

Production of hepatitis B surface antigen in transgenic plants for oral immunization

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Here we present data showing oral immunogenicity of recombinant hepatitis B surface antigen (HBsAg) in preclinical animal trials. Mice fed transgenic HBsAg potato tubers showed a primary immune response (increases in HBsAg-specific serum antibody) that could be greatly boosted by intraperitoneal delivery of a single subimmunogenic dose of commercial HBsAg vaccine, indicating that plants expressing HBsAg in edible tissues may be a new means for oral hepatitis B immunization. However, attainment of such a goal will require higher HBsAg expression than was observed for the potatoes used in this study. We conducted a systematic analysis of factors influencing the accumulation of HBsAg in transgenic potato, including 5' and 3' flanking elements and protein targeting within plant cells. The most striking improvements resulted from (1) alternative polyadenylation signals, and (2) fusion proteins containing targeting signals designed to enhance integration or retention of HBsAg in the endoplasmic reticulum (ER) of plant cells.

Keywords: hepatitis B surface antigen, transgenic plants, edible vaccine, potato, oral vaccine, protein engineering

Two key factors have enabled the development of the hepatitis B virus (HBV) vaccine (as reviewed in ref 1.): (1) identification of an immunogenic HBV protein that would stimulate the human immune system to produce protective antibodies, and (2) establishment of a quantitative measure of the success of vaccination (minimum protective antibody level), which allowed optimization of dosage levels and timing. Both factors are now well accepted; this resulted in the licensure of a commercial parenteral (needle-delivered) vaccine that is safe and efficacious, albeit too expensive for adoption in many developing countries where HBV infection is a major health problem.

The discovery of a suitable immunogen for HBV vaccination came from studies of the surface antigen (HBsAg), which is produced in copious amounts in liver cells of infected individuals². Some of this envelope protein is secreted from liver cells and can be recovered in serum in the form of viruslike particles (VLPs). The VLPs, consisting of HBsAg and membrane lipids, were found to be highly immunogenic and would elicit antibodies specific for the authentic HBV. Using the tools of molecular biology, the gene encoding HBsAg was expressed in yeast cells, from which immunogenic VLPs could be harvested using protein isolation/refolding technology³. Chimpanzees immunized with yeast-derived HBsAg were protected from infection when challenged with HBV⁴. Later, it was determined that protective immunization in humans was indicated by serum anti-HBsAg antibody at a level of ≥ 10 mIU/ml (refs 5,6). This correlate-of-protection is the basis for licensure of the current injectable vaccine.

There have been numerous studies of morphogenesis of HBV and HBsAg particles in mammalian cells⁵. A crucial step in this process is the integration of HBsAg into the membrane of rough endoplasmic reticulum (ER). This involves co-translational transport mediated by a noncleavable signal peptide in the first hydrophobic domain that interacts with signal recognition particle⁷. HBsAg has at least two and as many as four membrane-spanning domains; both N and C termini are exposed to the ER lumen.

Nanometer-scale biological structures assemble by polymerization of similarly folded subunits using a small number of well-

defined bonding contacts⁸. The driving force for polymerization is the formation of favorable bonding interactions as free subunits are incorporated into the growing polymer. For envelope proteins such as HBsAg, two essential steps in the polymerization process are correct integration of the polypeptide into the ER membrane, and establishment of disulfide cross-links among the protein subunits. HBsAg expressed in yeast cells is chemically reduced after extraction to cause disulfide bond formation³.

With over two billion people worldwide infected with HBV (ref. 9), there is great need for an inexpensive vaccine that would allow vaccination of large segments of the population. Plants are a potential source of HBsAg that is not dependent upon process technology to ensure protein folding and particle assembly. In addition, a plant-based HBsAg expression system makes possible the testing of an oral immunization strategy by simply feeding the plant samples. Expression of HBsAg in transgenic tobacco leaves yielded VLPs averaging 22 nm in size¹⁰. When used for parenteral immunization of mice the tobacco VLPs provoked B- and T-cell immune responses comparable to yeast-derived vaccine¹¹. However, because of possible degradation of proteins in the gut, presentation of vaccine antigens by an oral route requires much higher levels of immunogen than parenteral delivery¹². Increasing the levels of HBsAg in plant tissues would enhance the utility of plants as a source of recombinant antigen. The present study was designed to determine if oral delivery of HBsAg produced in transgenic plant tissue could stimulate serum anti-HBsAg, and to develop expression vectors that would increase expression of HBsAg in plants.

Results and discussion

Oral immunization with raw potatoes. We previously showed that HBsAg extracted from transgenic tobacco leaves contains VLPs, and that parenteral immunization of mice with this extract elicited B- and T-cell responses similar to those of the commercial vaccine^{10,11}. We developed transgenic potatoes that express HBsAg in tubers for use in oral delivery. The gene construct pPAT-HB (Fig. 1A) contains the tuber-specific patatin promoter¹³ to drive transcription of

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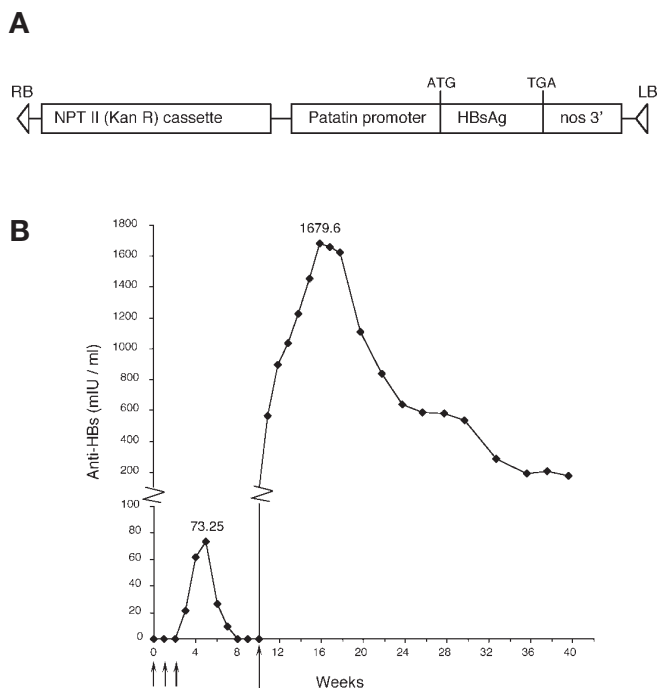


Figure 1. Oral immunization of mice with transgenic potatoes expressing HBsAg. **(A)** Structure of T-DNA region of pPAT-HB used to create the transgenic line PAT-HB-7. LB, RB: left and right border elements of the T-DNA that is mobilized into plant cells by *Agrobacterium tumefaciens*; ATG, start codon; TGA, stop codon. **(B)** Serum immune responses of mice that were fed raw tubers of potato line PAT-HB-7 expressing 1.1 μg HBsAg/g tuber. Animals were fed 5 g potatoes plus 10 μg CT per mouse per feeding. Small arrows indicate the feeding schedule: day 0, 7, and 14. Large arrow indicates a single intraperitoneal boost at week 10 with alum-adsorbed recombinant HBsAg (0.5 μg /mouse). Data are means of values for 10 animals. Numbers above peaks indicate the anti-HBsAg titer.

HBsAg. Transgenic potato line PAT-HB-7 was selected for oral immunization studies; it accumulated 1.1 μg HBsAg per g fresh tuber. Mice fed three weekly doses of PAT-HB-7 potato tubers (containing a total of 5.5 μg HBsAg per dose) plus 10 μg cholera toxin (CT) added as adjuvant developed a primary serum antibody response that peaked at 73 mIU/ml three weeks after the last dose, then declined over the next three weeks (Fig. 1B). Intraperitoneal injection of a subimmunogenic dose (0.5 μg) of commercial vaccine resulted in an immediate high-level recall antibody response. Mice fed control tubers with CT showed neither primary nor booster responses (data not shown). We conclude that immune memory cells were established following oral delivery of HBsAg in potato tuber and that a recall response was elicited by parenteral immunization.

Oral immunization results from the activation of lymphoid tissue in the gut. Specialized M cells recognize particulate structures, such as bacteria or viruses, and transport them to underlying B and T cells to initiate an immune response¹⁴. Soluble proteins are poorly immunogenic; therefore VLP formation by the HBsAg subunits is likely required for an effective oral vaccine. We determined by sucrose gradient sedimentation that the HBsAg in transgenic potato tubers is particulate¹⁵. The present experiment was limited by the total amount of tuber tissue that mice can ingest in 24 h and the amount of HBsAg produced in the tubers. Higher doses would likely cause a more robust immune response, perhaps without the use of an added oral adjuvant, although the tuber tissue itself may be acting as an adjuvant because it contains proteinase inhibitors¹⁶. To address these possibilities, we need edible plant tissues that produce higher levels of HBsAg, and we thus focused efforts on increasing HBsAg accumulation in plants.

Analysis of new expression vectors. We constructed a series of HBsAg expression vectors (Fig. 2) using the nominally constitutive cauliflower mosaic virus (CaMV) 35S promoter. We examined the 5'-untranslated regions (5'-UTRs) from tobacco etch virus (TEV) and tobacco mosaic virus (TMV), the signal peptide from soybean vegetative storage protein (VSP), a plastid transit peptide, and three different 3'-flanking sequences. Because transgene position and copy numbers vary, mRNA levels in individual transgenic lines can be quite different. To allow comparisons among vectors, we generated multiple individual potato lines for each construct and analyzed leaves for HBsAg and mRNA (Fig. 3). We accounted for transcriptional effects by comparing the levels of HBsAg accumulated per unit of mRNA in independent lines. Thus the expression effects of the different vectors can be discerned among random transcriptional variation using the slope of the plot of HBsAg protein vs. mRNA (Fig. 3), denoted P/R (Table 1). We appreciate that any individual line could have altered protein metabolism due to a T-DNA insertion mutation. However, we consider this possibility to be remote, and thus the effect of a single data point on the P/R slope would be minimized in the regression analysis of multiple transgenic lines.

Polyadenylation signals and 5'-UTRs. pHB104 and pHB114 were designed to test alternative 3' sequences linked to the HBsAg gene, since polyadenylation affects stability of mRNA¹⁷. pHB104 uses the 3' end of the soybean *vspB* gene and pHB114 uses the 3' element from the potato *pinII* gene. Both constructs produced several lines that showed more HBsAg mRNA than HB103 lines containing the nopaline synthase (NOS) 3' element (data not shown). pHB104 gave the highest P/R value in potato leaves (1.077, Table 1), and pHB114 the second highest (0.652). At ~0.25% of total soluble protein (TSP) in leaves of tissue-cultured plantlets, the amount of HBsAg produced in several HB104 and HB114 lines was much higher than the best lines from any of the other constructs (Figs 3 and 4). We conclude that posttranscriptional effects contribute strongly to the enhanced expression.

Some of the best HB104 and HB114 lines grew poorly in the greenhouse or had poor tuber yield, indicating that HBsAg at high levels may be phytotoxic. However, such effects could result from insertional mutagenesis. The best line for production of HBsAg in greenhouse-grown plants (HB114-16, 16 μg HBsAg per g tuber, Table 1) contained four insertions of the pHB114 cassette as determined by Southern blot (data not shown). The maximum HBsAg level in tubers of HB104 lines was 6.5 μg /g (Table 1); however, the best HB104 lines (by leaf expression) yielded fewer tubers in the greenhouse.

We compared vectors pHB103 and pHB111 to determine whether the TEV or TMV 5'-UTR would provide more effective

Table 1. Analysis of HBsAg protein vs. mRNA^a

Construct name	Slope of protein vs. mRNA (P/R)	Correlation coefficient R ²	Tuber HBsAg level ($\mu\text{g}/\text{g}$)
pHB103	0.079	0.1818	0.33
pHB104	1.077	0.3711	6.50
pHB105	0.423	0.7085	1.25
pHB106	0.443	0.4431	0.80
pHB107	0.501	0.6042	2.40
pHB110	ND ^b	ND ^b	ND ^b
pHB111	0.196	0.3052	0.33
pHB114	0.652	0.2353	16.00

^aData from Figures 3 and 4 were used for regression analysis done as described in the legend to Figure 3. The slopes (P/R) of the derived regression lines are shown with the correlation coefficients. The HBsAg level in the tuber tissue of the best line for each construct is shown as micrograms of antigen per gram fresh tuber weight.

^bNot determined; HB110 lines did not produce detectable HBsAg protein, although mRNA was detected (see Fig. 5).



Figure 2. Structures of expression cassettes. All constructs are driven by the CaMV 35S promoter with dual enhancer (green). TEV, Tobacco etch virus 5'-UTR (light blue) used in all constructs except pHB111; Ω , tobacco mosaic virus 5'-UTR; NOS, 3' region from *A. tumefaciens* nopaline synthase gene (dark purple); VSP, 3' region from soybean vegetative storage protein gene *vspB*; pin 2, 3' region from potato proteinase inhibitor II gene; *vsp* α S, signal peptide from soybean vegetative storage protein *vspA*; *vsp* α L, *vspA* signal peptide plus a putative vacuolar targeting signal; SEKDEL, hexapeptide ER retention signal; TPSS, transit peptide from the small subunit of Rubisco.

translational enhancement. HB111 plants showed a slightly higher P/R value (Table 1), but maximal HBsAg expression in tubers was the same. Thus there was no striking difference in these 5' elements in this context.

Signal peptides and ER retention. We constructed pHB106 and pHB107 in order to test the idea that a cleavable plant signal peptide added to the N terminus of HBsAg may mediate more efficient ER targeting in plant cells than the noncleaved HBV signal peptide. pHB106 contains the soybean VSP " α S" signal peptide, and pHB107 contains the VSP " α L" signal peptide (α S plus a putative vacuolar targeting sequence)^{18,19}. HBsAg protein levels in leaves of HB103 (Fig. 4A), HB106 (Fig. 4C), and HB107 (Fig. 4D) were in a similar range. However, P/R values for HB106 and HB107 plants were much greater than that of HB103 plants, showing that HB106 and HB107 lines accumulated more HBsAg per unit mRNA. The VSP signal peptide is normally cleaved in soybean¹⁸; however, we do not know whether cleavage occurred with our fusion proteins. The highest HBsAg expression in tuber tissue was 2.4 μ g/g in a HB107 line. pHB105 encodes the ER retention signal "SEKDEL" (refs 20,21) at the C terminus of HBsAg. We reasoned that ER retention may allow concentration of HBsAg subunits and drive more efficient subunit interaction and disulfide cross-linking. The HB105 plants showed a higher P/R value than the HB103 plants, similar to HB106 and HB107 plants (Table 1), indicating that the SEKDEL extension mediates better accumulation of HBsAg in plant cells. Leaf and tuber expression was improved in HB105 lines compared to HB103 lines (Fig. 4, Table 1).

In mammalian cells, HBsAg undergoes extensive cross-linking in a post-ER, pre-Golgi compartment that is devoid of protein disulfide isomerase (PDI)²². Thus shuttle vesicles that salvage and recycle resident ER proteins displaying C-terminal KDEL may represent a similar compartment in plant cells^{23,24}. A combination of both the signal peptide and KDEL may result in further enhancement, and these experiments are in progress.

Plastid targeting. pHB110 contains the N-terminal chloroplast transit peptide from the small subunit of ribulose biphosphate car-

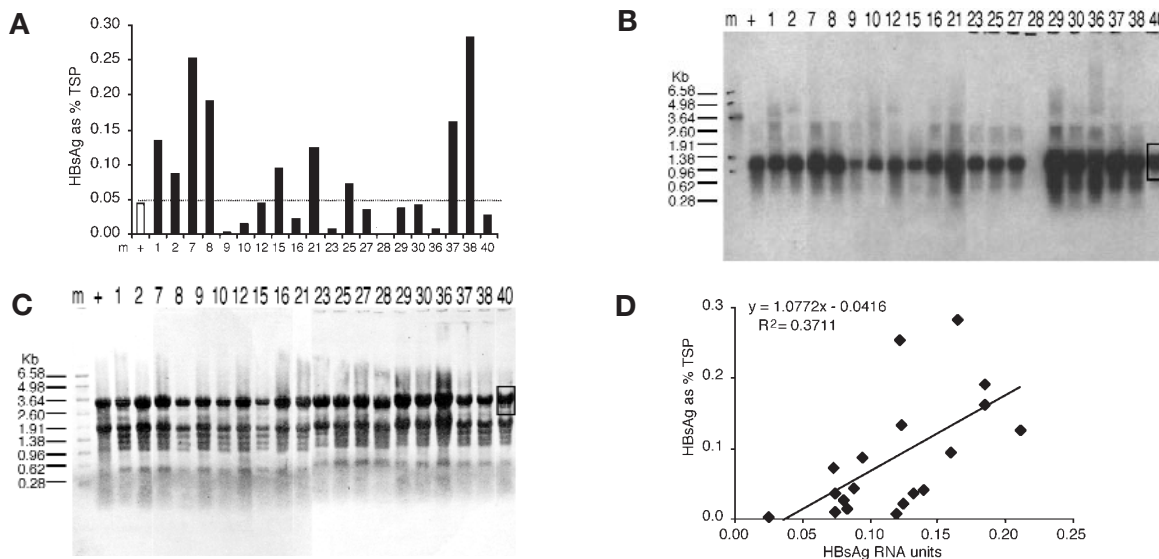


Figure 3. Analysis of HBsAg protein and mRNA from leaves of potato lines transgenic for pHB104. (A) HBsAg protein determined by ELISA is plotted as percentage of TSP. The dashed line indicates 0.05% TSP. +, Control sample from line HB107-16 measured in a separate experiment. Numbers indicate individual lines transgenic for pHB104, and apply to panels A, B, and C. *, HBsAg protein was not determined for line HB104-28. (B) RNA analyzed by northern blot hybridized with HBsAg coding sequence. Lane marked + contains 5 μ g total RNA from line HB107-16. The signals from sample HB107-16 were arbitrarily assigned a value of 1 total RNA unit and 0.1 HBsAg RNA unit, and all other samples were adjusted to this scale. m, RNA size standards; boxes in lane 40 of B and C represent the area of each lane that was quantified. (C) Methylene blue staining of the RNA membrane to indicate loading density. (D) Plot of HBsAg protein vs. HBsAg mRNA. HBsAg mRNA values are standardized by calculating the value for northern blot signal divided by the value for the 25S rRNA signal on the methylene blue-stained blot, and presented in arbitrary units. The regression equation and R^2 value for the data points are shown at upper left.

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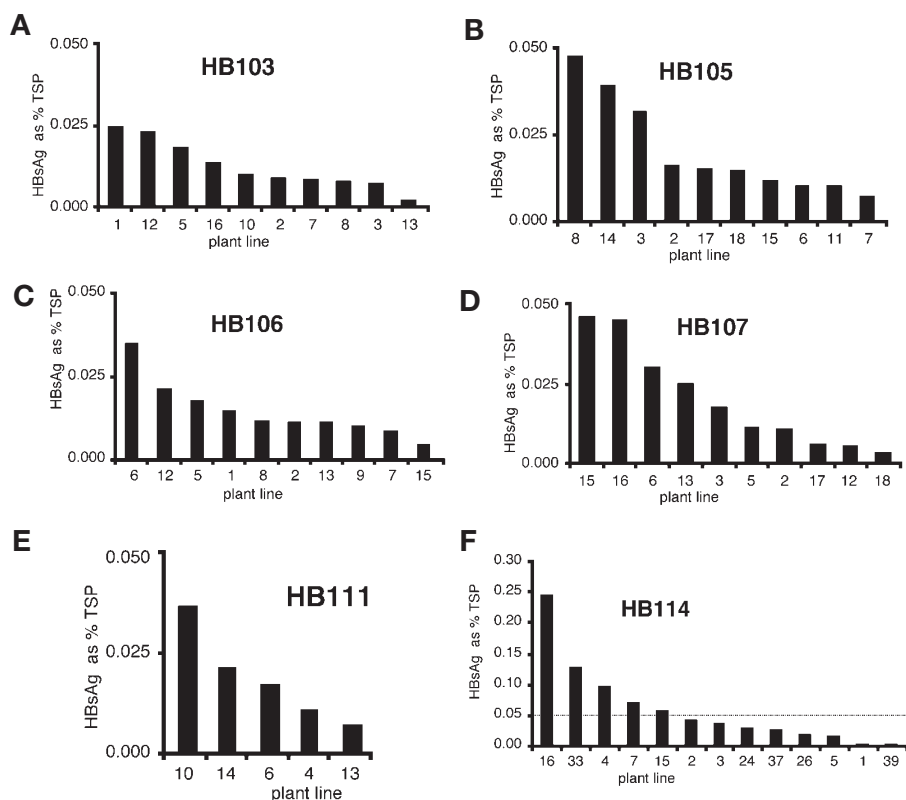


Figure 4. HBsAg expression (% TSP) in leaves of potato lines transgenic for various constructs. Numbers indicate independent transgenic lines for each construct. (A) HB103 (B) HB105 (C) HB106 (D) HB107 (E) HB111 (F) HB114. Note that the ordinate scale in (F) is different from the other plots; dashed line indicates 0.05% TSP, which is the top of the scale for the other plots.

boxylase-oxygenase (Rubisco)²⁵. We hypothesized that this construct might allow HBsAg insertion into the outer membrane of the plastid. Plants containing this construct accumulated mRNA, as seen in Figure 5, but no measurable HBsAg. As expected, the HBsAg mRNA for HB110 lines was larger than that of the other constructs by the additional 0.27 kb encoding the transit peptide. It is possible that the transit peptide was either poorly utilized or inefficiently cleaved in HB110 plants, resulting in misfolded subunits that were rapidly degraded. Alternatively, integration into the outer membrane of the plastid may result in subunits unable to form cross-links that are then degraded. This result suggests that proper HBsAg subunit folding and stable disulfide cross-linking requires ER insertion, and that simply being targeted to any membrane does not suffice.

Conclusions. In this study, we answered two major questions that relate to use of transgenic plants for oral hepatitis B vaccines. First, we showed that an antigen from a non-enteric human pathogen (HBV) could be orally immunogenic when produced and delivered in plant tissue without any process technology. Second, we identified strategies to increase the HBsAg content of plant tissues. The resulting information opens the opportunity for future preclinical and clinical studies of HBsAg as an oral vaccine, with more detailed focus on communication between humoral and mucosal branches of the immune system, which may be very important for the commercial development of plant-based vaccines. Combinations of oral and parenteral delivery may eventually prove to be most efficient, and thus should be studied. Using transgenic potato plants developed as part of the current study, we are continuing evaluation of oral immunogenicity in mice. Although these studies are not yet complete, they suggest that increased protein levels correspond to a stronger and more prolonged immune response in both primary immunization and boosting regimes.

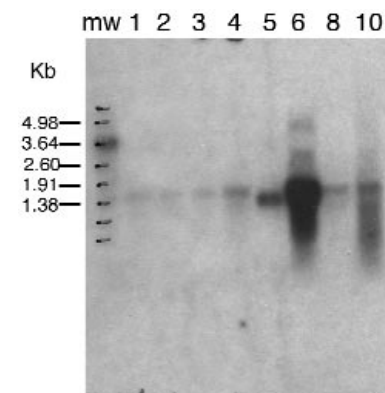


Figure 5. RNA from leaves of potatoes transgenic for pHB110 analyzed by northern blot hybridized with HBsAg coding sequence. Numbers at left indicate size in kilobases of RNA size markers. Lane mw, RNA size markers. Lane numbers indicate individual transgenic lines. Lines 3, 4, 5, 6, and 10 did not produce measurable HBsAg protein. Lines 1, 2, and 8 were not assayed for HBsAg protein.

Experimental protocol

Mouse feeding experiment. Tubers of transgenic potato line PAT-HB-7 expressing HBsAg at 1.1 µg/g fresh tuber weight were used to feed Balb/C mice that were fasted overnight before feeding peeled, cubed raw tuber. Ten mice for each experiment were fed either PAT-HB-7 tuber or control non-transgenic tuber plus 10 µg CT (Sigma, St. Louis, MO) per mouse per feeding, once per week for three consecutive weeks. Each mouse was housed separately for the feedings, and the amount of tuber weighed before and after feeding to monitor the amount ingested. Tuber tissue was offered until all of it was consumed or 24 h later, whichever was earlier. Mice were boosted intraperitoneally with 0.5 µg yeast-derived vaccine (Recombivax; Merck Research Laboratories, West Point PA). Serum was analyzed using the AUSAB Kit (Abbott Diagnostics, Abbott Park, IL) with the AUSAB quantitative panel standards for conversion of optical density values to mIU/ml.

Construction of plasmids. pHB101 and pHB102, containing the gene encoding the HBsAg S-protein, are described¹⁰. pPAT-HB was made by ligation of the 2.2 kb patatin promoter obtained by *HindIII/BamHI* digestion of pPS20A-G (ref. 13) into pHB101. pHB103 was made by first inserting the HBsAg gene from plasmid pKSHBs (ref. 10) amplified by polymerase chain reaction (PCR) with the oligos HBNco (5'-CATGCCATGGAGAACACAA-CATCAGG-3') and HBSac (5'-GCCGGAGCTCAAATGTATACCCCAAGACA-3') into pIBT210.1 (ref. 26) to make pHB201. pIBT211.1 was made by ligation of the NOS terminator fragment from pBI101 (Clontech, Palo Alto, CA; ref. 27) into *SacI/EcoRI*-digested pIBT210.1. The *XhoI/SacI* fragment from pHB201 was subcloned into pIBT211.1 to form pHB203. The *HindIII/EcoRI* fragment from pHB203 was subcloned into pBI101 to make pHB103. pHB104 was obtained by subcloning the *SacI/EcoRI* fragment from pHB201 into pHB103. To make pHB105 an intermediate clone was constructed by a three-fragment ligation consisting of the *XhoI/AclI* fragment from pHB201, the *AclI/SacI* fragment obtained by annealing two oligonucleotides (aSEKDELS-F, 5'-ATACTCTGAGAAAGATGAGCTATGAGAGCT-3' and aSEKDELS-R, 5'-CTCATAGCTCATCTTTCTCAGAGT-3'), and the *XhoI/SacI* fragment of pBluescript SK+ (Stratagene, La Jolla, CA). The resulting pHB305 was subcloned as described above for pHB103 to give pHB105.

pHB106 and pHB107 were made by first subcloning the *XhoI/SacI* fragment from pHB201 into pBluescript SK+ to create pHB303. pHB303 was digested with *NcoI* and ligated with the 67 bp *NcoI* fragment from pVSP-alpha-Sig-GUS (ref. 19) to make pHB306, or the 106 bp *NcoI* fragment from pVSP-alpha-Leader-GUS (ref. 19) to make pHB307, which were subcloned as described above for pHB103 to give pHB106 and pHB107. pHB110 was made by PCR of pUC-TPSS; ref. 25) with primers 5'-TPSS (5'-GGATC-CATGGCTTCTATGATACTT-3') and 3'-TPSS (5'-GGATCCATG-GAATCTCTGGTCAATGGTGG-3') and insertion into pHB303 after digestion with *NcoI*. The resulting pHB310 was subcloned by inserting the *XhoI/SacI* fragment into pBT211.1, and then the *HindIII/EcoRI* fragment was subcloned into pGPTV-Kan²⁸ to give pHB110. pHB111 was made by first subcloning the *EcoRV/XhoI* fragment from pBSG660 (Biosource Technologies, Inc., Vacaville, CA) into pHB203 to make pHB211.1, which was digested with *XhoI* and *NcoI*, followed by mung bean nuclease and ligation. The resulting pHB211 subcloned by inserting the *HindIII/EcoRI* fragment into pGPTV-Kan to give pHB111. pHB114 contains the 400 bp potato *pin2* terminator²⁹ subcloned from pDP687 (Pioneer Hi-Bred International, Johnston, IA) into *SacI/EcoRI*-digested pHB103.

Plant transformations and growth of tubers. These were performed as described³⁰. Putative PAT-HB transformants were subcultured on microtuberization media¹³.

Northern blots. These were performed as described³⁰. Nylon membranes were stained with 0.4% methylene blue/0.03 M sodium acetate, pH 5.2, and scanned with a Microtek ScanMaker III (Redondo Beach, CA). NIH Image (developed at NIH, available at <http://rsb.info.nih.gov/nih-image/>) was used to quantify the RNA within each 25S rRNA as shown within the box in Figure 3C. The ³²P-labeled random-primed probe used a 0.7 kb *NcoI/SacI* fragment of pHB201 containing the HBsAg gene. The washed blots were quantified using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager or by exposing to Kodak XAR film and scanning as above for stained blots.

Protein extractions and enzyme-linked immunosorbent assay. Tissue culture leaves or microtubers were homogenized with a conical tissue grinder (Bel-Art Products, Pequannock, NJ) in 1.5 ml Eppendorf tubes with cold extraction buffer containing phosphate-buffered saline (PBS), 10 mM EDTA, 0.1% Triton X-100, 5 mg/ml sodium ascorbate, and 10 µg/ml leupeptin. Soil-grown tubers were sampled as described³⁰ and extracted in ice-cold buffer (PBS, 10 mM EDTA, 0.1% Triton X-100, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride). HBsAg in the 12,000 g supernatants was quantified using the Auszyme Monoclonal kit (Abbott Diagnostics) with human serum-derived HBsAg as standard. Total soluble protein was quantified using the BioRad assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

Acknowledgments

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