [16, 28, 29], and Norwalk virus capsid protein (NVCP) [15, 30] were among the few antigenic structures with some degree of complexity that have been stably expressed in plants. Preliminary results of the expression of the P1 polyprotein of FMDV in alfalfa plants was the only complex antigenic structure published until now [31].

We expressed the HAV ORF without any leader signal. Our objective was to drive the polyprotein into the cytosol of the tobacco cell and then accumulate HAV particle in this compartment, considering that the HAV polyprotein is processed in the cytosol of hepatocytes [8]. Here we did not examined the proteolytic processing of the HAV polyprotein by the viral protease 3C nevertheless, the similarity of the plant produced HAV particles and the original virus suggested the possibility that the 3C protease is responsi-

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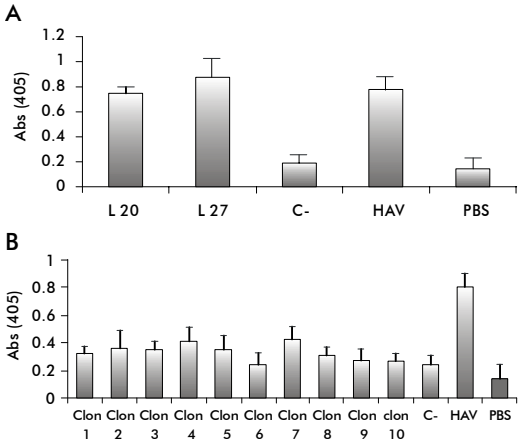


Figure 3. Detection of purified particles from tobacco cells by ELISA assay. Protein extract from plant were purified and concentrated by a combination of ultracentrifugation and the use of a centrisart filter. Each value corresponds to the mean ± the standard deviation of three independent tests. A. *L 20*, tobacco cell culture line 20; *L 27*, tobacco cell culture line 27; *C-*, untransformed NT1 cell. B. *Clons* 1-10, transgenic tobacco plants; C-, untransformed tobacco plant; *HAV*, dilution 1/20 purified HAV antigen (0.30 ng/μL); *PBS*, Phosphate Buffers Saline.

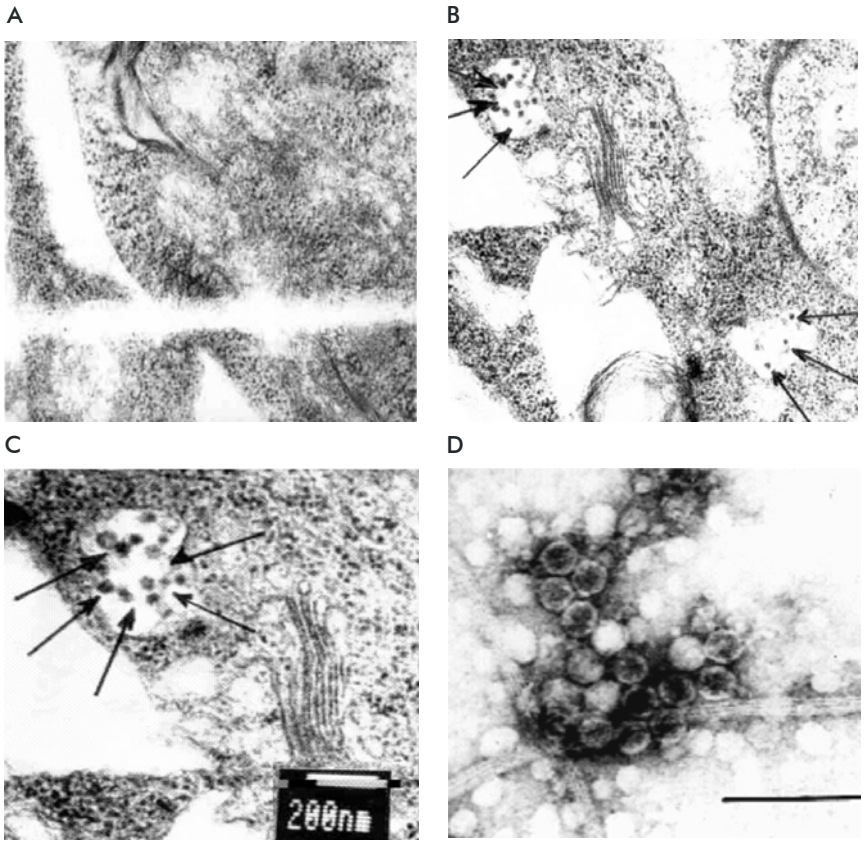


Figure 4. Transmission electron microscopy of HAV particles expressed in tobacco cell suspension. (A) Image of an untransformed tobacco cell. (B) Image of a HAV in transgenic tobacco cells. Arrows indicate the vesicles containing 27 nm putative HAV particles. (C) Enlarged image of vesicles containing putative HAV particulate structures indicated by arrows. (D) Particles purified by a combination of ultracentrifugation and concentration with centrisar filter. (Bar= 200nm).

ble for polyprotein processing. The 3C protease is the only one that has been implicated in the nine protein cleavages of the HAV polyprotein [32]. Other authors reported that the HAV particle expression in *E. coli* is processed by the *E. coli* protease, but the size and shape may not be the same as those of 70S HAV particles [33]. The expression in tobacco cell suspensions of particles that were similar to HAV, support the idea that plant cells can manufacture functional capsids from polyprotein. This evidence suggested that the 3C protease is presumably functional in plant cells.

The expression levels of recombinant HAV particles in our studied lines were low (0.0015% of the TSP). Similar results were previously obtained with NVCP and HBsAg [15, 18].The HAV particles were not detected in leaf extract probably due to their lower stability in the green tissue. Proteolytic degradation represents a significant barrier to the efficient production of several recombinant proteins in plants [34]. Generally, levels of pharmaceutical proteins produced in nuclear transgenic plants have been less than the 1% of TPS [35, 36]. The low expression of these proteins seems to be the main problem to be solved to develop any vaccine in plant in the future.

However, the expression of HAV particles might be increased by several modifications, including the use of stronger promoters [37], targeting the protein for accumulation in different compartments [38] or producing transplantomic plants. High levels of antigen expression have been achieved with chloroplast transformation [39].

These results demonstrate, the possibility of using transgenic plants to express complex antigenic structures for vaccine production

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