Expression of Recombinant gD2 Protein in Transgenic Tomato plants for development of a plant-derived vaccine against *Human herpes virus 2*

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ABSTRACT

Human herpes virus 2 (HSV) is a DNA-viral genome and cause infection for both human and animal. HSV-1 and HSV-2 are causing infection to human. HSV-2 is a sexually-transmitted virus. HSV-2 is prevalence in European countries and also in Egyptian-closed societies like Upper Egypt. Egyptian isolate of HSV-2 was isolated and Glycoprotein D (HSV-2gD) subunit was amplified using specific PCR primers.PCR product was molecularly cloned in t *E.coli* cells using PCR2.1/TOPO/ TA cloning vector. HSV-2gD fragment was liberated from 1 µg recombinant plasmid by using the restriction endonuclease *EcoRI*. HSV-2gD was successfully sub-cloned into binary plant expression vector PBI-121. HSV-2gD subunit-containing binary vector PBI 121 were transformed into *Agrobacterium tumifaciens* LBA 4404 strain for Agro-inoculation. Tomato plants were transferred into regeneration medium and genetically transformed with the HSV-2gD-PB21 binary vector through Agrobacterium co cultivation. Agro-inoculated tomato plants were tested for the HSV-2gD insertion and transcription using both PCR and RT-PCR and the results were successful in getting insert-containing tomatoes against HSV-2gD. Specific expression of the introduced antigen construct at the RNA level was detected by RT-PCR, using specific primers for the HSV-gD2 transcript. Expression of the HSV-2 gD antigen was assayed by ELISA on protein extracts isolated from same transfected plant tissue samples as for the RT-PCR assays. Results showed that the HSV-gD2 construct was expressed at both the RNA and protein levels in the transgenic tomato plants. Finally, we report here, the use of stable transformation to develop transgenic tomatoes expressing the biologically active HSV-2 gD antigen (gD2). Thus, our results may have implications for the development of plant based vaccine production systems against HSV-2.

Key words: *Human herpes virus 2*, Polymerase chain reaction (PCR), Transformation, edible vaccine, tissue culture, antigen expression, food vaccines.

INTRODUCTION

Human herpes virus 2 (HSV-2) is an enveloped virus which a highly prevalent sexually transmitted infection (STI), and considered as the most common cause of genital herpes in developing countries (Looker *et al.*, 2008. Wald and Link, 2002; Corey *et al.*, 1998; Fields, 2001). The genital lesions produced by latent infection of HSV-2 can facilitate the entrance of infectious agents such as human immunodeficiency virus (HIV) (Dickerson *et al.*, 1996, Wald and Corey, 2003). Considering the complications of this virus, some synthetic antiviral compounds based on guanosine analogues, such as acyclovir, penciclovir and vidarabine were developed for treatment of active herpetic infections, but they are not effective for the treatment of latent infections (Naesens and Clercq, 2001).

Many considerable efforts have been made to develop a vaccine against genital herpes (Sara *et al.*, 2005). Different genital HSV model systems were used for the experimental investigations for several candidate vaccines (Chu *et al.*, 2008). The

candidate vaccines which developed using the traditional methods such as inactivated viruses, protein subunit, recombinant glycoprotein, plasmid DNA, and attenuated live viruses have been shown to confer variable levels of protective immunity; however, the application of these vaccines is precluded in humans due to safety concerns (Koelle and Corey, 2003). Individual recombinant HSV proteins such as immunodominant envelope glycoprotein D (gD) is one of the best studied examples, that have shown promise as prophylactic or therapeutic vaccines in animal models when administered in combination with an adjuvant (Sara *et al.*, 2006). In humans, the administered HSV protein subunit vaccines have displayed poor protective immunity. Thus, there remains a need to develop new strategies to prevent genital herpes infection (Bourne *et al.*, 2003).

Plants have been used to produce recombinant proteins, including subunit vaccines antigens for human diseases (Aboul-Ata *et al.*, 20014; James *et al.*, 2013). The use of plants to produce vaccine has several practical advantages compared to fermentation or cell-culture facilities such as delivery of edible vaccines by the oral route, the ease of agricultural scale production, safety from contaminating human or animal pathogens, and the possibility of growing and manufacturing vaccine materials locally (Nemchinov *et al.*, 2000 and El-Attar *et al.*, 2004). The benefits of increased safety and cost-effectiveness make vegetable crops, such as tomato, inexpensive biofactories and appropriate systems for the production and delivery of oral vaccines in the developing countries (Maxim, 2011 and Rice *et al.*, 2005). The field of crop plant biotechnology is now at an exciting stage of development and advancing rapidly due to novel developments in genetic and genomic tools (Maria *et al.*, 2013 and Sarw *et al.*, 2013). The list of plant-derived vaccinogens continues to grow and includes viral, bacterial, enteric and non-enteric pathogen antigens (Ahmad *et al.*, 2010). Transgenic plants have been found to have many advantages like, development of high yielding varieties of crop plants and disease resistant (Ahmad and Umar, 2011; Ahmad and Prasad, 2012).

In this research we focused on the development of transgenic tomato plants to express the HSV-2gD antigen as a step forward to be used as edible vaccine against the HSV-2. The stable transformation utilizing the Agrobacterium cells with binary plant expression vector carrying the Egyptian HSV-2gD gene was used instead of chimeric virus-based vector strategy.

MATERIALS AND METHODS

Genomic Material and Polymerase Chain Reaction

HSV-2 DNA was prepared from the HSV-2using the NucleoSpin virus kit (Clontech Laboratories Inc.). The HSV-2 infected serum was collected from Egyptian patients and processed at the faculty of medicine, El Azhar University. Normal serum collected from healthy patient was used as negative control for the PCR analysis.

The total DNA extracted from HSV-2 infected serum was used as a template for PCR to amplify the gD gene. The PCR mixture for each reaction contained 20 ng of template DNA, 1x polymerase reaction buffer provided with 2.5 mM MgCl2, 200 µM of each dNTP, 25 pmol of each primer, 1.25U of dream-Taq polymerase (Fermentas) and sterile water to a final volume of 25 µl. The specific Primers were designed to bracket a well-conserved region in Glycoprotein D. The forward primer sequence was GD-Xba-F: 5′CCC TCT AGA GGC ATG GGG CGT TTG ACC TCC3′ and reverse primer sequence was GD-Xba-

r:5′ GGG TCT AGA TTA GAT CAG GCC CGG GTT GCT GGG GGC 3′. Amplification program started with denaturation step at 94°C for 5 minute followed by 35 cycles starting with denaturation at 94°C for 30 second, annealing at 65◦C for 1 minute and primer extension at 72◦C for 2 minute with a final extension step at 72°C for 10 min. The PCR products were stained with gel star (Lonza, USA) and analyzed by electrophoresis in 1.0% agarose gel and visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA).

Molecular cloning

All enzymatic digestions, ligations, cell transformations, and other manipulations with DNA were done according to (Sambrook *et al.*, 1989). The fresh PCR product was molecularly cloned in One Shot Top 10 chemically competent *E.coli* cells using PCR 2.1/TOPO/ TA cloning vector (Invitrogene, USA) and the clones were identified by restriction analysis after plasmid purification, and results were confirmed with DNA sequence analysis. The standard mini-prep protocol was used to isolate pure super-coiled plasmid DNA. The DNA insert fragment was liberated from 1 µg recombinant plasmid by using the restriction endonuclease *EcoR*1. Automated DNA sequencing reactions in MWG in Germany were performed using the universal M13 forward and reverse primers.

Molecular sub cloning

Each purified clone (TOPO- HSV-2 gD), which gave a 100% identity after alignment with the gD sequence published on GenBank, and the purified binary vector PBI 121 were digested using the restriction endonucleases *XbaI.* The fragment of HSV-2 glycoprotein D sequence was extracted and purified from the agarose gel using QIAquick Gel Extraction kit. Linearized PBI 121 binary vector was purified as well using PCI. The digested insert was ligated into PBI 121 binary vector using T4 DNA ligase enzyme and transformed in DH5α competent *E.coli* cells using 1 min. heat shock. The clones were tested using PCR colony amplification using specific primers compatible with PBI vector and HSV-2 glycoprotein D insert. The forward primer, 30B-Gd-f: 5′ CCC TTT AAT TAA TGG GGC GGT TCC3′ and the reverse primer, 30B-gD-r: 5′ CCC TCG A-GT TAG ATC AGG CCC GCT 3′ (thermo scientific).PCR file was the same.

***Agrobacterium* preparation and Tomato transformation**

Preparation of explants

Tomato seeds of MP1 and Castle Rock varieties were sterilized and inoculated in an autoclaved- germinate medium (4.3 g/l MS, 2 mg/l glycine, 15 g/l sucrose and 0.8% plant agar at pH 5.8) in sterile jars and were let in dark room for 5-7 days at 24°-26°C after that all germinated jars were kept at 24°C -26°C for 16 hours photoperiod in a growth chamber for 10 days.

The well-developed cotyledons were taken out of agar and placed on a Petri dishes covered with sterile filter paper. The cotyledons were separated from the hypocotyls near the stem. Large cotyledons were divided into two pieces. Cotyledons were placed in the agar plates containing the regeneration medium (the upper face close to medium) and 40-60 explants/plate were put.

Preparation of Agro bacterium culture:

Agrobacterium tumifaciens competent cells were prepared as follow: Competent cells of *Agrobacterium tumifaciens* strain LBA4404, that was kindly supplied by prof. Ghandi Anfoka, at Al-Balqa Applied University, Jordan (other strains may cause *Agrobacterium* contamination for explants) was grown in 10

ml LB with 50µg/ml rifampicin for 48 hours at 28°C with shaking. Ten ml cells were centrifuged for 10 minutes at 4500 rpm. The pellets were re-suspended in 0.5 ml LB medium (without antibiotics) and were kept on ice. The *Agrobacterium tumifaciens* competent cells were aliquot in 100 µl sample and kept at -80°C for using it later.

To transform the PBI 121 into previously prepared *Agrobacterium tumifaciens* competent cell by heat shock; five µg from the PBI 121 vector containing HSV-2gDgene were added to *Agrobacterium tumifaciens* competent cells and left for 15 minutes on the ice and then were kept for 5 min in liquid Nitrogen and then were incubated at 37ºC for 10 minutes. One ml LB medium (without antibiotics) was added to the mixture and incubated for 4 hours at 28ºC with shaking. The cells were centrifuged for 5 minutes at 4500 rpm. Supernatant was discarded and the pellet was dissolved in 80µl of LB medium (without antibiotics). The mixture was kept on LB agar medium plates supplemented with 25 µg/ml kanamycin and 100 µg/ml rifampicin. The plates were incubated at 28ºC for 48-72 hours. Single colonies were picked up with a sterile tooth picks, Single colony was taken and was deposited in 20 µl sterile water. And 2 µl was used for PCR using HSV-2 gD specific primers (30B-Gd-f/30B-gD-r). This work was done to confirm that Agrobacterium tumifaciens was contained of PBI 121 binary vector carrying the HSV-2 glycoprotein D.

One *Agrobacterium* transformed colony was grown in 5 ml LB medium containing 50 µg/ml rifampicin and 25 µg/ml kanamycin and kept for 2 days at 28ºC with shaking (200 rpm). One ml of *Agrobacterium* culture was diluted in 30 ml LB medium which is containing previous antibiotics and was grown for 12-16 hours at 28ºC with shaking, until the OD_{600} is 0.3-0.5. The previous *Agrobacterium* cells were collected by centrifugation for 10 minutes at 4500 rpm and the pellet was re-suspended with 30 ml liquid germination medium. Acetosyringone was added to a final concentration of 375µM.

Co-cultivating explants with the transformed Agrobacterium:

Six ml of previous *Agrobacterium* was spread on regeneration-agar medium containing explants. The plates were incubated for 2 hours at 26 ºC in the dark. The excess *Agrobacterium* was removed from the plates with a pipette and the total treated explants were arranged in the plates so that they touch each other. The plates were left at 26 ºC in the dark for 2 days.

Regeneration and selection of transformed explants:

The cotyledons were transferred to the regeneration medium (4.3 g/l MS, 2 mg/l glycine, 3% glucose, 1mg/l Zeatine, 0.1mg/l Indol Acetic Acid (IAA) and 0.8% phyta-gel at pH 5.8) containing 500 µg/ml carbenicillin, 250 µg/ml claforan and 50 µg/ml kanamycin. The plates were incubated for 5 weeks in growth room (in the same previous mentioned condition) until 2 to 3 leaflets have regenerated.

Elongation

The regenerated explants (at the 2-3 leaves stage) were cut out gently from the callus, using a sharp scalpel. The explants were transferred to jars that containing elongation medium containing (4.3 g/l MS, 2 mg/l glycine, 3% glucose, 0.l mg/l Zeatine, 0.04 mg/l IAA and 0.25% phytagel at pH 5.8). The elongation medium was replaced every 10 days. During each transfer the callus is cut out around the shoots.

Rooting

After shooting (2-3 cm long), the plants were transferred to the rooting medium (4.3 g/l MS, 2 mg/l glycine, 1.5% sucrose, 2 mg/l Indol Butyric Acid (IBA) and 0.25%

phytagel at pH 5.8) that containing 500 µg/ml claforan and 500 µg/ml carbenicillin in addition to 50 µg/ml kanamycin as a binary-vector antibiotic. The plants were incubated for 30 days to have formation of a strong rooting system.

Planting in soil and hardening

The well-developed plantlets, with developed roots were taken out from the media, and were washed under running water. The washed plantlets were planted in pots containing wet compost supplemented with vermiculite and covered with plastic bags, then transferred into greenhouse for growing and hardening. It was checked every day. One to 2 holes were made in the plastic bags to allow air exchange, and these holes could be used to irrigate the plantlets as well. After 10-15 days the plastic bags were removed from the plants.

Selection of transformed plants using PCR and RT-PCR

A small piece of the transformed leaflets (about 2 mm) were cut from the explants. DNA was extracted using the Delleporta method. Small amounts of tissue were collected in microfuge tubes. Five hundred µl of Delleporta buffer was added to the samples and they were grinded well. Thirty three µl of 20% SDS was added to the samples, vortexed and were incubated for 10 minutes at 65 ºC. One hundred sixty µl of 5 M potassium acetate were added, vortexed and were centrifuged at 10,000 rpm for 10 minutes and 500 µl were removed from the supernatant of each sample to clean tubes (in case of large amount of tissue the last step was repeated). Equal volume PCI (phenol chloroform isoamyl alcohol) was added to the supernatant and the samples were shacked for 5 minutes. The samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant were transferred to clean tubes. Three hundred fifty µl ice cold isopropanol were added to the samples, vortexed and were centrifuged at 10,000 rpm for 10 minutes. The supernatants were discarded and 500 µl of 70% ethanol and were centrifuged at 10,000 rpm for 5 minutes twice. All samples were dry for 5 minutes in the speed vacuum. Finally the pellets were re-suspended in 50 µl TE RNase buffer. One µl each sample was tested with PCR using specific primers of the gene of insert (HSV-2 glycoprotein D).

Using GeneJet RNA purification kit (Fermetas, # K0731)RNA was extracted from transformed tomato plant tissue and the extracted samples were tested with RT-PCR. The RT-PCR mix was 12.5 µl master mix, 1.25 µl RT-enhancer, 3µl from RNA extraction, 1 µl from forward and reveres primer, 0.5 µl from reverse transcriptase enzyme and 5.75 µl water.

ELISA

Protein was extracted from tomato leaf samples previously transformed with the gD constructs and homogenized in 1X PBS containing 1% plant protease inhibitor cocktail (Sigma) and centrifuged 2x14000 rpm for 10 min at 4 ºC in an Eppendorf centrifuge. The supernatant fraction was used for ELISA experiments. Micro-titer plate wells were coated with anti-gD antibodies (Mabs). After incubating for 2h at 37ºC the wells were blocked for 1 h at 37ºC with 1% BSA solution containing 0.1 % Tween20. The plates were then incubated overnight with the protein extracted from the transformed tomato samples (plant extracts) at 4ºC, followed by incubation with anti-gD antibodies at 37ºC for 2 h. Washings were performed 5 times with 1X PBS-Tween buffer between the different incubations. Anti- mouse IgG-AP conjugate was then added and after incubation at room temperature for 2h, alkaline phosphatase substrate was added to the wells. Plates were read at 405 nm using an ELISA reader (ELX 800, Biotek Instruments, Inc.).

RESULT

HSV-2gD constructs engineering and nucleotide sequencing:

Polymerase chain reaction:

PCR amplification of HSV-2 Glycoprotein D Egyptian isolate was carried out. Glycoprotein D gene isolated from total viral DNA is shown in Fig. 1 as a single band at1021 bp. The serum collected from healthy patients didn't show any bands after PCR.

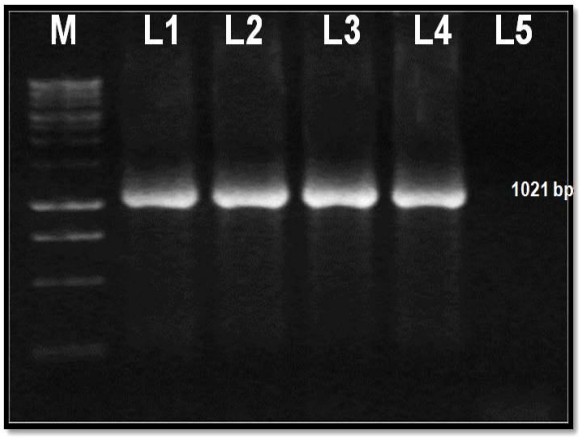

Fig.1: Electrophoresis for PCR product of HSV-2 gD. M: 1kb DNA ladder. L1, L2, L3 and L4 PCR products amplified from 4 different samples infected withHSV-2. L5: Negative control.

Restriction enzyme digestion:

The PCR product, which give the correct size, was directly cloned into pCR2.1-TOPO. Restriction digestion of TOPO/HSV-2 gD plasmid using *EcoR*I restriction endonucleases, after plasmid purification, showed a clear band at the expected size of the insert (1021 bp) in addition to another band specific forthe digested vector at the expected size (~ 4000 pb) as shown in Fig. (2).

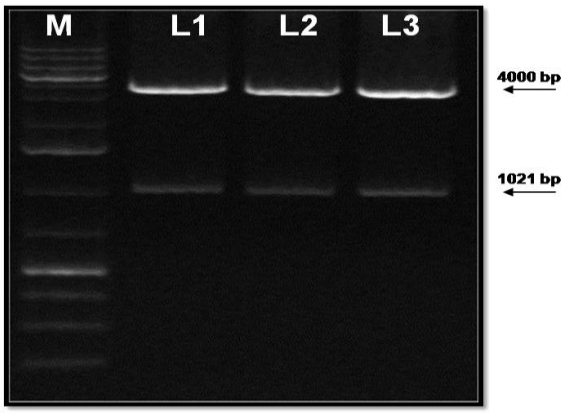

Fig.2: Shows the *EcoRI* digestion of the TOPO-HSV-2gD clones. L1 to L3are different clones; M is 100 bp plus DNA marker.

Sequence analysis and cloning of the expression construct:

Nucleotide sequence alignment between the sequence data obtained from the DNA automatic sequence for the pre checked plasmids and the designed consensus sequence for HSV-2 glycoprotein D and were shown 100% identify with the Egyptian isolate.

The HSV-2 glycoprotein D fragment was release from the TOPO/HSV-2 glycoprotein D plasmid, which gives 100% alignment with the Egyptian isolate, using *XbaI* restriction endnuclease. The digestion mixture (50 µl total volume) from glycoprotein D and PBI 121 vector were electrophoresed on agarose gel to check for completion of digestion and to verify the size of insert. The HSV-2 glycoprotein D fragment (1021 bp) digested with *XbaI* restriction endnuclease was excised from the low melting agarose gel and purified using QIAquick gel extraction kit (QIAGene, Germany). The PBI 121 vector was linearized with the *XbaI* restriction endnuclease; electrophoresed and purified with PCI then rechecked for both vector and HSV-2 glycoprotein D with electrophoresis before sub cloning Fig.(3). The gel extracted HSV-2 glycoprotein D fragment was ligated

with PBI 121 vector and molecularly sub cloned in *E.coli* DH5 α strain. The PBI 121 / HSV-2 glycoprotein D recombinant construct was purified from the DH5 α *E.coli* bacterial cells and checked for the insert which give correct insert size and orientation of HSV-2 glycoprotein D fragment using colony PCR through specific primers for The PBI 121 / HSV-2 glycoprotein D recombinant construct.

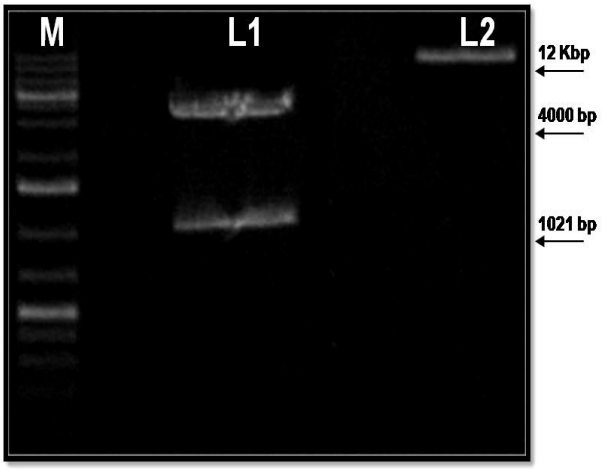

Fig. 3: L1: *XbaI* digestion for the TOPO /HSV-2 gD constructs. L2: The linearized PBI 121 vector digested with *XbaI* enzyme digestion. M; 100 pb (plus) DNA marker.

Colony PCR for testing the transformed Agrobacterium:

Agrobacterium Competent cells were transformed by PBI-gD construct using heat chock. The colony was tested by PCR colony and showed single band at the expected size of gD (1021 bp) Fig. 4.

Agrobacterium mediated transformation:

MP1 and Castle Rock tomato seeds were germinated in germination media as shown in Fig. (5A). The developed cotyledons were infected with *Agrobacterium tumifaciens* contacting the PBI/gD engineered construct and transferred to the regeneration medium that was supplemented with three types of antibiotics: Rifampicin, kanamycin, carbenicillin and growth hormones: Zeatin and Indol Acetic Acid (Fig. 5B).

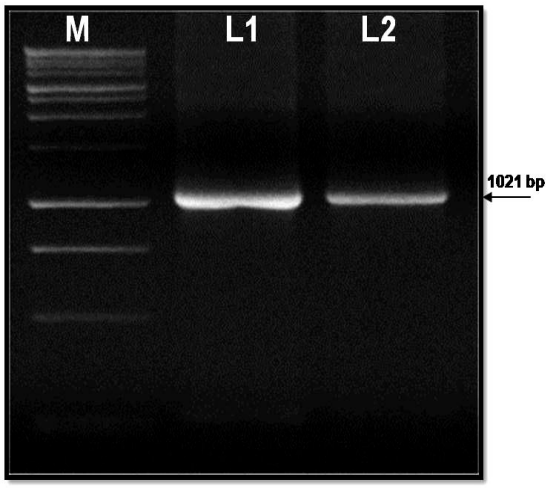

Fig. 4: Electrophoreses analysis for PCR colony amplification for *Agrobacterium* containing glycoprotein D

The regenerated explants were transferred into elongation medium contain the same growth factor and the same antibiotics (Fig. 5C). The regenerated explants were cut out gently from the callus and were transferred to jars that containing elongation medium which was supplemented with the same antibiotics and the same growth hormones and let for 1 month. The shoots were transferred to the rooting medium supplemented with the same antibiotic and Indol Butyric Acid (IBA) which stimulate the root's growth (Fig. 5 D).

Screening for the transformation and expression of gD gene in transgenic tomato plants:

The PCR results for the DNA extracted from the transformed plants showed the successful transformation of the HSV-2 gD into tomato cells. Figure 6 showed a single band at the expected size of gD gene when DNA was extracted from transformed plants and not when the DNA was extracted from non-transformed tissues.

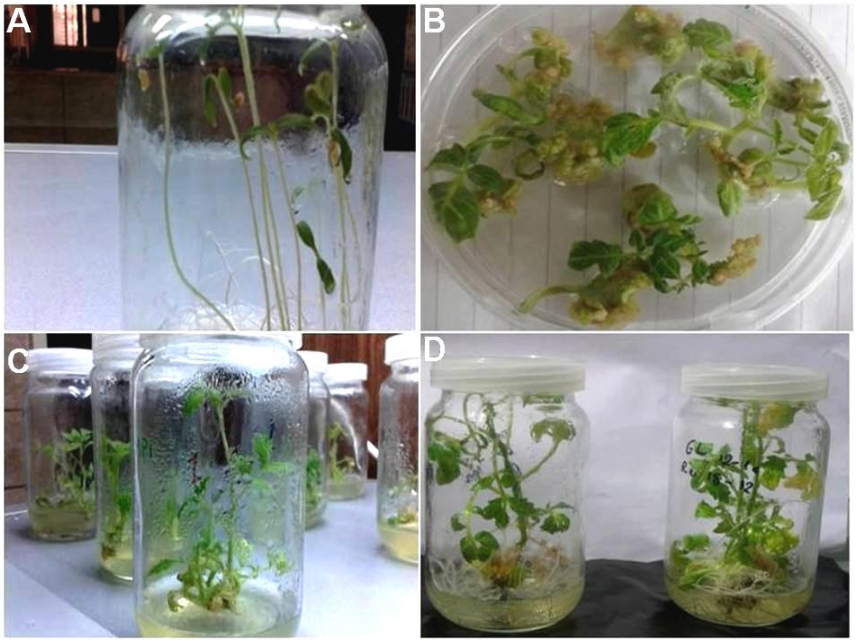

Fig. 5: Tissue culture line for the tomato plants transformed with the Agrobacterium. A: germinated tomato seeds15 days old; B: Agro-inoculated plants in the regeneration stage; C: transformed plants in elongation stage and D: transformed plants in the rooting stage.

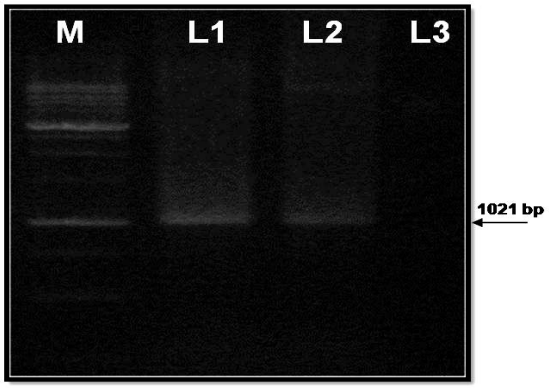

Fig. 6: Electrophoreses analysis for PCR amplification of the HSV-2/GD. M= 1kb DNA Marker. L1 and L2: DNA extracted from transformed plants, L3: DNA extracted from non-transformed plant.

To test the gD gene expression; RT-PCR was performed using RNA extracted from the transformed tomato plants. The single band at the expected size of gD that showed in fig. 7 clearly demonstrated the transcription of the messenger RNA representing the gD gene. The RNA extracted from non-transformed samples didn't show any bands after RT-PCR.

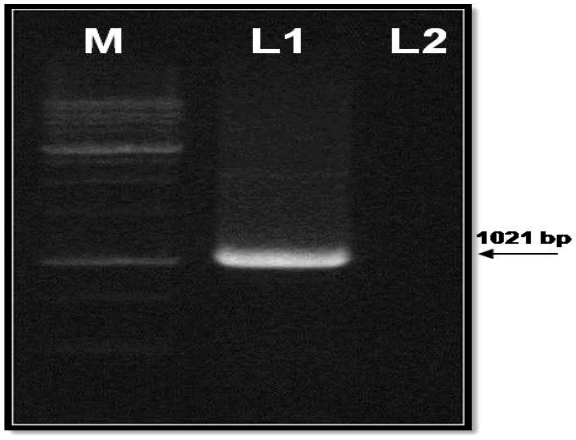

Fig. 7: Electrophoreses analysis for RT-PCR amplification of the HSV-2/gD. M: 1kb DNA Marker. L1: RNA extracted from transformed plant and L2: RNA extracted from non-transformed plant.

ELISA experiments

Tomato leaf samples were collected from the transformed plants at the regeneration stage. ELISA tests were performed on crude extracts using anti- HSV-gD antibodies. This confirmed that *tomato* plants transformed with the gD construct expressed the gD antigen. We first captured the gD protein molecules on ELISA plates coated with anti gD antibodies. The gD antigen in the plant extract was detected by means of anti-gD antibodies. This showed that only plants transformed with the gD construct were positive (Table 1), indicating that the gD is indeed expressed in the transgenic tomato leafs.

Table 1: The analysis of plant extracts from transformed plants using ELISA

Sample	Absorbance (*A*405)			
	1	2	3	4
Transformed	2.522	2.562	2.36	2.550
Non Transformed	0.169	0.653	0.255	0.338

DISCUSSION

Our work has focused on the development of HSV-2gD edible vaccine antigens throughout stable plant transformation, to express heterologous proteins in transgenic tomato plants. This technology leads to better understanding gene promoters, gene terminators and gene expression factors (Hooper, 2009). Plant is being the factory for not only subunit vaccines production but also for VLPs as well as plant bodies (Pnieweski, 2013). Because of enormous planta reactions, European Plant Science Organization (EPSO) has been initiated and established aiming at using plants not only as food and feed but also as pharmaceutical therapeutics and diagnostics of viral and non-viral infection.

In this work, we have used modern biotechnology by using agro-inoculation instead of virus-based vector with binary vector for inserting Egyptian HSV-2gD subunit sequence into tomato cotyledons. The single band at 1021 bp clearly verified the specific amplification of HSV-2 gD. The molecular cloning of the glycoprotein D gene fragment into TOPO vector was performed successfully and confirmed with the restriction enzyme digested using XbaI restriction site which was shown as a sharp bad at 1021 bp specific for the gD in addition to a single band at the expected size of the TOPO vector. The sequence analysis for the cloned gD fragment showed 100% alignment homology with HSV-2gD available in the GenBank. Data presented in Figure 4showed the linearization of the PBI 121 vector as well as the specific digestion of the TOPO-HSV-2gD construct with the XbaI restriction to create the specific cloning site for sub cloning In. Competent cells of *Agrobacterium tumifaciens* were prepared and transformed with the engineered construct. Figure 6confirmed the successful heat shock to transform the *Agrobacterium transformation* with the engineered PBI 121 construct carrying the glycoprotein D.

The tissue culture line for the transformed tomato plants was performed successfully to develop transgenic tomato plants in this study. The cotyledon of the germinated tomato seeds was used successfully for the agro inoculation step. The growth regulators used in the regeneration media positively affected the shooting of the transformed tissues. Data optained from the tissue culture experiments showed that, use of zeatin as cytokinin and IAA as auxin produced good regeneration of the inoculated plants. The differentiations of the plant cells as well as the organogenesis during the different steps of the tissue culture were controlled by the interaction between zeatin and IAA concentration. Several factors are involved in the improvement of the transformation efficiency; one of them is the addition of acetosyringone to the liquid

germination medium which facilitated the co-cultivation of explants with the *Agrobacterium* which agreed with Ahmed *et al.*, 2008. Another factor was the influence of overgrowth-control antibiotics on both regeneration and transformation efficiency (Wei and Gregory, 2001). Three different antibiotics (carbenicillin, claforan and kanamycin) were used in this study. The result of the tissue culture line showed that, these antibiotics had no negative effects on shoot regeneration from the cotyledons of tomato.

The PCR results presented in figure 6 using the DNA extracted from the inoculated plants confirmed the successful Agrobacterium transformation and the integration of the HSV-2gD gene into the plant genome. The results of RT-PCR using the RNA extracted from the regenerated plants provided that, the messenger RNA of the gD gene was transcribed in the transformed plant cells which indicate the successful expression of the HSV-2gD gene that can be used as an edible vaccine against the HSV-2 (Nemchinov *et al.*, 2000).

The Presence of gD protein in the transgenic tomato leafs was confirmed by ELISA using monoclonal antibodies against the gD protein. The results displayed in table 1 proved the successful expression of the HSV2-gD protein in the developed transgenic tomatoes using the stable transformation system that we used in this research.

Future prospects have been outlined by (Chen *et al*., 2011 and Dhama *et al.*, 2013) as transforming plants to carry vaccines is a new frontier medical technology that can prove to be very effective, if rightly implemented, in providing accessibility to developing and underdeveloped countries. Protein antigen expressed in plant tissue like seeds, tubers, and leaves makes delivery of the vaccine antigen easier and practically feasible application to large masses. These are also ideal for the vaccination of animals and birds living in the wild areas and also preventing the zoonotic diseases.

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