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Full Length Research Paper

Expression of hepatitis B surface antigen (HBsAg) gene in transgenic cherry tomato

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The plasmid carrying the gene (adr subtype) encoding the hepatitis B surface antigen (HBsAg) was constructed. Cotyledonary leaves of cherry tomato (*Lycopersicon esculentum* Mill.) were transformed using *Agrobacterium tumefaciens* strain LBA4404 harboring pCAMBIA-1301/HB constructs to express HBsAg. The transformed nature of the plants was confirmed by polymerase chain reaction (PCR) analysis and Southern blot hybridization. Expression of HBsAg was confirmed by western blotting and levels of expression were assayed by enzyme-linked immunosorbent assay (ELISA). Southern blot hybridization confirmed the stable integration of the target genes into the genomes of cherry tomato, while western blotting showed high levels of biologically active HBsAg in transgenic plants. ELISA assay showed that HBsAg in transgenic cherry tomato was 100.36 ng/g fresh weight (FW) in leaves and 127.54 ng/g FW in fruits, implying that recombinant HBsAg had natural epitope. This study indicates the feasibility of the expression of foreign antigens in cherry tomato plant for possible use as a raw and edible vaccine.

Key words: *Lycopersicum esculentum var. cerasiforme*, cherry tomato, HBsAg gene, transgenic, expression.

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most widespread viral infections of humans and causes acute and chronic hepatitis and hepatocellular carcinoma (Kane, 1995; Torbenson and Thomas, 2002). The number of chronic HBV carriers all over the world is estimated to be 350 million and the annual HBV morbidity rate is more than 1 million (Michel, 2002). Vaccination is the best method to prevent HBV infection. The major envelope protein, hepatitis B surface antigen (HBsAg), which represents the major component of the HBV subunit vaccines, induces a protective immune response against infection. At present, the development of "edible" vaccines based on transgenic plants is one of the most promising directions in novel types of vaccines. HBsAg gene has been mainly expressed in tobacco (Mason et al., 1992; Thanavala et al., 1995), potato (Richter et al., 2001; Liu , 1999), banana (Sunil Kumar et al., 2005b; Elkholy et al., 2009), tomato (Salyaev et al., 2007; Srinivas et al., 2008) and other fruits and vegetables (Marcondes and Hansen 2008; Pniewski et al., 2006).

Cherry tomato (*Lycopersicon esculentum* Mill.), a subspecies of common tomato varieties, is an annual or perennial herb of the Nightshade (Solanaceae), tomato genus (*Lycopersicon*) and tomato species. It has delicious taste and rich nutrition, and especially can be

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Abbreviations: HBsAg, Hepatitis B surface antigen; **HBV,** hepatitis B virus; **FW,** fresh weight; **6-BA,** 6-benzylaminopurine; **CaMV35S,** cauliflower mosaic virus 35S; **ELISA,** enzyme-linked immuno sorbent assay; **HBsAg S,** hepatitis B virus small antigen; **IAA,** indoleacetic acid; **MS,** Murashige and Skoog medium; **PCR,** polymerase chain reaction.

#Both authors contributed equally to this work.

eaten raw as a fruit. Therefore, it is an ideal candidate crop for the production and delivery of oral vaccines. There have been some reports of antigen expression in tomato (*L. esculentum*). The hybrid gene that encoded the chimerical protein consisting of antigenic determinants of HIV-1 and HBV protective proteins was expressed in transgenic tomato fruit (Shchelkunov et al., 2004). Six plant expression cassettes (pHBS, pHER, pEFEHBS, pEFEHER, pSHER and pEFESHER) were used to assay the possible expression levels of hepatitis B surface antigen in tomato by agroinfiltration (Srinivas et al., 2008). The gene encoding the modified HBV large surface antigen under the control of a fruit-specific promoter was constructed and expressed in transgenic tomato plants (Lou et al., 2007). Hepatitis B virus surface antigen was synthesized in tomato plants transgenic for the *preS*2-*S* gene (Salyaev et al., 2007). The subtype adw of HBsAg was studied in the above-mentioned researches. However, Hepatitis B surface antigen (HBsAg/adr) expression in tomato plants, has not previously reported in detail.

In this study, we constructed a plant expression vector carrying hepatitis B surface antigen gene (*HBsAg*/adr) transformed cherry tomato mediated by *Agrobacterium tumefaciens*, and obtained transgenic plants with an inserted HBsAg gene encoding the small protein. The novelty and priority of the research is that the object of this study was cherry tomato, a sub-variety of common tomato, which can be easily used as a raw and edible vaccine.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli DH5α strain and *A. tumefaciens* strain LBA4404 were employed. The plasmid pBI121/HB were previously constructed and maintained in our laboratory. The plasmid pCAMBIA1301 was kindly presented by CAMBIA Institute of Australian.

Plant materials

Seeds of cherry tomato (*L. esculentum Mill.*) was kindly provided by Deng J. J. (Shannxi, Xi'an, Vegetable Research Institute). After immersion in water for 2 h, the seeds were sterilized with 70% alcohol for 1 min and were washed with sterile distilled water several times. Then they were sterilized with 10% NaClO for 5 min and rinsed several times. Finally, the seeds were placed to germinate on 1/2 Murashige–Skoog (MS) (Murashige and Skoog, 1962) medium without any hormone. Cotyledonary segments from 10-day-old seedlings were excised and used for *Agrobacterium*-mediated transformation.

Construction of plasmids for plant transformation

The *HBsAg*-carrying genetic constructs for plant transformation were obtained by the standard methods of gene engineering (Sambrook, and Russell 2002). In this study, we used the plasmid pBI121/HB, which carried the synthetic 681-bp gene encoding the recombinant hepatitis B surface antigen of the adr serotype (*HBsAg*/adr). The HBsAg gene fragment containing CaMV35S promoter and nitric oxide synthetase (NOS) terminator was cut out from the plasmid pBI121/HB, cloned into pCAMBIA1301 after digestion with both *Hin*d III and *EcoR* I endonucleases, and transformed into DH5α and screened for recombinant plasmid named pCAMBIA1301/HB.

Plant transformation

Agrobacterium strain LBA4404 cells were transformed by the direct method (Horsch et al., 1985) with the plasmids prepared from *E. coli.* clones after the structure of the plasmid had been verified by restriction digestion. The cherry tomato plants (*L. esculentum* Mill.) growing under sterile conditions were used. The leaf discs were transformed by co-cultivating with *Agrobacterium* strains transformed with pCAMBIA1301/HB (Horsch et al., 1985; Draper et al., 1988). Shoots were generated from transformed callus selected on MS medium containing 20 mg/L Hyg and 300 mg/L cefotaxime. Shoots were rooted in medium containing 20 mg/L Hyg and 150 mg/L cefotaxime, and eventually transplanted to soil. The following identification and detection was conducted by using 20 subcultured plants every group after transformation.

DNA isolation and PCR identification

Isolation of DNA from cherry tomato leaves was conducted as described by Edwards et al. (1991). Polymerase chain reaction (PCR) conditions were those described by the *Taq* polymerase manufacturer (Life Technologies, Gaithersburg, MD). PCR conditions of reaction mixtures were as follows: an initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 30 s, 59°C for 40 s, and 72°C for 1 min; followed by a 10-min incubation at 72°C. Primer sequences designed to amplify the HBsAg gene was 5′ AACGGGATCCCGCACC ATGGAGAACACAACATCA 3′ and the reverse primer 5′ CCCGGAATTCCGGCTTAAATGTAT ACCCAAAGAC 3′.

Southern analysis of transgenic plants

The digested genomic DNA after gel separation was blotted into Hybond N+ nylon membranes (Amersham Pharamacia Biotech, UK). The 681 bp BamHI fragment from pCAMBIA1301/HB containing the HBsAg S gene was labeled using North2South® Direct HRP Labeling and Detection Kit from PIERCE (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions, and used for hybridization. The Southern blotting and subsequent hybridization were carried out as described in Sambrook and Russell (2002). Results were detected by the exposure of X-ray film.

ELISA analysis

Total protein was extracted from the single leave and fruit of untransformed control and transgenic plants as previously described (Mason et al., 1992). Each group contained 20 plants. The extracts were clarified and assayed in triplicates for the levels of HBsAg expression, and the mean values were taken as an estimate of the amount of HBsAg. An HBsAg enzyme-linked immunosorbent assay (ELISA) kit was purchased from Wantai Biotechnology Co., Ltd. (Beijing, China), and HBsAg standards were provided by Huamei Biotechnology Company. The positive control (human serum derived HBsAg) as a standard and negative

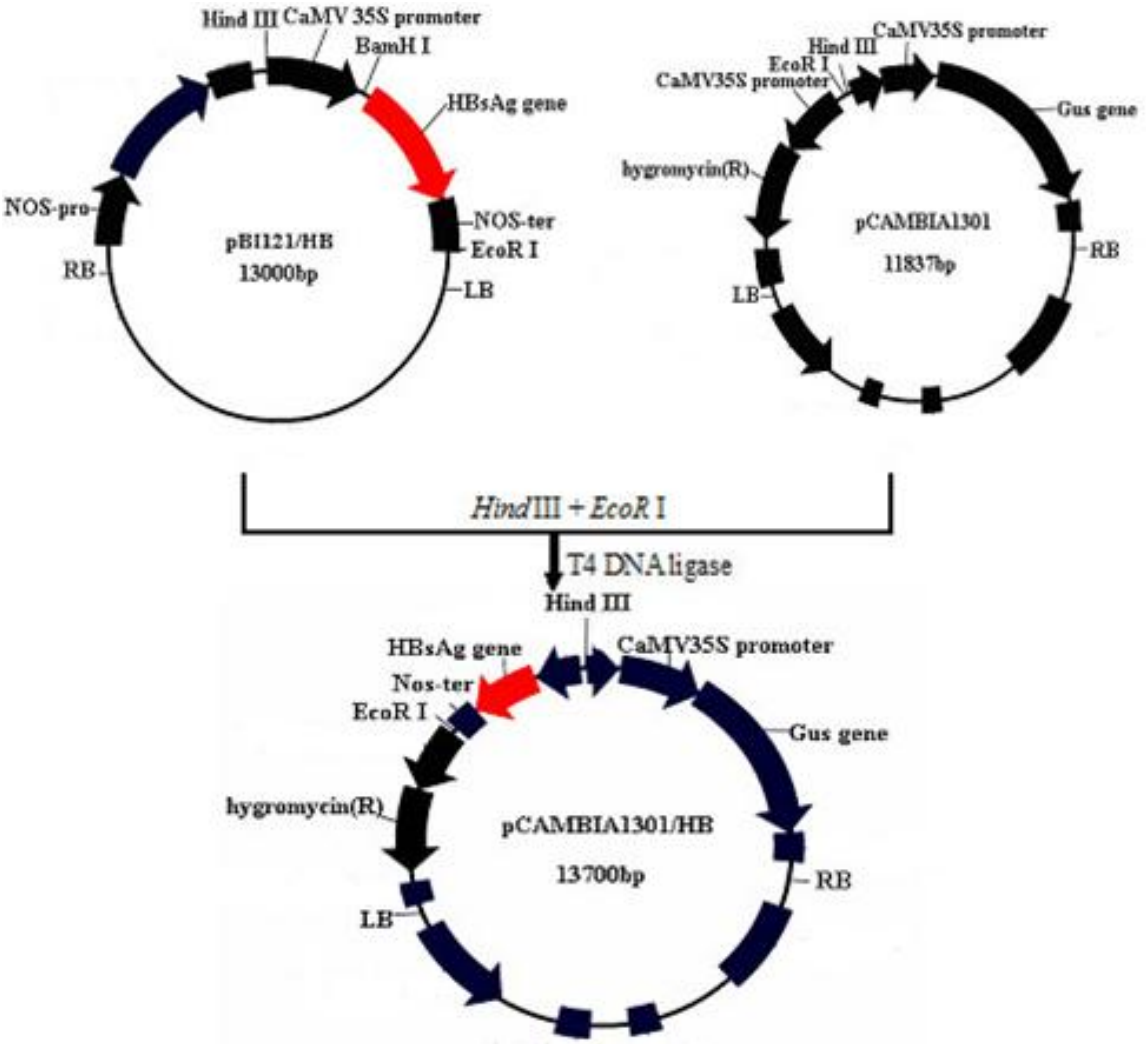

Figure 1. The construction of plant expressive vector. Plasmid pCAMBIA1301/HB with inserted HBsAg under the control of the cauliflower mosaic virus promoter p35S (CaMV 35S).

control (protein extracted from un-transformed control) were used. Total soluble proteins containing expressed HBsAg were detected by ELISA and quantified with the standard curve established by series dilution of HBsAg standards.

Protein extraction and western blot

Total protein of both untransformed and transgenic plants was obtained by centrifugation at 2000×*g*, and 5-fold concentration. Protein concentration was estimated by the procedure of Bradford (1976). Western blotting was carried out as described in Sambrook et al. (1995). Proteins were separated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 15% acrylamide), and transferred to a Polyvinylidene fluoride (PVDF) membrane (Amersham) using a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked for 1 h at room temperature with 150 mM NaCl, 10 mM Tris/HCl pH 8.0, 0.1% Tween 20 and 1% Triton X-100, and 5% (w/v) skimmed milk. Blots were incubated with specific rabbit anti-Fab IgG polyclonal antibodies, prepared as previously described (Ayala et al., 1995), and diluted to 10 ug ml^{-1} in Tris - buffered saline (TBS) containing 1% skimmed milk (1%TBS). After 2 h at 37°C, the membranes were washed and a goat anti-rabbit IgG alkaline phosphate conjugate (Boehringer Mannheim), after diluted 1:1600 in 1%TBS was added for 1 h at 37°C. The enzymatic reaction was developed with nitroblue tetrazolium (0.1 mg ml^{-1}), and 5-bromo-4-chloro-3-indolyl phosphate (0.06 mg ml^{-1}) in 100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris/HCl pH 9.5 was used. Apparent molecular weight of proteins was estimated with pre-stained markers (Rainbow Cold Markers, Amersham).

RESULTS

Identification of *Agrobacterium* transformation with pCAMBIA1301/HB

The HBsAg S gene fragment was amplified by PCR from plasmid pBI121/HB containing hepatitis B virus (adr subtype). Plant expression vector pCAMBIA1301/HB (Figure 1) in which HBsAg S gene was driven by the CaMV35S promoter and flanked downstream by the NOS-terminator was constructed. The vector was then

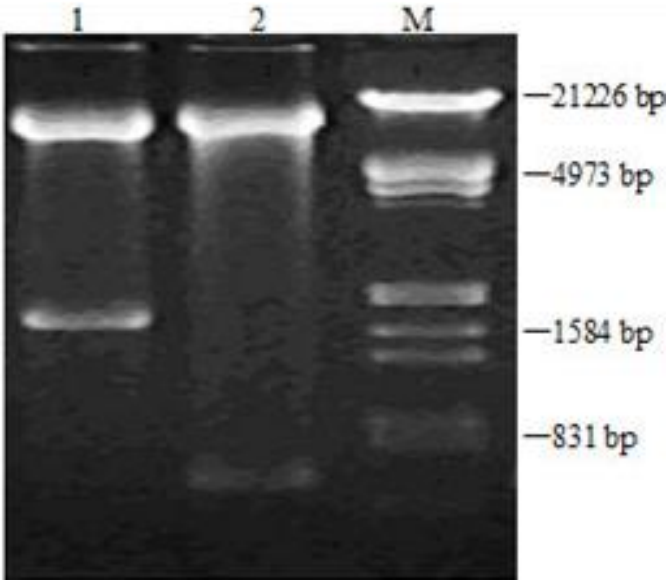

Figure 2. PCR products of pCAMBIA1301/HB. Lane 1, Digestion of pCAMBIA1301/HB with *EcoR* I + *hind* III; lane 2, digestion of pCAMBIA1301/HB with *Sac*I + *BamH*I; lane M, λDNA/*EcoR*I + *Hind*III Marker.

mobilized into *A. tumefaciens* LBA4404 by freeze-thaw method. Kanamycin-resistant *Agrobacterium* cells were confirmed by PCR and prepared for the transformation of cherry tomato. Plasmids in *A. tumefaciens* LBA4404 were extracted for double restriction enzyme digestion. Around 700 and 1900 bp bands were found, which were respectively consistent with HBsAg S gene and CaMV35S + HBsAg + NOS. The results show that the pCAMBIA1301/HB was successfully transformed into *A. tumefaciens* LBA4404 (Figure 2).

The cherry tomato leaf-sections transfected by *A. tumefaciens* LBA4404 were placed on selection medium of MS agar with hormones (2.0 mg/L 6-benzyladenine (6-BA) and 0.1 mg/L indole-3-acetic acid (IAA)) and antibiotics (Hyg 20 mg/L and cefotaxime 300 mg/L). Shoots began to develop on callus at the edge of the leaf sections two weeks later. The shoots 1 to 2 cm in height were cut off from the callus and transferred to MS agar medium supplemented with 0.2 mg/L of IAA and 20 mg/L of Hyg for rooting.

PCR and Southern blotting analysis of the transformed plants

The transgenic plants transformed with HBsAg were successfully obtained. The presence of the HBsAg S gene in the genomic DNA of the putative transgenic plants was initially examined by PCR. The transformed plants showed the expected HBsAg S gene band of around 700 bp in size. No band was amplified from wild-type plant. Figure 3 presents partial results of the PCR. To further confirm the integration of the HBsAg S gene in the transgenic cherry tomato genome, chromosomal DNA prepared from the PCR-positive plants transformed with pCAMBIA1301/HB were digested with BamHI/SacI followed by Southern blotting analysis. Plasmid pCAMBIA- 1301/HB DNA and genomic DNA of untransformed plant were also digested by HindIII/SacI and used as positive and negative control respectively. Southern hybridization showed the band with expected size in HBsAg S-transgenic lines, which confirmed the stable integration of the HBsAg S fragment into the cherry tomato genome. No hybridization band was detected in untransformed plant (Figure 3).

ELISA analysis

ELISA assay was performed on crude protein extracts prepared from leaves and fruits of transgenic cherry tomato plants. Cutoff values were calculated for leaves of untransformed plant, where cutoff = OD450/630 (negative average) × 2.1. A sample was positive when the OD450/630 was higher than the cutoff value and was negative when less than the cutoff value. Results show that the samples of transgenic plants was positive for HBsAg S protein (Figure 4), which indicated that there was HBsAg S protein expression only in transformed cherry tomato plants compared to the control pants.

ELISA analysis of the protein extracted from fruit and leaf tissues of the transgenic cherry tomato plants demonstrated that fruits and leaves of transgenic plants had much higher OD values than the untransformed plant control. The expression level reached 100.36 ng/g FW in leaves and 127.54 ng/g FW in fruits, respectively. This indicated that hepatitis B surface antigen could be expressed very well in cherry tomato, although these are not the maximal measures in single organs compared to relevant published data.

Western analysis

Western analysis was carried out to confirm the HBsAg expression in transformed cherry tomato. A 24 kDa HBsAg specific band was noted in the transformed cherry tomato plants, whereas it was absent in the control untransformed plants (Figure 5). The result is consistent with the results obtained by Ganapathi et al., (2007) and Shekhawat et al., (2007).

Development of stable transgenic plants

Transformed cherry tomato leaf discs showed the development of shoots on hygromycin selection medium (Figure 6A and B). The shoots were excised and cultured on rooting medium. The transformed shoots developed roots and grew into complete plantlets on the selection

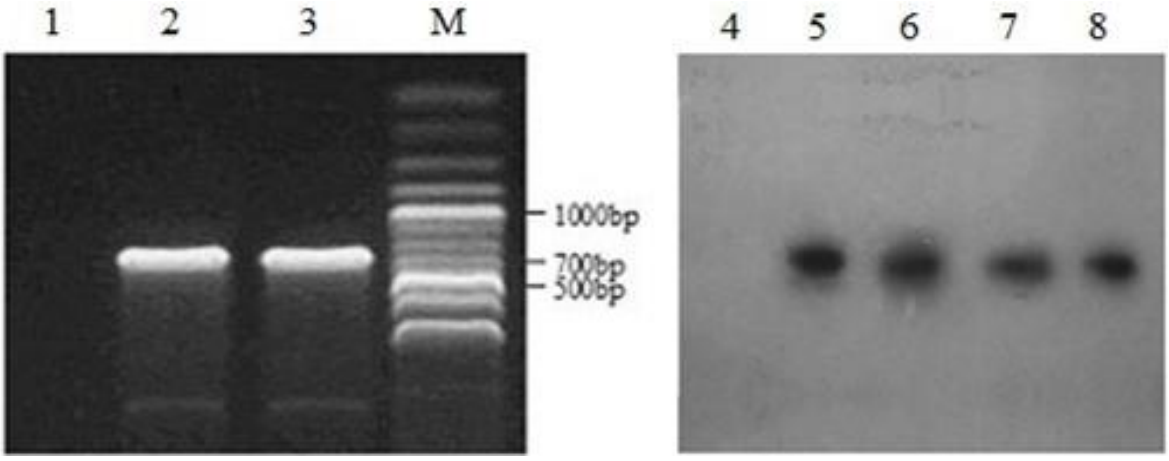

Figure 3. Result of PCR detection and Southern hybridization of partial transgenic cherry tomato plants transformed with pCAMBIA1301/HB containing HBsAg S fragment. Lane M, Marker; lane 1, PCR production of untransformed plants; lanes 2 and 3, PCR production of transformed plants; lane 4, Southern blotting of DNA of untransformed plants; lanes 5 to 8, Southern blotting of DNA of transformed plants.

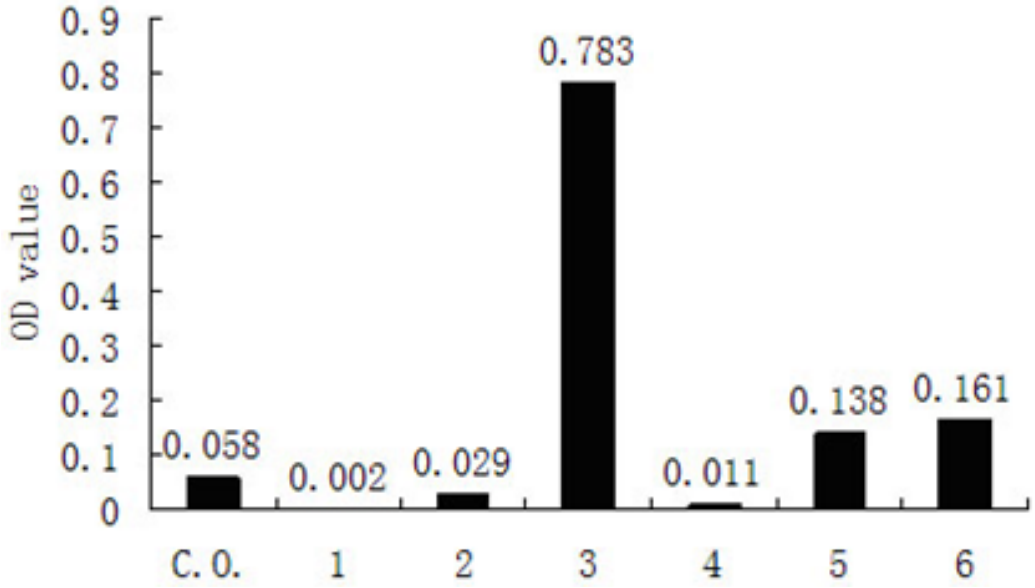

Figure 4. HBsAg-ELISA results of partial cherry tomato plants transformed with pCAMBIA1301/HB and un-transgenic plants (OD value). HBsAg S protein extracted from leaves and fruits of untransformed plant and transgenic cherry tomato plants. Cutoff (C.O.) value equals to multiplying the average OD450 value of non-transgenic plants by 2.1; 1. Blank; 2, negative control; 3, positive control; 4, negative control plants; 5, transgenic plant leaves; 6, transgenic plant fruits.

medium (Figure 6C). The 65 sterile transgenic seedlings with well-developed root system were obtained and transferred to the soil after culture of 28 days (Figure 6D and E). The transgenic tissue-cultural plantlets showed no significant differences compared to the control plants, apart from more green leaves. More obvious phenotypic alterations were gradually observed in the transgenic plants after their transplantation to the field, such as fleshy and dark green leaves, distinct notches on leaf edges, shorter and thicker internodes, more adventitious roots, round fruits, long-shaped or oval-shaped fruits with normal seeds (Figure 6F to H).

Quality of the transgenic cherry tomato fruit

We investigated the qualities of the transformed cherry tomato fruit after transplanted to the field (Table 1).

Compared with the wild cherry tomato fruit, the transgenic fruit had higher mean fruit weight, lycopene content, organic acids content, reducing sugar content and starch content. Although there were some changes of qualities in the transgenic fruit, both of the transgenic fruit and the wild one had no significant difference.

DISCUSSION

According to the different epitopes, HBsAg can be divided into eight pure subtypes and two mixed subtypes. Every subtype has different geographical distribution. The adr subtype mainly exists in Asia and the Pacific, especially in China. The current vaccine in domestic and international research is mainly adw subtype; therefore the research on the adr subtype of HBsAg in this study is of great practical value. The data in this study proved that a human HBV surface small antigen gene could be expressed in cherry tomato efficiently. Our results add a new example to the successful reports of HBsAg S gene expression in transgenic plants. In recent years, plants have been used as a safe, convenient and economical alternative to the expression system for bioreactor including antibodies, vaccines, biopolymers and so on (Schillberg et al., 2005). Due to its palatable fruit, attractive nutrition and the fact that it can be eaten raw, cherry tomato holds its own advantages in this research area.

Transgenic plants expressing recombinant antigens have successfully been in progress since the transformation method was first described by Mason et al. (1992). A deduction of this research is to develop virus vaccines which are produced in edible plants, so that the plant-derived vaccine can be ingested directly without purification or processing. And it appears to be a very promising alternative for expressing recombinant protein. However, a number of questions still remain to be solved. Its main disadvantages are low expression levels of antigen, potential impacts on the environment and human health. Therefore, much work need to be done such as studying the immunogenicity of the recombinant small surface antigen, the stability of foreign genes in future generations, the security of transgenic plants, and so on, some of which are going on.

In the process of callus induction and subculture, a variety of mediums of hormone combinations were tried, which caused the transformed tomato calluses seriously browning. Nevertheless, in the MS medium supplemented with 2.0 mg/L 6-BA + 0.2 mg/L IAA, transformed callus regenerated shoots very well. When reaching a certain height, shoots could be transferred to rooting medium after cutting and be rooting. After transplanted to the field, the transformed seedlings grew well and had obvious changes compared to non-transgenic plants. The transgenic cherry tomato plants showed short internodes, corpulent and dark green leaves, more inflorescence and flower buds, and rounded fruits.

At the same time, although there was no significant quality difference between the transgenic fruit and the wild one, the transformed cherry tomato fruit had some higher quality parameters. It is speculated that the insertion of exogenous DNA led to the related changes of transcription, translation and metabolism in the plants, thus showing differences in plant morphology. And its mechanisms of variation are now being explored.

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