Expression of Hepatitis B surface Antigen (HBsAg) gene in transgenic banana (*Musa* Sp.)

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Shereen F. Elkholy*; Roba M. Ismail*; Ahmed Bahieldin*; Atef S. Sadik and Magdy A. Madkour****
*Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, 12619 Egypt. **Faculty of Agriculture, Ain-Shams University.

ABSTRACT

The Hepatitis B virus (HBV) infection is one of the most widespread viral infections of humans and causes acute and chronic hepatitis and hepatocellular carcinoma. The world wide problem of HBV infection has necessitated the development of an effective vaccine. Currently, immunization programs for large segments of the population in many areas of the developing world are very expensive. This limitation led us to attempt the expression of the recombinant Hepatitis B surface antigen (rHBsAg) in plants with the hope of developing a less expensive production system and a way to present the rHBsAg in edible plant tissues in a form that would be useful as an oral vaccine. In this study, we attempted to express the HBsAg in cv. Williams banana plants to be used as an edible vaccine. Using a Biolistic Gene Gun, apical meristem explants of banana were bombarded with plasmid pBHsAg harboring the gene encoding the HBsAg and the bar gene as a selectable marker. Bombarded explants were selected on media containing 3 mg l Bialaphos. The HBsAg gene was detected using PCR analysis and its expression was tested via western blot analysis using specific polyclonal antibodies directed against human serum derived HBsAg. This study indicates the feasibility of the expression of foreign antigens in plants for possible use as an oral vaccine.

Key words: *Hepatitis B, Hepatitis B surface antigen, Transgenic banana, Molecular biofarming.*

INTRODUCTION

Exploiting plants as bioreactors for production and delivery of edible subunit vaccines is a promising application of biotechnology. In recent years, the tools of genetic engineering had allowed the development of transgenic plants that can express various recombinant biopharmaceutical compounds including viral and bacterial antigens, antibodies, and various therapeutic human and animal proteins. Vaccines against some serious diseases like Hepatitis B, cholera, and diarrhea have been produced in banana plants (Butetow and Korban, 2000; Giddings *et al.*, 2000; Daniell *et al.*, 2001). Selected proteins of disease agents are expressed in plant tissues and delivered as vaccines. The plant material containing the protein can be used directly as an edible vaccine, a Alternatively, the protein can be purified and delivered orally or by injection.

Several plant species have been used to express foreign proteins including vaccine candidates. The choice of species depends on experimental considerations, such as whether the plant can be easily transformed and the economics of large-scale production using the crop. Early work focused on tobacco and

potato since they are relatively easy to transform. However, from commercial standpoints, seed crops including maize are advantageous due to having high protein content and have been reported to express very high levels of foreign proteins (Stephan *et al.*, 2001). Furthermore, transgenic corn seeds can be preserved for long periods and can often be consumed raw, either whole or ground into flour, thus suggesting that they might be a good source of some edible vaccines.

The first stage in the development of plant-derived vaccine is selecting which antigen to express. Capsid proteins of many types of viruses can assemble into virus like particles (VLPs). Devoid of the viral genetic material, VLPs often resemble the native virions in their morphology, antigenic properties and stability. These particulate antigens generally elicit stronger mucosal immune responses than soluble antigens, which can often repress the immune response by inducing immunotolerance (Garside and Mowat., 1997). VLPs are therefore predicted to make excellent mucosal vaccines (Estes *et al.*, 1997). Hepatitis B virus is one of the most widespread viral infections of humans and causes acute and chronic hepatitis and hepatocellular carcinoma (Purcell, 1994). The infectious viral particle (Dane particle) of HBV is a 43 nm double-shelled sphere that consists of a core containing 3-2 kilobase DNA genome bound to the core protein, surrounded by the viral envelope containing phospholipids and the major hepatitis B surface antigen (HBsAg) (Fig. 1). Due to the limitation of the host range of hepatitis B virus to humans and chimpanzees, and since the virus cannot be propagated in cell culture, (HBsAg) was purified from the serum of infected individuals for use in vaccines until a recombinant form (rHBsAg) was produced in yeast and is commercially available for parenteral vaccination (Valenzuela P. *et al.*, 1982). HBsAg was also the first reported viral antigen to be produced in transgenic tobacco, banana and potato plants (Mason *et al.*, 1992). The protein assembles in plant tissues into subviral particles of 22 nm in diameter (VLPs), which are similar to those found in the sera of infected humans and in the commercial vaccine (Mason *et al.*, 1992). Intramuscular injection of serum-derived or yeast-derived (rHBsAg) resulted in effective immunization and protection from viral infection.

Delivering the vaccine in intact plant material, rather than in plant extracts, may enhance antigen immunogenicity, as bioencapsulation of the antigen within the tough plant cell wall and membrane compartments can provide increased protection from intestinal degradation (Qingxian *et al.*, 2001). High-level protein expression in seeds such as rice and maize may also concentrate the antigen and further reduce dosing requirements (Hood *et al.*, 1997).

The particle bombardment process is a method for the delivery of genes into intact cells and tissues through the use of DNA-coated microprojectiles (tungsten or gold). Genetic transformation of plants by direct DNA delivery *via* microprojectile bombardment has become an established procedure in recent years, and has been used to produce transgenic plants conferring resistance to biotic or abiotic stresses (Koziel *et al.*, 1993; Wan *et al.*, 1995; Songstad *et al.*, 1996; Zhang *et al.*, 1996; Brettschneider *et al.*, 1997; Bohorova *et al.*, 1999; Frame *et al.*, 2000; El-Itriby *et* ***al.,*** 2003).

In this study we aimed to engineer plants so that they can be used as inexpensive alternatives to fermentation system for production of subunit antigens. The gene encoding the HBsAg was successfully expressed in tobacco, banana and potato. The plant-derived HBsAg, delivered as food, is

orally immunogenic and elicit a primary antibody response (Smith *et al.*, 2001 and Kong *et al.*, 2003). We chose Banana (*Musa sp.*) to express the HBsAg as it is considered one of the most important fruit crops worldwide as well as in Egypt and it is eaten raw without cooking, thus avoid heat denaturation of the antigen.

MATERIALS AND METHODS

Construction of expression cassette encoding the HBsAg

The chimeric HBsAg gene (generously provided by Prof. Dr. J. Reimann, University of Ulm, Germany) was cloned into the *Bam* HI site of plant expression vector (pAB11) under the control of the cauliflower mosaic virus (*CaMV*) *35S* double constitutive promoter and terminated by the NOS terminator. The HBsAg gene includes *Bam* HI site, therefore, *Bgl*II site was added to the forward and reverse primers as compatible with *Bam* HI enzyme (forward primer with *Bgl*II site: GGAAGATCTATGGAGAACATCACATCA GGA, reverse primer with *Bgl* II site and an artificial stop codon: GGAAGATCTTTAAATGTATACCCAAAG ACAAAAG). The herbicide resistant (*Bar*) gene was cloned as a selectable marker gene into the *Hind* III site. The construct also included the alfalfa mosaic virus (*AIMV*) RNA4 translational enhancer leader sequence to increase translational efficiency. The pAB11 plasmid was constructed from pBI221 by excision of *GUS* gene using SacI and SmaI enzymes, and then the large fragment was filled up and religated. The final construct was designated as pBHsAg (Fig. 2a).

E.coli competent cells were transformed by pBHsAg as described by Ausbel *et al* (1987). The recombinant plasmid containing the gene of interest was verified by PCR using specific forward and reverse primers as shown in Fig. (2b).

Particle bombardment and selection

The gene gun (Bio-Rad Biolistic PDS-1000/He) was used for the transformation of banana apical meristems with the plasmid DNA pBHsAg harboring the gene encoding the HBsAg and the bar gene for herbicide resistance as a selectable marker (Fig.2a). Apical meristems explants of banana were bombarded once at 650 psi with sterilized gold particles coated with 5-µg plasmid DNA. Bombarded and non bombarded apical meristem explants were cultured for 3 days under shaking conditions at 28 °C on biolaphos-free liquid MS medium containing 3 mg/l PAB and 30 mg/l acetostrengen to avoid the phenolic compounds, then, after one week, transferred on MS solidified medium containing 3 mg/ml Bialaphos for one month in a controlled growth room. As shown in Fig (4). The biolaphos resistant explants as well as those grown under no selection pressure were transferred to the regeneration medium containing 3 mg/l bialaphos and left to regenerate for 3-4 weeks in a controlled growth chamber at 28±2 °C and a photoperiod of 8/16 hr (dark light). The plantlets were acclimatized in a controlled greenhouse into pots containing equal volumes of peat moss and sand (1:1, v/v).

Banana regeneration

After four rounds of selection, bialaphos resistant calli were regenerated according to El-Itriby *et al.* (2003), by transferring them to the regeneration medium RM1, followed by RM2 and incubated under fluorescent light. (RM1 and RM2 containing 3 mg/l bialaphos). The regenerated shoots were rooted on RM3 medium containing 3 mg/l bialaphos (Fig.3-B and C). Putatively transgenic plantlets were acclimatized in the biocontainment green house for 3-4 weeks, at 28 °C with a

photoperiod of 8/16-hr (dark/Light) in an aquarium containing a modified-Hoagland solution (Johnson *et al.*, 1957). Healthy rooted plantlets were transferred to pots containing a mixture of peatmoss : soil (1:1), (Fig. 3-D).

Herbicide testing

When the plants reached the four to six leaves stage, they were tested for resistance to the herbicide BASTA® by marking a 3 cm area of a young leaf and painting this area with 1% solution of the herbicide. Plants were scored for resistance one week after painting.

Evaluation of the putative transgenic plants

Detection of the insertion of the transgenes into the banana genome

Total genomic DNA from leaves of putative transgenic and untransformed plants (control) was isolated using DNeasy kit (Qiagen Inc.). PCR was conducted to detect the presence of the gene encoding the HBsAg in DNA of putative transgenic and control plants using two specific, forward and reverse primers. After denaturation for 5 minutes at 94°C, PCR reactions were carried through 35 cycles using the following temperatures sequence: 94°C for 1 min, 55 °C for 1 min and 72°C for 2 min. In addition, PCR was also conducted to confirm the presence of the bar gene in the putative transgenic plants using the specific forward and reverse primers for the *bar* gene. Products were size-separated on a 1% agarose gel containing ethidium bromide and viewed under UV light.

Protein extraction and Western immunoblot analysis

Transformed and untransformed banana leaves (300 mg) were ground in liquid nitrogen and resuspended in 500 µl of extraction buffer containing 200 mM Tris-HCl (pH 8.0); 100 mM NaCl; 10 mM EDTA; 2 mM Phenylmethyl sulfonyflouride (PMSF) and 5% of ß-mercaptoethanol was added to the buffer immediately before use. The tubes were shaken at 4°C for 20 min and then centrifuged at 13000 rpm for 15 min at 4°C. The leaf extracts were boiled in sample buffer (100 mM Tris-HCl (pH 6.8), 10% SDS, 20% glycerol, 1% bromophenol blue) to denature the proteins and electrophoresed in 12% (w/v) polyacrylamide gel with Tris-glycine electrophoresis buffer (25mM Tris-base, 250 mM Glycine and 1% SDS) for 3-4 hrs at 100 V. The separated proteins were transferred to a nitrocellulose membrane by electroblotting at 85 V for 1 hr. The membrane was blocked in 25 ml of 5% (w/v) non-fat dry milk in TBS buffer (20 mM Tris-base, 500 mM NaCl, pH 7.5) for two hours on a rotary shaker followed by washing in TBS buffer. The blocked membrane was incubated for 1 hour in 1:1000 dilution of rabbit anti-HBsAg antiserum in TBST (TBS buffer, 1% Tween 20) containing 1% non fat dry milk, then washed 3 times in TBST buffer followed by incubation in a 1:10,000 dilution of alkaline phosphatase conjugated mouse anti-rabbit IgG (Sigma) in TBST. The membrane was washed twice in TBST, followed by incubation in 100 µl alkaline phosphatase (AP) colour development reagent (BCIP/NBT) (Bio Rad) in 10 ml AP detection buffer (0.1 Tris-HCl, 0.1 M NaCl , 50 mM MgCl, pH 9.5) for one hour.

RESULTS AND DISCUSSION

Verification of the recombinant plasmids harboring the HBsAg gene

The first step in the production of plant-derived vaccines involves the identification of antigenic polypeptides and cloning their genes into a plant expression vector. The vector harboring the gene of interest (HBsAg gene) was fused to plant expression cassette including transcriptional and post-transcriptional regulation sequences (35 S promoter and NOS terminator). The construct

includes herbicide resistance (*bar* gene), a selectable marker gene, for screening and selection of the transgenic events. The construct might have to be optimized by using plant optimized codon sequence as described by Stephan *et al.*, (2001). They have utilized maize optimized codon of barley α amylase, a cell secretion signal, to optimize antigenic protein accumulation in transgenic plants. Optimization can also depend on targeting the protein to various possible sub-cellular compartments, for example, the lumen of the endoplasmic reticulum (Haq *et al.*, 1995). In the present study, *Escherichia coli* competent cells were transformed with the recombinant plant expression vector (pBHsAg) encoding the HBsAg and the different bacterial clones were screened by PCR analysis using specific forward and reverse primers in order to efficiently select the recombinant plasmids. The amplification of the 681 bp PCR product in some of the screened bacterial clones verifies the presence of recombinant plasmid (pBHsAg) as shown in Fig (2b).

Transformation and regeneration

In 1992, Arntzen and co-workers introduced the concept of transgenic plants as a production and delivery system for subunit vaccines (Mason *et al.*, 1992). They reported that production would be as cheap as agriculture, that distribution would be as convenient as marketing and that administration would be as simple and safe as eating. Plants are one of the cheapest sources of proteins and, therefore, potentially also the cheapest source for recombinant proteins. This is particularly true for large-scale production. Most of the experiments performed so far have employed tobacco and potato for antigen production. These two solanaceous plants are convenient experimental models because they can be easily transformed. However, tobacco cannot be a source of unpurified edible vaccines, and potato tubers, although readily eaten by mice and tolerated by volunteers, are not palatable when uncooked. Plants and plant parts (e.g. fruits) that can be consumed raw are preferable for human immunization, because cooking might inactivate the antigen (Arakawa *et al.*, 1998). Production of a foreign antigen has already been demonstrated in tomato fruits (McGarvey *et al.*, 1995). However, bananas might be the ideal source of edible vaccines because they are consumed raw even by infants and are a major crop in many developing countries. Thereby, in this study, we expressed HBsAg in *cv.* Williams banana plants to be used as an edible vaccine. The one disadvantage of both tomatoes and bananas is the low protein content of the fruit, which might limit the amount of antigen that can be expressed (Mason *et al.*, 1992).

Apical meristems are excellent targets for banana transformation and have been utilized previously in several studies (Persley and De Langhe., 1987; Novak, 1992 and Arntzen and Lam, 1992). In the present study, the plasmid pBHsAg (harboring the gene encoding HBsAg and the *bar* gene) was introduced into banana tissues *via* microprojectile bombardment system. Banana apical meristems were bombarded once at 1100 psi, and yielded eight transgenic plants (T_0) harboring the gene encoding the HBsAg as well as the *bar* gene. Herbicide resistance of putative transgenic banana plants was tested at biocontainment stage by painting the middle green parts of the banana leaf plants from both sides with 1 g/l BASTA®. Results in Fig (4) indicate that leaves of the herbicide-resistant banana plants showed a green healthy color while the others (either non-resistant herbicide or control non-transformed) turned yellow within two days. One week post leaf painting with BASTA®, 8 of 40 plants were still surviving. PCR was conducted to confirm the presence of the *bar* gene in leaves of putative

transgenic plants using two primers specific to the *bar* gene, PCR products with a size of about 561 bp were amplified as shown in Fig (5).

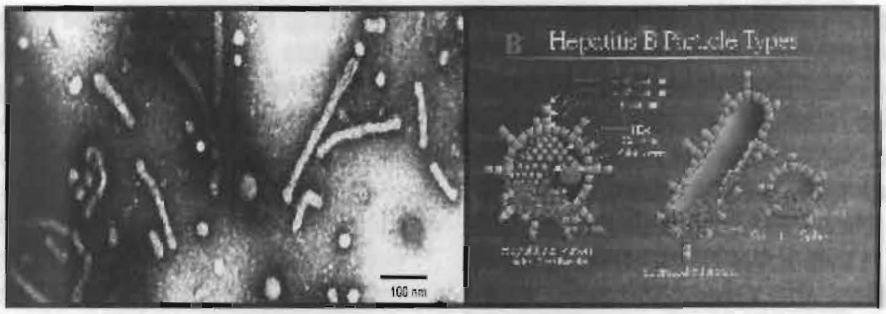

Fig. (1): (A) Electron micrograph of hepatitis B virus particles purified from virus-infected plasma showing spherical and filamentous form of the complete virion (Dane paritcles) with 42 nm. (B) The protein composition of the viral envelope composing the hepatitis B surface antigen (Mason et al., 1992).

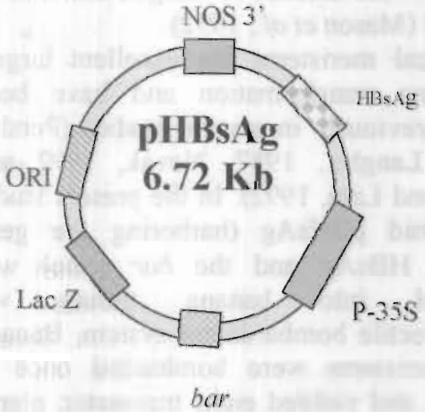

Fig. (2a): The expression cassette harboring the gene encoding the HBsAg and the bar gene as a selectable marker

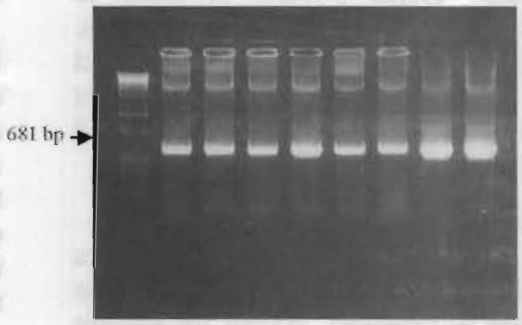

Fig. (2b): PCR detection of the recombinant plasmids using 2 oligonucleotides as specific primers to HBsAg gene. DNA isolated from different bacterial clones were used as templates for PCR amplifications at 55°C, annealing temperature. A fragment of 681 bp was obtained.

Detection of the presence of HBsAg gene in putative transgenic plants

PCR analysis was conducted to test the presence of the gene encoding the HBsAg in the putatively transgenic maize plants. The amplification of the 681 bp PCR product in the putative transgenics screened, confirmed the integration of the gene cassette into the plant genome (Fig 6). Moreover, PCR analysis carried out on the genomic DNA of putatively transgenic plantlets revealed the presence of

the 561 bp PCR fragment of the marker *bar* gene verifying transgenic events (Fig.5).

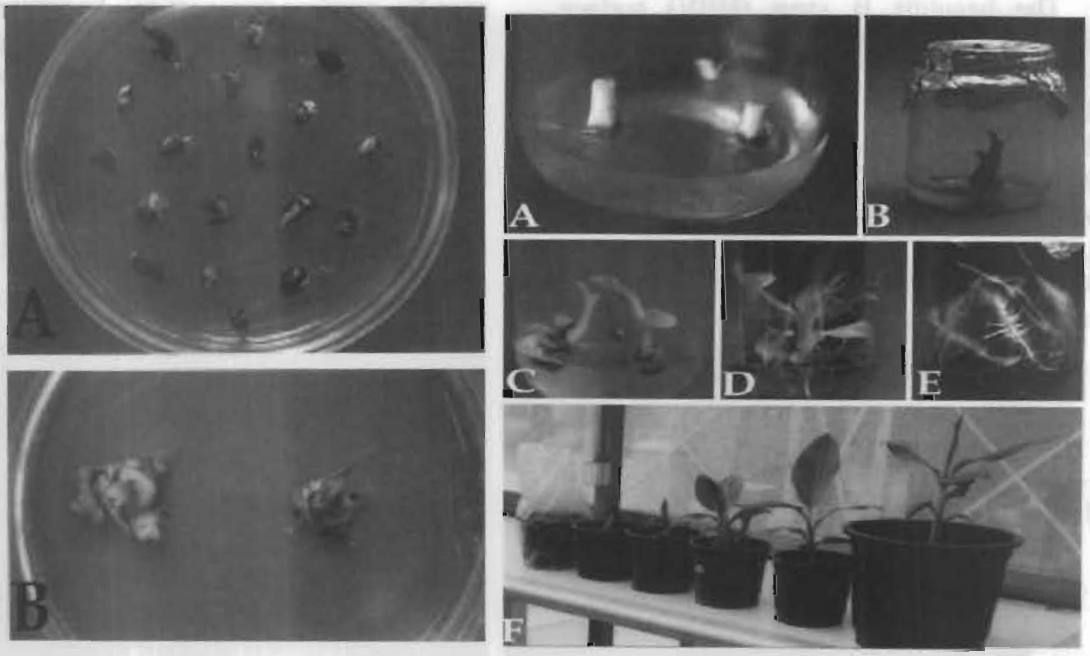

Fig. (3): A and B: Selection stages of transformed banana tissues. A: Six days old banana apical meristems prepared for bombardment. C, D and E: Banana plantlets regenerated on regeneration medium. F: Putatively transgenic banana plants acclimatized in the bio-containment green house.

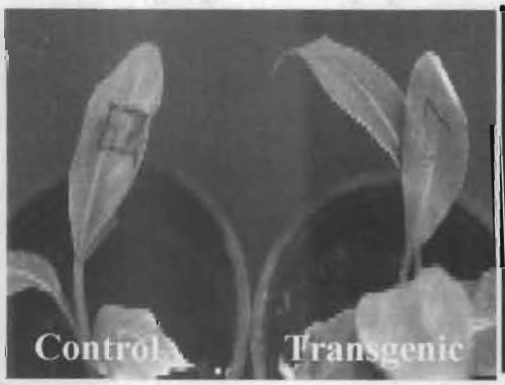

Fig. (4): Leaf painting of transformed banana plants.

Fig. (5): PCR detection for bar gene.

Detection of the expression of HBsAg

The hepatitis B virus (HBV) surface antigen (HBsAg) forms porous 20-30 nm lipoproteasomes composed of two hydrophobic domains separated by a hydrophilic one (Heermann and Gerlich., 1991). Extensive experimental and clinical experience is available for HBsAg, the antigen in the commercial vaccine against HBV. Protection against HBV induced by this vaccine is correlated with high levels of serum antibodies specific for HBsAg. The group-specific, immunodominant 'a' determinant is a conformational epitope containing a glycosylation site at (Asn-146), presented by the secreted non-glycosylated p24 and mono-glycosylated gp27 small S protein in almost all HBV isolates known (Prange and Streeck., 1995). It spans residues from 120 to 147 of the envelope protein and forms a loop between two transmembrane domains (Fig. 7).

Fig. (6): PCR detection of the presence of HBsAg gene in putative transgenic banana plants. PCR products with size of 681 bp were amplified in transgenic plant samples.

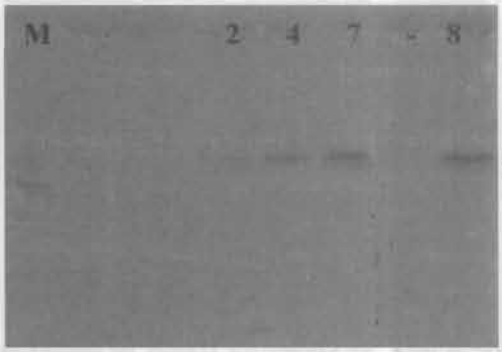

Fig. (7): Detection of the expression of HBsAg gene via western blot immunoassay using polyclonal specific rabbit antiserum.

The expression of the HBsAg gene was tested *via* western blot immuno-assay. The protein was extracted by homogenization from the putatively transformed samples (events) that showed positive results with PCR analysis. The expression of the gene was tested using a polyoclonal HBsAg specific rabbit antiserum as shown in (Fig. 7). The HBsAg gene (226 residues) was efficiently expressed as a non-glycosylated p24 and glycosylated gp27 S protein that mimic the native HBsAg. The expression level varies from one plant to the other. Sample number 8 showed higher level of expression and clear glycosylation (gp27 and p24) than the other samples (events) as illustrated in both Fig. (6 and 7). The variability in levels of expression that sometimes observed between different plants of the same line as shown in Fig. (8), and even within the same plant might be avoided by minimal processing of the plant material to create uniform batches as production of freeze-dried tomato batches. However, despite the progress achieved in the production of antigenic proteins in plants, the biggest obstacle remains which is the low level of accumulation of these foreign antigens in plant tissues. This problem becomes even more acute for some of the less immunogenic antigens. It would be useful if the expression

level could be predicted, directed and manipulated to allow maximal accumulation of the foreign protein without compromising the fitness of the transgenic plant. This could be achieved, for example, if the expression was inducible rather than constitutive or if it was tissue specific as using seed specific promotors to target the expression of the foreign antigens in the seeds as they have high protein content and have been reported to express very high level of antigenic proteins (Hood *et al.*, 1997).

Generally, the use of transgenic plants and in particular banana, for vaccine production has several potential benefits over traditional methods. Firstly, transgenic plants are usually engineered to express only a small

eliminating the possibility of infection or innate toxicity and reducing the potential for adverse reaction. Secondly, since there are no known human or animal pathogens that are able to infect plants, concerns with contamination are eliminated. Thirdly, the successful synthesis of foreign proteins in transgenic crops rely on the same established technologies to sow, harvest, store, transport and process plant material as those currently used for food crops, making transgenic plants biological bioreactors and very economical means of large-scale vaccine production. Fourthly, the expression of the HBsAg in banana is very convenient as it is considered one of the most important fruit crops worldwide as well as in Egypt and it is eaten raw without cooking, this avoid heat denaturation of the antigen, thereby, reducing the need for refrigeration and keeping transportation and storage costs low (Hood *et al.*, 1997).

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الملخص العربي

التعبير عن الجين المسئول عن إنتاج الانتجين السطحي لفيروس التهاب الكبد الوبائي (ب) HBsAg في نبات الموز المحور وراثيا

شيرين الخولي ، ربي اسماعيل ، احمد بهى الدين ، عاطف صادق ، مجدى مدكور
معهد بحوث الهندسة الوراثية الزراعية – مركز البحوث الزراعية

يعد التهاب الكبد الوبائى (ب) من اكثر الامراض الفيروسية انتشارا ويسبب تليف وسرطان الكبد المزمن لذا كان يجب إنتاج لقاح فعال ضد هذا المرض الخطير. إن برامج التطعيمات واللقاحات فى كثير من دول العالم الثالث حاليا باهظة التكاليف لذا كان يجب علينا محاولة استخدام النباتات المحورة وراثيا فى انتاج امصال ولقاحات للوقاية من العديد من الامراض وذلك لتقليل التكاليف باستخدام الوسائل الزراعية بدلا من استخدام الصناعات الصيدلية باهظة التكاليف والغير متوفرة فى معظم دول العالم النامى. فى هذه الدراسة قد تم التعبير عن الانتجين السطحى لفيروس التهاب الكبد الوبائى (ب) فى نبات الموز (CV Williams). بواسطة جهاز الدفع المباشر لنقل الجينات (Biolistic gene gun) قد تم قذف القمم النامية لنبات الموز بالناقل المسمى pHBSAg الذى يحتوى على الجين المشفر للانتجين السطحى للفيروس وكذلك جين المقاومة لمبيدات الحشائش الذى يستخدم لانتخاب النباتات المحورة وراثيا. النباتات التى قد تم قذفها قد تم انباتها فى وسط بيئى يحتوى على 3 mg/ml Bialaphos للتمييز بين النباتات المحورة وغير المحورة وراثيا. قد تم اختبار وجود الجين المسئول عن الانتجين السطحى للفيروس فى النباتات المنتخبة بواسطة تفاعل البلمرة المتسلسلة والتعبير عنها بواسطة اختبار التهجين الغربى Western Blot analysis باستخدام اجسام مضادة للانتجين السطحى. هذه الدراسة توضح امكانية التعبير عن كثير من الانتجينات فى النباتات لاستخدامها كلقاح مأكول ضد العديد من الامراض.