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Human hepatitis B virus (HBV) causes a communicable disease that spreads worldwide and has brought about considerable economic losses due to human mortality and morbidity. HBV fails to reproduce in both cell cultures and laboratory animals; however, it is known that excess virion surface protein named hepatitis B surface antigen (HBsAg) is produced during viral replication and circulates in the blood of carriers as noninfectious particles of 22-nm diameter. It had been shown that purified HBsAg particles induce an efficient systemic immune response after injection. Consequently, subunit HBV vaccines based on HBsAg synthesized in yeasts or mammalian cell culture are currently used. Taking into account that hepatitis B is a sexually transmitted disease, development of a mucosal HBV vaccine would be beneficial. In this article, we analyze the data on development of plant-based HBV vaccines.

**KEYWORDS:** edible vaccine • hepatitis B virus • plant virus • protective antigen • transgenic plant • vaccine

The etiology of ‘serum hepatitis’, as it was known for many years, was not elucidated until the 1960s. This human disease is caused by infection with hepatitis B virus (HBV) and is a global problem in public healthcare. It is one of the major killer diseases of mankind. According to the WHO estimates, more then 2 billion people worldwide have evidence of a past or a current HBV infection. The annual rate of HBV infection is approximately 4.5 million. HBV is a blood-borne pathogen also found in other body fluids, such as semen, vaginal secretions and saliva. The host range of HBV is limited to humans and chimpanzees. The virus is maintained in the human population mainly via transmission by exposure to contaminated needles or instruments, blood transfusion or sexual contact (mucosal route). The number of chronic HBV carriers in the world amounts to approximately 350 million. The risk of hepatocellular carcinoma development in chronic HBV carriers is 100–200-fold higher compared with uninfected individuals. The annual HBV-caused mortality is approximately 1 million cases [1–3]. This determines a high demand for a vaccine against HBV [4]. The complexity of HBV vaccine production is due to the impossibility of efficiently growing HBV in a cell culture or laboratory animals.

Human HBV is a small hepatotropic and highly infectious DNA virus from the family Hepadnaviridae. The genomic DNA of HBV is packaged into an icosahedral capsid approximately 30 nm in diameter, encompassed by a lipid bilayer where three surface (envelope) proteins are anchored (Figure 1A). In 1979, the HBV genomic DNA was sequenced and the virus genes were localized. HBV virions contain a circular, partially double-stranded DNA molecule (Figure 1B). The virus DNA comprises a long strand with a constant length of 3.2 kb in all molecules and a short strand which varies in different molecules in the range of 1.7–2.8 kb [5,6].

Production of the HBV virion surface proteins is controlled by one virus gene, env, which has three start codons. Thus, the HBV virion envelope contains three proteins – small (S; hepatitis B surface antigen [HBsAg], 226 residues), medium (M; preS2–HBsAg, 281 residues) and large (L; preS1–preS2–HBsAg, 389 residues) (Figure 1) [5,7]. The HBV S antigen is the major component of the virion surface. It exists in two main forms, glycosylated (gp27) and nonglycosylated (p24). Excess surface antigen is produced during viral replication and circulates in the blood of carriers as spheres or rods of 22-nm diameter that are of structured lipid and protein composition and are called HBsAg particles. The L and M forms are minor components of the structures formed by the S protein. The M antigen carries 55 additional amino acid residues at its N-terminal (preS2) and exists as mono- (gp33) and di-glycosylated (gp36) forms. The L antigen
contains an additional 108 or 119 amino acid residues of the preS1 sequence, and exists as glycosylated (gp42) and nonglycosylated (p39) forms [3].

It has been found that purified, noninfectious HBsAg particles are highly immunogenic and determine protection of humans from HBV infection [8]. HBsAg contains protective antigenic determinants, present in all the known HBV isolates [3,4] and, therefore, is appropriate for mass vaccination against this virus.

The first HBV vaccine approved for human use in 1981 contained multisubunit HBsAg particles purified from the blood plasma of chronically infected individuals [9,10]. This vaccine was inaccessible for mass population vaccination. Consequently, the advent of genetic engineering initiated numerous attempts to create subunit HBsAg-based vaccines in various gene-cloning systems.

Once the HBsAg coding sequence was determined, various laboratories attempted to construct bacterial producer strains for this virus antigen; however, these attempts were unsuccessful. Presumably, this is caused by toxicity of this virus protein to bacteria. The successful attempts were when the HBsAg gene was expressed in yeast cells or mammalian cell cultures [3].

The currently available hepatitis B vaccine approved for human use is the HBsAg produced by recombinant yeasts or Chinese hamster ovary (CHO) cells. The immunization course of three injections protects over 95% of infants and 90% of adults [11]. However, several factors limit a successful global administration of this vaccine. First, the price of this vaccine is still too high for developing countries. Second, the administration according to a certain scheme requires many sufficiently trained personnel. Third, a small proportion of the vaccinee population remains unprotected from HBV. Thus, new variants of anti-HBV vaccine that would be effective, safe and simple in administration are warranted. A mucosal vaccine could be such an alternative.

The experimental data accumulated so far suggest that mucosal vaccines could be effective in instances where the vaccine antigens are able to form multimeric particles, which are able to absorb on mucosal surfaces and to efficiently stimulate the development of immune responses [12]. To a considerable degree, HBsAg matches these criteria.

One of the newest approaches to development of oral (mucosal) vaccines is in the creation of transgenic plants that produce protective antigenic proteins of infectious agents [13–15]. Plant cell walls provide efficient protection of the enclosed antigen after ingestion and when passing through the stomach with its acid pH. Therefore, the antigen ‘packed’ in a plant cell effectively reaches the intestines, where it is presented to the mucosal immune system. An important specific feature of such oral (edible) vaccines is their potential inexpensiveness [15]. When transgenic plants are used as edible vaccines, no isolation and purification of the target protein is required, which decreases the price of immunogenic preparation. Other advantages of the edible vaccines based on...
transgenic plants are their biological safety (the absence of viral and other human pathogens in plants), simple storage, and simple and safe administration (needle-free immunization).

**Immunogenicity of the HBsAg produced in transgenic plants**

The concept of vaccine production in transgenic plants was first formulated in 1992 [16]. In this work, the recombinant HBsAg was isolated from transgenic tobacco plants by immunoaffinity chromatography and examined using an electron microscope. It was found that the HBsAg produced in plants was able to assemble into virus-like particles with a size of approximately 22 nm and to interact with monoclonal antibodies to the HBsAg. In 1995, the HBsAg isolated from transgenic tobacco plants was used for injection immunization of mice; it was found that the virus protein synthesized in plants stimulated a specific immune response similar to that induced by the commercial genetically engineered yeast subunit HBV vaccine [17]. This study demonstrated feasibility of constructing transgenic plants for production of recombinant virus proteins displaying a normal immunogenic activity.

Attempts to use leaves of the transgenic tobacco plant to produce HBsAg as an oral vaccine in a mouse model have demonstrated that low, but the most robust, antibody response was obtained at the lowest antigen and plant-tissue dose of this toxic plant [18]. These effects could be caused by oral tolerance to the target antigen or by a toxic effect of the secondary metabolites of this plant. Therefore, the development of oral vaccines on the basis of edible plants could be more productive.

In 1999, the first variants of edible immunogens against HBV were created based on transgenic lupine and lettuce plants [19]. Oral immunization of mice with the transgenic lupine induced antibodies specific to HBsAg. Specific humoral immune response to this virus protein also developed in the volunteers who ate the transgenic lettuce; however, in these reports, protective levels of immune responses were not quantified.

Transgenic potato producing HBsAg was then constructed; when these raw potato tubers were fed to mice, together with the choler toxin B subunit (CTB) as a mucosal adjuvant, the animals developed a specific immune response to HBV [20]. Raw transgenic potato tubers without adjuvant were used in experiments with volunteers immunized earlier by injection of a subunit vaccine against HBV. After eating the raw transgenic potato tubers twice (100 g each time), a considerable growth in the serum antibodies to HBsAg was observed in 52.9% of volunteers; and after triple oral immunization, in 62.5% [21]. Oral immunization of mice previously immunized by injection with recombinant HBsAg using transgenic cherry tomatillos expressing HBsAg boosted the immune response to HBsAg [22].

Thus, a promising application of edible plant vaccines can be an easy and inexpensive revaccination of the population previously vaccinated against a certain infection with another format of the vaccine.

It had been discovered that the antibodies to synthetic preS2 peptides are capable of neutralizing HBV [23,24]. A receptor binding site for hepatocytes was found within amino acid residues 21–47 of the preS1 sequence. Antibodies to a synthetic preS1 (21–47 amino acid) peptide were able to neutralize HBV by blocking the virus attachment to cells and to protect chimpanzees from HBV infection [25]. Therefore, it was assumed that improvement of the HBV vaccine involving the recombinant surface antigen HBsAg could be caused by incorporation of additional env gene regions, preS1 and preS2, into the expression cassette [26–28]. Mammalian cell-derived recombinant HBV vaccine containing preS2 and preS1 antigens shown safety and immunogenicity in children at a reduced dose in comparison with regular HBsAg vaccine [29] and in nonresponders and low responders to conventional vaccine [28].

It was proposed that a plant vaccine based on M (preS2-S) antigen could be more immunogenic as compared with the classical vaccine involving S antigen. Transgenic potato plants producing the HBV M antigen were constructed, and their oral immunogenicity was demonstrated in experiments with mice [10]. Animals were fed the potato tubers together with CTB as an adjuvant. However, the level of specific antibody production was rather low. Only subsequent boosting with the injection of recombinant yeast HBsAg led to a determined activation of the antibodies to the virus antigen. In these studies, the immunogenic properties of the HBV S and M antigens in oral administration within edible vaccines have not been compared.

Recently, transgenic carrot plants producing the S or M antigen of HBV were created [30]. An advantage of carrot is that it requires no thermal processing before consumption by humans. Raw storage roots of these transgenic carrot plants were used without any adjuvant for oral immunization of Balb/C mice. The animals were immunized by three feeding with 2-week intervals between each feeding. Analysis of mouse peripheral blood mononuclear cells starting from day 14 of the experiment demonstrated the induction of an efficient HBsAg-specific T-cell-mediated immune response in all the animals fed the transgenic carrot, producing either HBV S or M antigen. However, the antibodies to HBsAg in blood plasma were only detectable in 11% of the animals immunized with the S antigen-producing carrot and were undetectable in the case of the M antigen-producing carrot. Antibodies to HBsAg were detected in the intestines of animals in the groups that received the carrots producing S antigen (28%) and M antigen (11%). No antibodies to HBsAg were found in any blood or intestine samples of the control animals fed the initial carrot or the carrot with inserted vector T-DNA. Note that both the systemic and intestinal/mucosal HBsAg-specific antibody responses were considerably more pronounced in the case of the plants producing HBV S antigen. Presumably, this results from a lower stability of the M antigen as compared with the S antigen in the case of transgenic carrot oral administration. It was found earlier that the HBV M antigen was more sensitive to the action of several proteases compared with the S antigen [31]. Apparently, it is necessary to introduce amino acid substitutions into the potential proteolysis sites within the natural sequence of the HBV M antigen.

Rice transgenic plants producing the recombinant protein SS1, comprising HBsAg lacking three amino acid residues at the C-terminus and fused with the preS1 (21–47 amino acid) sequence,
were created [32]. The SS1 protein was produced in seeds of transgenic rice and formed virus-like particles characteristic of HBsAg. A triple (with 2-week intervals) intraperitoneal immunization of mice with the preparation of total protein from transgenic rice seeds in the presence of Freund’s adjuvant induced preSI- and HBsAg-specific antibodies in the blood of experimental animals. The authors believed that this rice-derived SS1 protein could be a promising candidate as an alternative HBV vaccine for preventing hepatitis B.

**Candidate bivalent vaccines against HBV & HIV**

Hepatitis B virus and HIV-1 infect humans by similar routes. Frequently, HIV-1-seropositive individuals are also HBV positive. Hence, an efficient HBV–HIV-1 bivalent vaccine would be useful, especially for developing countries [33].

Unlike HBV, where the immune response towards HBsAg alone is sufficient for protection, HIV-1 protection requires an immune response to a wide panel of viral proteins or peptides representing relevant immunogenic epitopes selected from the major antigenic viral proteins. Shchelkunov et al. constructed transgenic tomato plants producing the chimeric protein T- and B-cellular immunogen (TBI)–HBsAg [34]. This protein contains the sequence of an artificial polypeptide composed of nine immunogenic epitopes of the HIV-1 Env and Gag proteins (TBI). TBI was fused with the HBsAg at its N-terminus. In the first experiments, mice were fed raw transgenic tomato fruits four-times with 1-week intervals, and induction of the antibodies specific to HBsAg and HIV-1 were detected in the blood and feces of these animals [35]. Furthermore, when studying the potential of these transgenic tomato fruits as an edible vaccine, it was decided to use freeze-dried and powdered fruits, as it provided for a long-term preservation and standardization of the preparations [36,37]. Before feeding animals, the powder was suspended in water and the resulting paste was administered three-times with 2-week intervals. The mucosal antibodies to HBV and HIV-1 were detectable in the feces of experimental animals 2 weeks after the first feeding and for the remaining period of the experiment (56 days). Synthesis of the serum antibodies to both viruses was initiated only after the second feeding and was stably maintained over the observation period. Intraperitoneal injection of the DNA vaccine directing synthesis of the same protein, TBI–HBsAg, boosted the antibody response to HIV-1 in the blood serum; however, it had no effect on the high level of antibodies to HBV achieved. Thus, a candidate transgenic tomato-based edible vaccine against both HBV and HIV-1, the viruses of a global healthcare concern, was obtained for the first time.

A similar approach to creation of a bivalent HBV–HIV-1 vaccine was used by other researchers, who constructed transgenic Nicotiana tabacum and Arabidopsis thaliana plants producing chimeric proteins composed of a polypeptide sequence of HIV-1 Gag and Pol antigens fused with HBsAg [33]. It was demonstrated that the chimeric proteins synthesized in these plants were able to form virus-like particles. The HSB mice previously intramuscularly immunized with a polypeptide HIV-1 DNA vaccine and fed the powder of freeze-dried transgenic tobacco leaves displayed an activation of anti-HIV-1 specific CD8+ T cells in mesenteric lymph nodes that drain the mucosa lymphoid tissue.

**Increase in the immunogenicity of plant HBsAg-based vaccines**

The experimental data accumulated so far suggest that mucosal vaccines can be efficient when their antigens resemble active mucosal pathogens (i.e., they must be multimeric and/or arranged in supramolecular particles) and are able to absorb on mucosal surfaces [12]. HBsAg, to a considerable degree, matches these criteria. Different research groups using various plants have demonstrated that both an individual HBsAg and its chimeric variants with additional amino acid sequences attached can assemble into supramolecular structures [32,33].

A more pronounced immunogenic effect of edible vaccines is attainable when using mucosal adjuvants, such as heat-labile enterotoxin B subunit, CTB or saponins [11,38]. Note that the only licensed adjuvant used so far in medical practice is aluminum hydroxide, weakly stimulating the cell-mediated immune response. Saponins are the most attractive as nontoxic mucosal adjuvants, because these compounds are of plant origin and used in food the industry. Of interest among the edible plants are tomatoes, whose fruits contain a saponin, tomatin [39]. Presumably, the presence of saponin is the particular factor that provided for an efficient immune response to HBsAg in the animals fed with transgenic tomatoes [36,37].

**Increasing HBsAg production in plants**

The studies performed so far demonstrate that transgenic plants produce HBsAg at a rather low level; correspondingly, their oral administration induces a low level immune response to the target antigen in the majority of cases (Table 1). To increase the efficiency of edible vaccines, it is necessary to solve the problem of how to elevate the production of the target antigen in the developed transgenic plants [40,41].

Among the promising approaches to increasing the yield of the target protein is fusing recombinant proteins with signal sequences, which provide for their transport to the cell compartments with a decreased activity of proteolytic enzymes, for example, lumens of the endoplasmic reticulum (ER) [42] or vacuoles [43,44].

The accumulation level of the virus protein preS2–S (M antigen) was recently estimated in the leaves of two groups of carrot plants, in one of which the studied M antigen was fused with amino acid sequences that determined its transportation into ER lumens (sigER-preS2–HBsAg-HDEL) and in the other group, the antigen lacked such signals (i.e., the M antigen was destined for synthesis and diffuse distribution in the cell cytoplasm) [45]. Analysis of leaves of the transgenic carrot plants from both groups demonstrated a wide interindividual variation in the level of target antigen (HBsAg) production (2–45 ng/g of fresh weight). It was demonstrated that the presence of the signal sequences directing the transport of M antigen to the ER lumens correlated with an increase in the rate of individual transgenic plants, with a relatively high accumulation (more than 20 ng/g) of the target protein (43.6%) as compared with the plants displaying a diffuse distribution of M antigen in the cell cytoplasm (18.5%).
### Table 1. Examples of candidate hepatitis B virus plant-based vaccines.

<table>
<thead>
<tr>
<th>Expressed protein or peptide</th>
<th>Plant expression system†</th>
<th>Maximal expression level in plants</th>
<th>Vaccine type</th>
<th>Immunization pathway</th>
<th>Immunized subjects</th>
<th>Immunogenicity of vaccine</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>HBV</strong></td>
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<tr>
<td>Major virion surface protein (HBsAg, S antigen)</td>
<td>Tobacco</td>
<td>70 ng/mg TSP</td>
<td>Protein fraction containing 3% HBsAg</td>
<td>Intraperitoneal injection</td>
<td>Mice</td>
<td>Specific serum antibodies and T-cell-mediated response</td>
<td>[17]</td>
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<tr>
<td>Lupine</td>
<td>150 ng/g FW</td>
<td>Callus tissue</td>
<td>Feeding</td>
<td>Mice</td>
<td>Specific serum antibodies</td>
<td>[19]</td>
<td></td>
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<tr>
<td>Lettuce</td>
<td>6 ng/g FW</td>
<td>Leaves</td>
<td>Feeding</td>
<td>Human volunteers</td>
<td>Specific serum antibodies</td>
<td>[19]</td>
<td></td>
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<tr>
<td>Potato</td>
<td>8 µg/g FW</td>
<td>Raw tubers with applied mucosal adjuvant (CT)</td>
<td>Feeding</td>
<td>Mice</td>
<td>Specific serum antibodies</td>
<td>[20]</td>
<td></td>
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<tr>
<td>Cherry tomatillo</td>
<td>10 ng/g FW</td>
<td>Raw fruits</td>
<td>Feeding</td>
<td>Mice</td>
<td>Increase in the titer of specific serum antibodies in the animals previously once injected with HBsAg preparation</td>
<td>[22]</td>
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<tr>
<td>Potato</td>
<td>8 µg/g FW</td>
<td>Raw tubers</td>
<td>Feeding</td>
<td>Human volunteers</td>
<td>Increase in the titer of specific serum antibodies in 53–62% volunteers previously vaccinated against HBV</td>
<td>[21]</td>
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<tr>
<td>Carrot</td>
<td>20 ng/g FW</td>
<td>Raw storage roots</td>
<td>Feeding</td>
<td>Mice</td>
<td>HBsAg-specific T-cell-mediated response and antibodies in serum and intestines</td>
<td>[30]</td>
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<td>Tobacco</td>
<td>103 µg/g DW</td>
<td>Freeze-dried leaves</td>
<td>Gavage</td>
<td>Mice</td>
<td>HBsAg-specific IgAs and IgGs</td>
<td>[18]</td>
<td></td>
</tr>
<tr>
<td>Tobacco mosaic virus vector/ tobacco</td>
<td>295 µg/g FW</td>
<td>HBsAg particles partially purified in sucrose gradient</td>
<td>Intraperitoneal injection</td>
<td>Mice</td>
<td>Specific serum antibodies</td>
<td>[40]</td>
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<tr>
<td>preS2–HBsAg (M antigen)</td>
<td>Potato</td>
<td>900 ng/mg TSP</td>
<td>Raw tuber with mucosal adjuvant CT</td>
<td>Feeding</td>
<td>Mice</td>
<td>Serum and fecal anti-S and anti-preS2 IgGs after boosting with yeast-derived HBsAg</td>
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<td>Carrot</td>
<td>28 ng/g FW</td>
<td>Raw storage roots</td>
<td>Feeding</td>
<td>Mice</td>
<td>HBsAg-specific T-cell-mediated response and antibodies in intestines</td>
<td>[30]</td>
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†Transgenic plants if not stated otherwise.

CT: Cholera toxin; DW: Dry weight; EIA: Enzyme immunoassay; FW: Fresh weight; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; TBI: T- and B-cellular immunogen; TSP: Total soluble protein.
Table 1. Examples of candidate hepatitis B virus plant-based vaccines (cont.).

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<tr>
<td><strong>HBV (cont.)</strong></td>
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<tr>
<td>HBsAg (1–223 amino acids)– preS1 (21–47 amino acids), recombinant protein SS1</td>
<td>Rice</td>
<td>32 ng/g DW</td>
<td>Total protein of freeze-dried seeds</td>
<td>Intraperitoneal injection</td>
<td>Mice</td>
<td>Serum anti-S and anti-preS1 antibodies</td>
<td>[32]</td>
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<tr>
<td><strong>HBV and HIV-1</strong></td>
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<tr>
<td>Chimeric protein TBI–HBsAg</td>
<td>Tomato</td>
<td>300 ng/g DW (according to HBsAg EIA)</td>
<td>Freeze-dried fruits</td>
<td>Feeding</td>
<td>Mice</td>
<td>Serum and mucosal antibodies to HBV and HIV</td>
<td>[35–37]</td>
</tr>
<tr>
<td>Chimeric protein preS2–polHIV– HBsAg</td>
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<td>Mice after polHIV DNA vaccination</td>
<td>Activation of anti-HIV-1 specific CD8+ T cells</td>
<td>[33–44]</td>
</tr>
</tbody>
</table>

*Transgenic plants if not stated otherwise.

CT: Cholera toxin; DW: Dry weight; EIA: Enzyme immunoassay; FW: Fresh weight; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; TBI: T- and B-cellular immunogen; TSP: Total soluble protein.

It is also possible to elevate the level of synthesis of a foreign protein by increasing the gene dose; however, multiple repeats are unstable in chromosomal DNA. This hindrance can be bypassed using a transgenic (transplastomic) system of plastids (chloroplasts).

Plastids are polyploid and are present in large amounts in various plant organs and tissues. Therefore, insertion of a single copy of a transgene into plastid DNA can, via selection of transplastomic plant, eventually lead to an increase in the dose of a foreign gene of 1000–10,000 copies per cell. The experiments on obtaining transplastomic tobacco plants, performed in different laboratories, have demonstrated that it is possible to elevate the production of target foreign protein to 1–25% of the total soluble plant protein [46]. This multifold exceeds the levels of synthesis of recombinant proteins in transgenic plants (Table 1). An important specific feature of plastids is that they are inherited in a maternal manner and are usually absent in pollen. Therefore, the transplastomic plants are considerably safer for the environment compared with common transgenic plants, because an uncontrolled spread of the transgene into other plants is prevented. The transformation of potato, tomato, carrot and other plant plastids have been reported recently [35,47,48].

When using plants as alternative expression systems for accumulation of antigens, the work is also directed towards an increase in the yield of recombinant protein in plant tissues [40,47,49]. In this case, the transient expression system using plant virus-based vectors have the advantage of rapid high-level expression of the recombinant protein as compared with stable transgenic plants. Recent work has demonstrated that tobacco leaves infiltrated with MagnICON viral vectors produced HBsAg at a high level (~300 ng/g fresh leaf weight) on day 10 after infection [40], which exceeds the best results achieved for transgenic plants by several times (Table 1). It was demonstrated that transiently expressed HBsAg was correctly assembled and was immunogenic in intraperitoneally injected mice.

**Expert commentary**

The subunit HBsAg vaccine produced in yeasts or mammalian cells is an injection vaccine and, therefore, it does not induce the mucosal immunity against HBV, which is important for protection against sexual transmission of this virus. Plant-based mucosal HBV vaccines can be a useful supplement to the currently used recombinant injection vaccines as an additional mucosal vaccine expanding the range of protective responses in vaccinees. Moreover, a plant oral vaccine is potentially appropriate for simple and safe revaccination of humans [21,22].

Since HBsAg produced in an adequate expression system is able to self-assemble into supramolecular structures with high immunogenic potential that provide protection from HBV infection, the first genetically engineered viral vaccine was designed using this protein, produced in yeasts. When developing candidate vaccines based on transgenic plants, the majority of research projects have utilized the HBsAg gene. It has been proven that the HBsAg synthesized in plants forms virus-like particles displaying high immunogenic activity. Glycoforms of the plant-produced HBsAg have not yet been studied.

An important feature of some plants is that their secondary metabolites can play the role of mucosal adjuvants and, thereby, enhance the immunogenic properties of the corresponding edible vaccines.

One of the problems of oral plant vaccines is variability of target antigen production in individual transgenic plants. Therefore, freeze-dried and powdered plants, or their edible parts could be used for long-term preservation and standardization of the antigenic preparations.

Another problem in using edible plant vaccines could be the induction of oral tolerance to the target antigen. This question should be deeply investigated in any case of plant-based vaccine. Furthermore, possible allergy to edible plants themselves once associated to target immunogenic antigen should be experimentally excluded.
A recent development of a highly efficient plant virus-based vector systems allows plants to be considered as an alternative for the existing systems (i.e., yeast or mammalian cells) of large-scale HBsAg production and the use of plant-derived purified protein for classical injection vaccination.

Five-year view

An important research direction is creation of oral HBV vaccines using the plants consumable without thermal processing (e.g., tomatoes, carrot, bananas or lettuce). For better preservation without refrigerators and standardization of the target recombinant antigen content, it is expedient to freeze-dry the fruits and storage roots of transgenic plants, grind them into powder, and pellet or encapsulate them. Before administration, such dry preparations can be suspended in water and converted into either a paste or juice. Since recombinant proteins can be stably preserved for a long time in grain and plant seeds, it is expedient to create transgenic maize, rice and other cereals producing HBsAg and its chimeric variants.

To provide a higher efficiency of edible vaccines, it is necessary to solve the problem of increasing the production level of protective HBV antigens by designing transplastomic plants. In principle, if HBsAg synthesis in plastids is not prohibited, it will be possible to design such vaccines.

To elevate the efficiency of edible plant-based vaccines, it is necessary to introduce into plants not only the target HBsAg transgene, but also the genes encoding mucosal adjuvants of a heat-labile enterotoxin B subunit or CTB type. In addition, it is important to select the edible plants producing endogenous saponins (efficient mucosal adjuvants), such as tomatoes and so on, for production of a HBV vaccine.

After successful preclinical trials, it is necessary to organize pilot production of a standard edible HBV vaccine based on a transgenic or transplastomic plant and perform extended clinical trials to test such candidate vaccine for primary immunization and/or additional mucosal immunization to a standard scheme of injection vaccination with the recombinant HBsAg. The combined use of injection and oral variants of HBV vaccine is likely to provide the best protection from this viral infection.

Upscaling of controlled process for manufacture of the HBV S, M or L antigen in the system of plant viral vectors can represent an alternative method for the manufacture of a subunit HBV vaccine, more economic as compared with the currently used yeast or mammalian cell systems.

Taking into account the ability of chimeric HBsAg proteins with attached additional amino acid sequences to self-assemble into virus-like particles, it is reasonable to continue the development of bivalent plant-based vaccines involving this HBV antigen.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- The immune response towards hepatitis B surface antigen alone is sufficient for protection from hepatitis B virus (HBV) infection.
- The HBV M and L antigens can provide additional potential in protection from HBV as components of a subunit injection or an oral plant-based vaccine.
- It is important to introduce the genes encoding mucosal adjuvants into transgenic plants used for creation of edible HBV vaccines and/or use plants containing the secondary metabolites capable of playing the role of mucosal adjuvants.
- Highly efficient systems of transplastomic plants or transient expression systems using plant virus-based vectors can be an alternative for production of HBV antigens for an inexpensive and safe subunit vaccine.
- Combined use of injection and mucosal vaccines against HBV can provide the most reliable protection against this viral infection.

References

Papers of special note have been highlighted as:
- of interest
- of particular interest


- Oral delivery of plant-derived M antigen (preS2-hepatitis B surface antigen [HBsAg]) to mice resulted in fecal anti-S and anti-preS2 as well as serum IgG.

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5. **Kapusta J, Modelska A, Figlerowicz M.** Without increasing levels of regulatory antigen-specific IgAs and IgGs in mice, doses of plant-based HBsAg induced hepatitis B virus.


- First study to describe a transgenic lupine producing HBsAg as an edible vaccine for human volunteers.


17. **Neurath AR, Seto B, Strick N.** Antibodies to synthetic peptides from the preS1 region of the hepatitis B virus (HBV) envelope (Env) protein are virus-neutralizing and protective. *Vaccine* 7, 234–236 (1989).


- Tomato fruits producing the chimeric T- and B-cellular immunogen—HBsAg were fed to experimental mice and high levels of HBV- and HIV-specific antibodies were present in the serum and feces of the test animals.


- Describes a plant viral expression system that gave the highest level of HBsAg production in plants.


• Summarizes successful examples of signal peptides used for direction of target proteins to vesicular cell compartments in transgenic plants.


• Hyperexpression of vaccine antigens or therapeutic proteins in transplastomic plants containing integrated target genes in chloroplasts.


• Comprehensive analysis of developments in using plants for production of vaccine antigens.