Expression of the Human Hepatitis B Virus Large Surface Antigen Gene in Transgenic Tomato Plants

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Hepatitis B virus (HBV) is a major cause of acute and chronic hepatitis. HBV DNA was found in most of the newborns from hepatitis B surface antigen (HBsAg)-positive mothers (27). The current HBV vaccine is a biotechnology product that falls in the category of “subunit vaccines” and is made from yeast cells grown by fermentation. The vaccines have prevented numerous infections. However, administration of the vaccine intramuscularly causes some pain, and it is not widely accepted, especially for children. In addition, current vaccines have the safety limitations of syringe-and-needle vaccination programs. In recent years, a novel production system of vaccines—‘edible vaccines’ or ‘oral vaccines’—has been developed. Compared to traditional vaccines, oral vaccines can serve multiple immunization priorities and offer advantages, including simplicity of use, lesser expense, greater convenience for storage, an increase in compliance (as a result of increased comfort of delivery), enhanced immune responses at mucosal sites, and stimulation of humoral immunity.

So far, much has been achieved in production of oral vaccines and proteins by using transgenic plants (1, 4, 17). The results of tests involving humans who ingested transgenic potato and lettuce expressing recombinant HBsAg (rHBsAg) have been reported, and the special antibody was detected in the serum of volunteers who were given edible tissues of transgenic plants (14). As we know, potatoes and lettuce cannot be eaten raw in certain populations. However, the cooking process would destroy the target protein and affect its immunogenicity. Transgenic edible raw crops can be produced at low cost. The agricultural infrastructure of any given country can accommodate a program to grow the vaccine-producing plant, and food-processing technology would be used for product manufacture. In our study, we chose the tomato as the plant system for producing the recombinant antigen.

HBV is a mostly double-stranded DNA virus in the Hepadnaviridae family. The HBV genome includes four genes: pol, env, precore, and X, respectively, which encode the viral DNA polymerase, envelope protein, precore protein (which is processed to viral capsid), and protein X. The most recent vaccine is based on the cloned copies of the env gene in yeast. Studies of mRNA transcription suggest that this gene has the potential to code for three related proteins: (i) a protein of 226 amino acids, identified as a major protein constituent of the HBV envelope, termed the S protein; (ii) a protein with 55 additional amino acids at the N terminus, encoded by a portion of the env gene upstream of the S gene (pre-S1); (iii) a protein corresponding to the entire env gene (pre-S2). Three different proteins are produced by combining these proteins in different formations: large protein on the envelope (combination of the pre-S1, pre-S2, and S genes); middle-sized proteins on the envelope (pre-S2 and S genes); and the major component of HBsAg, the S protein. The extension polypeptides have been claimed to enhance the immunogenicity of the S antigen in eliciting anti-S antibody (9, 19, 20, 21, 22), suggesting that they may play an important role in the process of virus infection and the induction of a defensive host response.

Although many previous studies have shown that plant cells...
TABLE 1. Proteins with application for human HBV vaccine and expressed by transgenic plants

| Year of expression | Protein or peptide expressed | Plant expression system | Maximum recorded protein level in plants<sup>a</sup> | Reference |
|--------------------|-----------------------------|------------------------|-----------------------------------------------|-----------|
| 1992               | HBsAg                       | Tobacco                | <0.01% TSP                                    | 17        |
| 1997               | M and S protein             | Potato                 | Not given                                     | 7         |
| 1999               | HBsAg                       | Lupine (Lupinus spp.)  | <0.15 μg/g FW                                 | 14        |
| 2000               | HBsAg                       | Potato                 | <0.01 μg/g FW                                 | 23        |
|                    | HBsAg                       | Tomato                 | 8 μg/g FW Fruit                              | 29        |
| 2002               | HBsAg                       | Kelp                   | 6.67 μg/g FW                                 | 13        |
|                    | L protein                   | Tomato                 | Not detected                                  | 29        |
| 2005               | HBsAg                       | Peanut                 | 0.032 TSP                                    | 3         |
|                    | HBsAg                       | Banana                 | 38 ng/g FW                                   | 16        |

<sup>a</sup> TSP, total soluble protein; FW, Fresh weight.

**MATERIALS AND METHODS**

**Chemical synthesis of HBV large surface antigen gene** PRS-S1S2S. The original HBV large surface antigen gene was synthesized based on the sequence of Genbank accession no. S20745. Oligonucleotides were synthesized using the oligo 1000 M DNA synthesizer (Beckman), and each oligonucleotide fragment was about 90 bp long. A total of 18 primers (Table 2) were designed according to the gene’s synthetic strategy (Fig. 1), and about 20 nucleotide residues were overlapped between two neighboring primers (Table 2). Sequences encoding the tobacco pathogenesis-related protein S (PR-S) signal peptide (5) were fused to the 5’ end of the modified HBV large surface antigen gene. The sequence encoding amino acids S, E, K, D, E, and L was placed at the 3’ end of the HBV large surface antigen gene. The SEKDEL sequence was found to be sufficient for driving fruit-specific protein expression. The aim of our work was to investigate the feasibility of producing a new HBV oral vaccine with the HBV large surface antigen gene.

**Expression of HBV large surface antigen gene in tomato**. The entire nucleotide sequence of the synthetic HBV large surface antigen gene PRS-S1S2S (Genbank accession no. S20745) is 1,245 bp in length. To improve protein secretion, a 72-bp sequence encoding the tobacco PR-S signal peptide was added to the 5’ coding regions of the modified HBsAg genes, and an 18-bp sequence encoding the ER retention signal SEKDEL was added to the C-terminal regions of modified HBsAg. For modified HBsAg, all codons were designed to be preferential to tomato plants. The G+C and A+T contents in the synthesized gene were balanced, and the predicted hairpin structures and motifs containing six consecutive A/Ts were eliminated (Fig. 3).

**Plasmid transformation**. The pYPF9616 plasmids were transformed into Agrobacterium strain LBA4404 cells by using the electroporation method (6). Tomato plants were transformed by a modified leaf disk cocultivation method using Agrobacterium tumefaciens harboring pYPF9616 (11). Shoots were generated from transformed calli selected on medium containing 0.05 mg of kanamycin per ml and 0.25 mg of carbenicillin per ml. Shoots were rooted in medium containing 1 × 10⁻⁴ mg of 3-indole butyric acid per ml and 0.125 mg of carbenicillin per ml, transplanted to soil, and grown to maturity.

**Screening of transgenic plants for PRS-S1S2S gene**. The transformed tomato plants were screened by PCR for the partial PRS-S1S2S fragment (300 bp) using the following primer pairs: forward, 5’-CTCTGTACATGAGTCGTGTTG-3’; reverse, 5’-ACGGAAGCCATTCCTCCAAAGAAC-3’. The accuracy of the PCR was performed at 94°C for 5 min for the first cycle, followed by 30 cycles of 94°C for 30 s, 62°C for 40 s, 72°C for 1 min, and 15 s.

**Analysis of total RNA extracted from transgenic plants**. Total RNA from leaves of transformed plants with pYPF9616 was isolated as described previously (18). The PRS-S1S2S mRNA was detected by reverse transcriptase PCR (RT-PCR) amplification using the Access RT-PCR system (Promega) (partial PRS-S1S2S amplification product, 300 bp; forward, 5’-CTCTGTACATGAGTCGTGTTG-3’; reverse, 5’-ACGGAAGCCATTCCTCCAAAGAAC-3’). The amplified RNA was ligated into the vector pYF9616 between BamHI and PstI to form the HBV large surface antigen expression vector pYPF9616 (Fig. 2).

**Analysis of protein from transformed tomato**. An HBsAg enzyme-linked immunosorbent assay (ELISA) kit was purchased from Huawei Biotechnology Co., Ltd. HBsAg standards were kindly provided by Y. Lu, Fudan-Yueda Biotechnology Company. Transgenic tomato plants were grown with liquid nitrogen and then incubated in phosphate-buffered saline (pH 7.0). Total soluble proteins containing expressed HBsAg were detected by ELISA and quantified with the standard curve established by series dilution of HBsAg standards.

**Transmission electron microscopy** (TEM). TEM was viewed on only the mature tomato fruit extract but also the mature tomato fruit sections with immunogold labeling. On the one hand, the tomato fruit extract was bound with 1% bovine serum albumin onto copper grids. The trapped HBsAg particles were probed with monoclonal antibody to BALB/c mouse HBsAg (1:10) and secondary gold-labeled (5 nm) anti-mouse antibody (1:20). The grids were stained with phosphotungstic acid and viewed using a Philips Tecnai 12 transmission electron microscope. On the other hand, mature tomato fruit were excised, cut into 1-mm squares with a razor blade, fixed in 2.5% glutaraldehyde for 4 h at 4°C, washed, and dehydrated in a graduated ethanol series at 4°C. 100% acetone. The tissue was infiltrated with epon resin at 37°C and cured at 37°C for 12 h and then at 45°C for 48 h. Sections of about 60 nm in thickness were obtained by using a Reichert-Jung microtome. The sections were viewed by immunogold labeling, stained with uranyl acetate and lead, and viewed on a Philips Tecnai12 electron microscope.

**RESULTS**

**Chemical synthesis of HBV large surface antigen gene** PRS-S1S2S. The entire nucleotide sequence of the synthetic HBV large surface antigen gene PRS-S1S2S (Genbank accession no. S20745) is 1,245 bp in length. To improve protein secretion, a 72-bp sequence encoding the tobacco PR-S signal peptide was added to the 5’ coding regions of the modified HBsAg genes, and an 18-bp sequence encoding the ER retention signal SEKDEL was added to the C-terminal regions of modified HBsAg. For modified HBsAg, all codons were designed to be preferential to tomato plants. The G+C and A+T contents in the synthesized gene were balanced, and the predicted hairpin structures and motifs containing six consecutive A/Ts were eliminated (Fig. 3).
Transformation and regeneration of tomato. The plasmid for expression of HBV large surface antigen in plants (pYF9616) (Fig. 2) was introduced into Agrobacterium tumefaciens and used in transformation experiments. Kanamycin incorporated into tissue culture medium allowed selection of transformed callus tissue, from which shoots and thereafter mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months. Thirteen independent kanamycin-resistant lines of mature tomato plants were regenerated over a period of about 4 months.

RNA analysis. RT-PCR analysis was carried out to show the presence of an amplified product of the expected size (300 bp) in genomic DNA of tested PC- and glucuronidase-positive plants (data not shown) which was absent in nontransformed plants. According to the protocol of a reverse transcription system kit (Promega), the sls2s gene was proved to be transcribed in all transgenic plants but not in nontransgenic plants (Fig. 4).

Immunodetection of HBV large surface antigen. The ELISA method was used to detect and quantitatively analyze the target protein. rHBsAg levels in leaves and fruits from a representative tomato plant line of several tactic lines are presented in Table 3. The results indicated that the expression levels of rHBsAg varied in leaves and fruits in the same plant. Apparently, rHBsAg showed high levels in mature fruit, while leaves and immature fruit had low levels. The amount of HBsAg in mature fruit was found to be 65- to 171-fold higher than in small or medium fruits and leaf tissues. rHBsAg protein levels in immature fruits and leaves were in a similar range, and variation was observed among the mature fruits in different plants. The expression level of rHBsAg also appeared much lower in other organs, including roots, flowers, and stems, than in mature fruit (Table 4).

**TABLE 2. Oligonucleotides used to synthesize the PRS-S1S2S gene**

| Oligonucleotide       | Nucleotide sequence               |
|-----------------------|----------------------------------|
| HBSAG0                | 5'-TTG TTG GTC TTG CAG GCT GGT TTC TTG TTG ACC AGA ATC TTG ACC ATC CCT CAG TTC TTG GAG TCC TGG TGG ACC TTC TTG AAC TCC-3' |
| HBSAG1                | 5'-ATC TTC AGA ATC GGT GAT CCT GTG TTG AAC ATG GAA AAC ATC ACC TCC GGT TTC TTG CCT TTG GTG TTC ATC GAG GTG GCC CTG GAG-3' |
| HBSAG2                | 5'-GAG GTA GGA GAG TGG TTG GAG GTA GGG GAT TGG GAG GAT TGA CCA CCC AAG CAG ACG TGA GTA CCT CCC AAG AAC ATG GAA TAC TCT CTG GAG-3' |
| HBSAG3                | 5'-CCC TGC TTG GAG TTC CTC CTC CGG TGT CGT CAA CCC TGT GCT CAG CAG CCG TCC TTC GTC CTC TAT CCT CTC CAG AAT CGG, TGA, TCC-3' |
| HBSAG4                | 5'-AAG, ATG, AAC, AGG, AAA, ATG, ATG, AAT CTT CTC AAG CAC ATC CAT CTG TAA CCA GGG CAG GAT GGA GGG CAA GAG GTA GGA GAG TGG TGG GAG-3' |
| HBSAG5                | 5'-AGC TAT GGA ATG GAA CTG CAC CCT CCA GAC CTT GCA AGA CCC TAG AGT CAG AGG TTG GCA CTT CCC TGC TGG TGG TTC CTC CTC-3' |
| HBSAG6                | 5'-AAG GCC AGA CAG CCA ACA TAC CCT GGT AGT CCA ACA AGA CCA ACA AGA TCA AGC ACA AGA TGA AGA AAA TGA TCC-3' |
| HBSAG7                | 5'-TCC ACC AAC AGA CAG TCC GGT AGA CAA CCT ACC CCT TTG CCT TTG ACC ATC ACC AAC CAC CCT CAA GCT ATG CAA TGG AAC ACC ACC-3' |
| HBSAG8                | 5'-AGT ACC TTG AGC AGT GGT CAT GAG CTG TCT GCA AGG ACC AGT AGA GTG GGA ACC ACC AGG GAT CAA GCA GAC AGG CAA CAT ACC-3' |
| HBSAG9                | 5'-TTT GCT GGG TTG GTC TTC TCA TCA GGC TGG TAT CTT GTA GAC GCC CCT TGC TAA ACC TTC TCC TTG TTC TGC TTC CAA CAG ACA TAG CCG-3' |
| HBSAG10               | 5'-GAA GGG ATG TGA ATG CAG TGG CAG TTA CCG TGG GAA GGC TTG GGT CAA CAG CAG GAA GGA TAC ATA GGAG ATG CAG GTG CAG-3' |
| HBSAG11               | 5'-ACC TGG CCT GAC GCT AAC AAG GTC GGT GCT GGT GCT TTC TTG TTG TTT ACC CCT CAC GGT GTG TTG CCT GTC CAG-3' |
| HBSAG12               | 5'-ACC AGC AAC GAC AAC CAG GAG CAG GAT CTA GCG GAA GCC CAT CAC AAC AAG TTA CGG AAA GCC CAG GAG GAA GGT AGG ATG GTG-3' |
| HBSAG13               | 5'-CAC CAG TTG GAC CCT GCT TTA GCT AAC ACC GCT AAC CCT TGC GAC GTC ACC TAC CTC CGG TAA ACC TGG GAC CCT TGC GAT AAG-3' |
| HBSAG14               | 5'-TAC CAC ATC ATC CAG ATG ACG AAC ACG AAC AGC TGG ACC AA TGG ACG GAA ACC AAC ACG AAC ACC AAC GAG AAC GAC GAG-3' |
| HBSAG15               | 5'-TAC TTC TGT GGT GTG ACT CAC CCT GCT GAG CAG ATG TTG ACT CTC AAT CCT TTG GGT TTC GTC CTT CCT GAC CAG CAC CAG TTC TTG ATC CTC-3' |
| HBSAG16               | 5'-TGA GCT CCT ACA AGG CCA AGA AGC TAG CAG GTC TTG GAG-3' |
| HBSAG17               | 5'-AAG GAT CCA ACA ATG GAC TTC TTG AAA TCT TCT CCA TTC TAC GCT TTC TTG TGC TTC GGT CAA TAC TTC GTC GCT GTG ACT CAC GGT-3' |

**FIG. 2.** Linear map of T-DNA region of pYF9616.
The foregoing results suggested that HBsAg can assemble into particles which are about 17 nm in diameter. To determine whether the human HBV surface large antigen in tomato plants also assembled into similar particles, the protein

![Image of TEM](image_url)

**FIG. 3.** Nucleotide and deduced amino acid sequences of the HBV large surface antigen gene.

**TABLE 3.** Expression of HBsAg in different transgenic tomato lines

| Plant no. | Organ       | Amt of HBsAg (ng/ml) | TSPa (mg/ml) | HBsAg/TSP ratio (10⁻⁵) | Amt of HBsAg (fresh wt) (ng/g) |
|-----------|-------------|----------------------|--------------|------------------------|-------------------------------|
| 1# Small fruit | 2.12 | 1.49 | 1.42 | 2.25 | 1.71 |
| Medium fruit | 2.35 | 0.30 | 7.81 | 2.23 | 0.47 |
| Mature fruit | 156.84 | 2.12 | 73.90 | 266.63 | 1.71 |
| Leaf | 1.76 | 4.29 | 0.41 | 2.78 | 5.10 |
| 2# Small fruit | 1.43 | 1.40 | 1.02 | 1.75 | 1.38 |
| Medium fruit | 1.55 | 0.28 | 5.52 | 1.80 | 0.28 |
| Mature fruit | 101.29 | 0.51 | 200.00 | 168.82 | 1.38 |
| Leaf | 1.43 | 5.70 | 0.56 | 3.82 | 5.70 |
| 3# Small fruit | 2.23 | 1.50 | 1.48 | 3.04 | 1.50 |
| Medium fruit | 2.47 | 0.29 | 8.46 | 2.96 | 0.29 |
| Mature fruit | 381.43 | 2.66 | 143.00 | 523.53 | 2.66 |
| Leaf | 3.18 | 4.81 | 0.56 | 3.82 | 4.81 |
| CK Small fruit | 1.42 | 1.40 | 1.02 | 1.75 | 1.42 |
| Medium fruit | 0.47 | 2.23 | 1.50 | 3.04 | 0.47 |
| Mature fruit | 1.71 | 2.47 | 0.29 | 3.04 | 2.47 |
| Leaf | 5.10 | 3.18 | 0.56 | 3.82 | 3.18 |

a Note that 1#, 2#, and 3# were different plants of the same transgenic tomato line; CK was a nontransformed plant grown in the greenhouse.

b TSP, total soluble protein.
was immunotrapped in the plant extracts using the immunogold labeling method. Gold particles can be observed attached to plant-produced particles, binding to the putative capsomers, which are about 17 nm in diameter (Fig. 5A). None of these kinds of particles was observed for nontransgenic control extracts (Fig. 5B). It was proved that the recombined HBV large surface antigen could retain the capacity for self-association and has the physical properties of human serum-derived HBsAg, which is highly immunogenic in the particle form.

In order to determine where the recombined particles assemble in the plant cell, we observed the immunogold labeled tomato fruit section by TEM. Gold particles were observed near the ER. This is similar to the production of rHBsAg in the leaves of transgenic potato (15). Microscopic examination of cells of nontransgenic tomato mature fruit did not reveal any gold particles near the ER. We therefore conclude that transgenic plant cells may accumulate recombinant HBV surface large antigen near the ER.

### DISCUSSION

The data presented in this paper prove that a human HBV surface large antigen fusion gene could be expressed in tomato efficiently. To our knowledge, this is the first report to demonstrate that the human HBV surface large antigen can be synthesized in plants.

For transgenic plants, a large proportion of capsomers can be observed by electron microscopy in extracts and fruit sections. This result is different from that reported by Imamura et al., who thought that the large S gene could not be assembled into particles in Saccharomyces cerevisiae (12). Zhao et al. also attempted to express the human HBV surface large antigen in tomato (29). No spherical particles 22 nm in diameter were observed by electron microscopy, nor were the specific proteins examined by the ELISA method in the extracts and sections of transgenic tomato fruit. This may have been caused by an inefficient system of expression of the human HBV surface large antigen gene.

Transgenic plants expressing recombinant antigens have been developed successfully since the method was first described by Mason et al. (17). A corollary of this research is to develop subunit vaccines which are produced in edible plant products, such that the plant-derived vaccine can be ingested directly without prior purification or processing. This appears to be a very promising alternative to other methods for expressing recombinant protein. Nevertheless, a number of questions and challenges still remain to be solved. Also, the method’s main disadvantage is low yields of expressed antigen. Next are several risks during the production and delivery stages of this technology, with potential impacts on the environment and on human health. Risks to the environment include gene transfer and exposure to antigens or selectable marker proteins. Risks to human health include oral tolerance, allergy, inconsistent dosage, worker exposure, and unintended exposure to antigens or selectable marker proteins in the food chain. These risks are controllable through appropriate regulatory measures at all stages of production and distribution of a potential plant-made vaccine. Successful use of this technology is highly dependent on stewardship and active risk management by the developers of this technology and through quality standards for production, which will be set by regulatory agencies.

Various approaches other than use of a fruit promoter have been suggested to increase the expression levels in transgenic plants. These include codon optimization and use of a specific promoter. Mason et al. reported that the HBsAg concentration in engineered tobacco was 0.01% of the total soluble protein (17). In this report, we describe the use of the fruit-specific promoter 2A11 to increase the level of foreign antigen protein and to show greater expression efficiency (26). The highest value was 0.02% of total soluble protein (Table 3). It was also very efficient for the expression of heterogeneous protein in transgenic plants to target the protein to different subcellular compartments, such as the chloroplasts of the ER, or potentially membrane-anchoring regions, where there is better accumulation and usually less proteolytic activity (2). The KDEL is commonly found at the C terminus of soluble proteins in the ER, and it contributes to protein localization by interaction with a receptor that recycles between the Golgi complex and the ER (8). The KDEL signal is sufficient for stable protein accumulation in the plant ER (23). Stoger et al. (25) found that single-chain antibody fragment levels were 6 to 14 times higher in cells transformed with the single-chain antibody fragment gene containing the KDEL than those transformed with the same gene without the KDEL. In our study,
the tobacco PR-S signal (7) was added in the N terminus and the KDEL ER retention signal at the C terminus encoded by the HBV large surface antigen gene.

The results of this work suggest the possibility of producing a new, alternative vaccine for human HBV. This alternative vaccine will be less expensive and more convenient to store than traditional vaccines. This work also gave us properties dedicated to developing technologies for a high-immunogenicity and low-cost oral vaccine for the developing world, though this is only a successful first step in a long-term project. Much more work needs to be done: for example, studying the immunogenicity of the recombinant large surface antigen, the stability of foreign genes in further generations, the security of transgenic plants, and so on. In future work, we will start orally immunizing mice with transgenic tissues to test the immunogenicity of the tomato-derived recombinant human HBV large surface antigen and study the stability of foreign genes in further generations.

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