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(54) **Title**: Expression cassettes, T-DNA molecules, plant expression vectors, transgenic plant cells as well as the use thereof in the production of a vaccine

(57) **Abstract**: Binary vectors with an expression cassette containing S-HBs protein Ag or M-HBs Ag or L- HBs Ag or HBc Ag under the control of the constitutive 35S promoter or the organo- specyfic leg A promoter, as well as transgenic lettuce, peas and tobacco plants producing the S-HBs Ag or M-HBs Ag or L-HBs Ag or HBc Ag proteins, which facilitate the production of plant material for the production of an improved oral, parenteral or nasal vaccine against viral Hep B

Expression cassettes, T-DNA molecules, plant expression vectors, transgenic plant cells as well as the use thereof in the production of a vaccine

The present invention relates to the production of a vaccine containing the individual HBV antigens: S-HBsAg, M-HBsAg, L-HBsAg, HBcAg; directed against viral hepatitis type B (viral HepB) for oral application in the form of preserved plant material as well as parenteral or nasal application in the form of a purified preparation extracted from plant material.

The production of a recombinant vaccine against viral hepatitis type B (viral HepB) in the early 80s of the 20th century was a considerable success of medicine and vaccine science, since it made mass vaccinations against viral HepB possible. Despite the large, 90 to 95%, effectiveness of the vaccine the global number of the chronically ill due to HBV infection increases annually and currently exceeds 400 million. It is estimated that close to 100 million of these patients can die due to diseases and other complications, such as cirrhosis or liver cancer. At the same time, the HBV chronic carrier population constitutes a large reservoir for fresh infections. Epidemiological data show that close to one third of the global population has undergone infection with the type B hepatitis virus. And although in developed nations the incidence of type B hepatitis is rather low, less than 0.5%, in developing nations and China, the incidence indicator is from 5 to 50% (Hollinger 1996, Young 2001). The occurrence of viral hepatitis B has been and continues to be a serious epidemiological problem in developed nations as well, where up to 20% of the population exhibits markers indicating infection with HBV. In Poland, over 50% of virus-borne liver diseases, are caused by HBV (Walewska-Zielecka 1996).

The number of chronically ill and carriers of HBV is on the rise, despite the introduction of recombinant vaccines (for example Engerix-B®, Euvax B®) based on the small, S, surface antigen (HBsAg, Hepatitis B surface Antigen). Briefly, the so-called small surface antigen of the virus, S-HBsAg, is produced in yeast cells (rHBsAg.). Although these vaccines have made mass vaccinations possible, they still do not ensure adequate protection. It has been shown that infections and virus replication occurs as a result of the appearance of mutated HBV strains in a number of persons who have been vaccinated. These strains are characterized by an altered amino-acid sequence within the "a" epitope of the S-HBs antigen, which is a neutralizing factor for all virus subtypes described so far. It's thought that mutated epitope "a" variants appear as a result of the selective pressure exerted by the antibodies resulting from the vaccination, against which the vaccinated organism does not produce antibodies. A chronic illness develops as a result of the lack of adequate immunological protection, despite the previous response in excess of 10 mIU/ml anti-HBV

antibodies, a level which in most nations is thought to be sufficient to impart immunological protection against viral infection (Cooreman 2001, Huang 2004).

Furthermore, a number of patients fail to mount an immune response following the parenteral application of a vaccine containing the small surface antigen S-HBsAg, or the response is insufficient. Particularly the elderly, smokers, the obese, then, persons infected with immunosuppressive viruses (i.e. HIV), or the immunodeficient due to other factors as well as patients with renal failure react significantly worse to the classical vaccine. In extreme cases, the efficacy of vaccination in these groups barely reaches several percent, despite multiple vaccinations with increased doses of the preparation (Singh 2003). As a consequence, these persons remain susceptible to the disease. In light of the above, research efforts have been conducted for some time with the aim of producing a novel vaccine, a so-called III generation vaccine against viral HepB, containing S-HBsAg and one or both of the remaining surface antigens of HBV: the medium (M-HBsAg) and large (L-HBsAg) antigens (Madaliński 2002, Yamada 2001, Young 2001). The above-mentioned antigens are highly immunogenic, which means that III generation vaccines (i.e. Hepacare®, Bio-Hep-B™) induce the production of a much larger spectrum of antiviral antibodies in relation to vaccines based solely on S-HBsAg. Clinical studies performed both on adults and children as well as neonates confirm the strong immuno stimulant properties of preparations containing the full surface antigen set of HBV. The anti-HBV titer following a double immunization with such a preparation was up to three times larger than that resulting from the triple application of the classical vaccine containing only the S-HBs antigen. Moreover, this preparation also induced a immune response in a significantly larger percentage of patients with a poorer response to HepB vaccines: the elderly, men, smokers and the obese (Young 2001). In other research, it was shown that persons who previously did not respond to a vaccine containing only the small surface antigen produced appropriate antibodies following the administration of a preparation which also contained the medium, M-HBs, and large, L-HBs, antigens. Following a single dose, almost 70% (Zuckerman 1997) of patients underwent seroconversion, whereas almost 80% did so following a double application (Yap 1996). The increased efficacy of the vaccine containing all the capsid antigens is also confirmed by research in which the use of severalfold smaller dose of such a preparation (2.5 μg) than the standard vaccination dose based on S-HBs induced the production of appropriate antibodies in 100% of vaccinated children (Madaliński 2002).

In addition to research on the production of an improved prophylactic vaccine against viral HepB, efforts are also being made to design agents for the treatment of the chronic forms

of the disease, which affects up to 10% of adults, up to 40% of children and up to 90% of neonates. Interferon therapy, currently in use, yields modestly positive effects in about 30 to 50% of patients (Juszczyk 2000, Martin-Vivaldi 2000), but a considerable percentage of patients exhibit strong side effects (Deres 2003). In light of these problems there, is much interest in the approach based on attempts to modulate the immune response using preparations containing all of the HBV surface antigens and/or the Hepatitis B core antigen. Extant attempts at the immunotherapy of chronic viral HepB performed using preparations of the S-, M-, and L-HBsAg antigens have yielded promising results, because a 100 fold increase in immunogenicity in relation to the standard vaccine has been achieved (Yuen 2001) or the induction of a humoral response against HBS antigens has been observed along with a cytotoxic response in 50% of chronic HBV carriers (Couillin in 1999, Pol 1998).

In addition to the envelope proteins, a significant role in the future therapeutic vaccine is assigned to the HBcAg core antigen of which the capsid of the hep B virus is built. The HBc antigen is a strong immunogen, both T-lymphocyte dependent and independent, and for this reason is an excellent candidate for the design of therapeutic vaccines as it induces both a strong cytotoxic as well as humoral response with anti-HBc antibodies (Heathcote 1999, Bocher 2001).

The increasing number of HBV carriers, the need for mass vaccination, as well as the insufficient efficacy of extant preparations fuel the need for the design of inexpensive and highly effective vaccines against viral HepB

Basing on extant data, one can assume that the immunogenicity of the preparation containing all the antigens of the envelope, HBsAg, as well as the core antigen, HBcAg, will be very similar to that elicited by complete HBV virions, the so-called Dane particles. Such a preparation will be a "vaccine analogue" of a complete virus and would induce a strong immunological response through eliciting antibodies against all antigens of the virus. Vaccination with this sort of preparation would cause a situation similar to immunization with a native virus, but at the same time safe because the preparation lacks genetic material and is thus noninfectious and would not cause disease symptoms. The production of a series of transgenic plants producing the full set of HBV antigens from various strains of the virus will make it possible to design an alternative vaccine of plant origin, both for prophylaxis and therapy of many strains (haplotypes, serotypes) of HBV.

The main principle behind the production of a prophylactic and therapeutic vaccine against viral HepB to the administered orally, parenterally or nasally is the expression and efficient production of highly immunogenic subunits of the surface antigen, S-HBsAg, M-HBsAg

and L-HBsAg as well as the HBc core antigen of various strains of HBV in an edible transgenic plant in the case of the prophylactic vaccine, or one characterized by rapid growth, large biomass and ease of extraction in the case of parenteral or nasally applied vaccines. Research efforts in this area have been conducted for a number of years by several research teams (see literature) throughout the world. The transgenic plants or cell/tissue cultures produced thus far has been characterized by resistance to antibiotics, particularly kanamycine. At the same time, the HBsAg protein expression level was low and for S-HBsAg oscillated from 1 to 3 μg/g fresh weight (FW) and sporadically reached a maximum of 16 μg/g FW. For M-HBsAg the range was 0.01 - 1 μg/g FW, whereas for L-HBsAg it averaged 0.01 μg/g FW. Moreover, in oral vaccination studies on animals, possibly on volunteers, only raw plant material was used.

The goal of the present invention is the production of an alternative oral, parenteral or nasal prophylactic or therapeutic, III generation vaccine against viral HepB, designed with the use transgenic plants.

In particular, the first goal of the present invention is the expression of the HBsAg and HBcAg antigens of various haplotypes in edible transgenic plants, for example in lettuce or peas, which would make it possible to design a vaccine to be applied orally, i.e. in the form of a suspension, a syrup, a granulate, a tablet or capsule. An oral vaccine in this form would indeed be easier to use on a mass scale, both as a primary vaccine and a booster vaccine for the previously immunized, in whom the anti-HBs antibody titer had decreased or in whom anti-HBs antibodies are no longer detectable.

In particular, the second goal of the present invention is the efficient expression of the HBsAg and HBcAg antigens of various haplotypes in tobacco, which is a species characterized by rapid growth and large biomass, facilitating the effective extraction and purification of the antigens as the basic components of a vaccine applied parenterally as an injection or nasally in the form of an aerosol or drops. The use of transgenic plants in the production of vaccine components would be less expensive than the currently used industrial methods of microbiological production.

Unexpectedly, the goals described above have been achieved by the present invention. The subsequent subjects of the present invention have been defined in the attached claims.

The subject of the present invention are expression cassettes comprising sequences encoding the small, medium and large subunits of the surface antigen (S-HBsAg, M-HBsAg, L-HBs e.g.) or a subunit of the core antigen (HBcAg) of HBV of the strains *awy4* or *adw4* for, as well as regulatory sequences controlling their expression, preferably the 35S RNA promoter of the cauliflower mosaic virus (CaMV) or the legumine A promoter

(*legA*) as well as the nopaline synthase terminator (NOSt). Preferably, that they contain the sequences shown as SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14 and SEQ ID No. 15.

The next subject of the present invention is a T-DNA molecule comprising two flanking T-DNA sequences and two expression cassettes located between them:

- an expression cassette comprising a sequence encoding the S-HBsAg or M-HBsAg or L-HBsAg or HBcAg proteins and regulatory sequences controlling its expression, preferably the 35S RNA promoter of CaMV with a single enhancer or the *legA* promoter and a nopaline synthase transcription terminator (NOSt),

and

- an expression cassette comprising a sequence encoding a herbicide resistance gene, preferably the *bar* gene, and regulatory sequences controlling its expression, preferably a nopaline synthase promoter sequence (PNOS) and the g7 transcription terminator, shown as a SEQ ID No. 16.

Preferably, a T-DNA sequence according to the present invention contains at least one sequence selected from among: SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16.

The next subject of the present invention are plant expression vectors containing an expression cassette according to the present invention, as defined above, preferably contained in a T-DNA molecule according to the present invention, as defined above. In an example embodiment these are the binary vectors pKHBSsdwBAR, pPLAHBSBAR, pPLAHBSadwBAR, pKHBMBAR, pKHBMadwBAR, pPLAHBMBAR, pPLAHBMadwBAR, pKHBLBAR, pKHBLadwBAR, pPLAHBLBAR, pPLAHBLadwBAR, pKHBCBAR, pKHBCadwBAR, pPLAHBCBAR, and pPLAHBCadwBAR.

The next subject of the present invention are strains of *Escherichia coli* and *Agrobacterium tumefaciens* containing expression vectors according to the present invention.

The next subject of the present invention is the use of plant expression vectors according to the present invention in the production of transgenic plants, preferably lettuce, peas and tobacco as well as the use of the vector pKHBSBAR according to invention P.382769 in the production of transgenic peas and tobacco.

The next subject of the present invention is a transgenic plant cell containing a plant

expression vector according to the present invention possessing resistance to phosphinotricine and its derivative herbicides, which is capable of expressing HBV proteins of the strains *ayw4* and *adw4*. In particular, the subject of the present invention are transgenic cells and regenerated plant progeny of the subsequent generations of lettuce, peas and tobacco, as well as the vegetative clones thereof, characterized in that as a result of transformation with one of the vectors according to the present invention they express: the small surface antigen S-HBsAg at a level greater than or equal to 5 μg/g fresh weight (FW) or the medium surface antigen M-HBsAg at a level greater than or equal to 4 μg/gram FW or the large surface antigen L-HBsAg at a level greater than or equal to 2 μg/g FW or the HBcAg core antigen at a level greater than or equal to 15 μg/g FW. The plant material from lettuce or peas can be used as an oral vaccine against HBV and, as a consequence, against viral HepB following dehydration by lyophilisation, plant material according to the present invention maintains the native structure of the antigenic proteins at room temperature as well as its immunogenicity for a period of at least 12 months and may be used in the production of an oral vaccine against viral hep B in the form of a suspension, a syrup, and granulate, tablets or capsules. The plant material originating from tobacco can be used as a raw material for the extraction and purification of HBV antigens as components of an anti-HBV vaccine and, as a consequence, against viral hep B both parenterally (as an intramuscular, intraperitoneal or subcutaneous injection), or as a nasally applied vaccine in the form of an aerosol or drops.

The next subject of the present invention is a method of vegetative replication of transgenic lettuce plants producing HBV proteins for vaccination, so as to ensure the reproducible and copious proliferation of clone plants, where in at least 75% of plants the differences in the levels of HBV vaccine proteins do not exceed 20%; and therefore these clones are characterized by a statistically balanced vaccine protein content, and furthermore, in at least 64% of the clones the HBV protein content does not change in comparison to the initial plant. This method of proliferation facilitates the industrial production of plants with a high and stable level of the individual HBsAg or HBcAg proteins as raw materials for the production of an oral vaccine.

The next subject of the present invention is the use of a lettuce or pea plant cell according to the present invention in the production of an oral vaccine against type B viral Hepatitis.

Lyophilized plant material containing the S-HBsAg antigen *ayw4* according to invention number P.382769 administered orally in the form of a suspension to experimental animals induced an immune response of the mucous membranes characterized by the production of IgA-class anti-SHBs antibodies as well as a systemic response characterized by the

production of IgA and IgG anti-SHBs antibodies. In particular, the lyophilized plant material according to the present invention administered orally to animals that had been previously orally immunized induces a boost of the immune response in the mucous membranes characterized by the production of IgA anti-SHBs antibodies as well as a boost of the systemic response characterized by the production of IgA and IgG anti-SHBs antibodies. It is, therefore, very likely that a similar immunizing effect will be achieved using plant material containing S-HBsAg *adw4* or M-HBsAg *ayw4/adw4* or L-HBsAg *ayw4/adw4* or HBcAg *ayw4/adw4*.

Preferably, the oral vaccine produced is in a form selected from among: a suspension, syrup, granulate, tablet or capsules. Preferably, the granulate, tablets and capsules are formed from pulverized lyophilisate of transgenic lettuce according to the present invention, and maintain the native structure of antigenic proteins at room temperature as well as their immunogenicity for at least 12 months.

The next subject of the present invention is the use of a tobacco plant cell according to the present invention in the production of a parenteral or nasal vaccine against viral hepatitis type B.

Based on extant knowledge, it can be safely assumed that extracted and purified HBV antigens, preferably from plant material according to the present invention, administered via a parenteral injection (intramuscular, intraperitoneal, subcutaneous) will induce a systemic immunological response characterized by the production of anti-SHBs antibodies, chiefly of the IgG class and of other classes as well, and that HBV antigens administered nasally in the form of an aerosol or droplets through the mucous membranes of the nasal cavity will an induce immunological response of the mucous membranes characterized by the production of IgA-class anti-SHBs antibodies as well as a systemic response characterized by the production of anti-SHBs antibodies, chiefly of the IgG class and of other classes as well.

In relation to the solutions known from prior art, the present invention represents significant novelty and substantially innovative approach in the production of an oral, parenteral or nasal III generation vaccine against viral HepB, comprising the following elements:

- transgenic lettuce plants producing S-HBsAg at a level in excess of 5 μg S-HBsAg/g of fresh weight (FW) and reaching 100 μg/gram FW, M-HBsAg at a level in excess of 4 μg M-HBsAg/g FW and approaching 35 μg/g FW as well as L-HBsAg at a level in excess of 2 μg L-HBsAg/g FW. and approaching 20 μg/grams FW, and not containing antibiotic resistance marker genes but resistant to the phosphinotricine herbicides, i.e. Basta, and thus

they do not increase the risk of the possible increased antibiotic resistance of microflora in the human,

- transgenic lettuce leaves producing HBcAg at a level in excess of 50 μg HBcAg/g FW and approaching 500 μg/g FW, and not containing antibiotic resistance marker genes but resistant to the phosphinotricine herbicides, i.e. Basta, and thus they do not increase the risk of the possible increased antibiotic resistance of microflora in the human,
- a method of vegetative propagation of transgenic plans producing HBV proteins for vaccination in such a way as to consistently and repeatedly obtain plant clones, and which in at least 75% of individuals the differences in the content of HBV vaccinating proteins do not exceed 20%, and furthermore in at least 64% of clones the HBV protein content does not change in comparison to the ancestral plant. The vegetative clones thus produced are characterized by a statistically stable content of vaccine proteins which facilitates the industrial production of plants with a high and stable content of the individual HBsAg or HBcAg proteins as raw materials for the production of an oral vaccine
- transgenic pea plants which produce HBV antigens in the seeds at a level of several ng/grams fresh weight as well as ones which do not contain antibiotic resistance marker genes but are resistant to phosphinotricine herbicides, i.e. Basta, whereby they do not constitute a risk of potentially increasing the antibiotic resistance of microflora in the human
- the initial production of a prototype oral vaccine in condensed, lyophilisate form or an initial component for an oral vaccine from transgenic lettuce producing the individual HBV antigens, in the form of: a lyophilisate suspension, syrup, granulate, tablets, or capsules
- transgenic tobacco plants producing: S-HBsAg at a level in excess of 20 μg S-HBsAg/g FW and reaching 200 μg/g FW, M-HBsAg at a level in excess of 4 μg M-HBsAg/g FW and approaching 35 μg/g FW as well as L-HBsAg at a level in excess of 2 μg L-HBsAg/g FW and approaching 20 μg/g FW, as well as HBcAg at a level in excess of 50 μg HBcAg/g FW and approaching 500 μg/g FW, and not containing antibiotic resistance marker genes but resistant to the phosphinotricine herbicides, i.e. Basta, and thus they do not increase the risk of the possible increased antibiotic resistance of microflora in the human,

Detailed description of the invention

One of the preferable embodiments of the present invention are the following vectors: pKHBSadwBAR, pPLAHBSBAR, pPLAHBSadwBAR, pKHBMBAR, pKHBMadwBAR, pPLAHBMBAR, pPLAHBMadwBAR, pKHBLBAR, pKHBLadwBAR, pPLAHBLBAR, pPLAHBLadwBAR, pKHBCBAR, pKHBCadwBAR, pPLAHBCBAR, and

pPLAHBCadwBAR (Fig. 1) for the production of plants resistant to phosphinotricine herbicides such as Basta and at the same time which constitutively express the heterologous proteins S-HBsAg, M-HBsAg, L-HBsAg and HBcAg, mainly in the leaves or tissue-specifically in the seeds.

An important characteristic of the vectors is T-DNA containing two expression cassettes which determine the expression of the immunogenic protein subunits S, M and L of the surface antigen of HB or the core antigen protein HBcAg (subtypes *ayw4*, *adw4*) of HBV as well as for phosphinotricine acetyltransferase which gives resistance to phosphinotricine which itself is a component of a number of non-selective herbicides, for example Basta. The first cassette contains a sequence encoding one of the antigenic HBV proteins under the control of the following regulatory sequences: the CaMV 35S RNA promoter with a single enhancer or the *legA* promoter and nopaline synthase transcription terminator (NOSt). The second cassette contains a sequence including a gene under the control of the nopaline synthase promoter (PNOS) and the g7 transcription terminator (g7t). The next significant property of the constructed T-DNA is the occurrence of the above-mentioned coding or regulatory sequences only in one copy and in particular, their orientation with regard to one another (Fig. 1). The absence of repeated sequence motifs eliminates recombination within the introduced T-DNA as well as the balanced transcription of the individual transgenes. The structural characteristics of the T-DNA limits gene silencing and thereby increases the stability of the T-DNA within the transgenic plant genome, which is particularly significant in the lettuce, whose genome is relatively variable and subject to rearrangements (McCabe 1999). As a result, the structural characteristics of the T-DNA play a role in the stable expression of the transgenes, S-HBs, M-HBs, L-HBs and HBc as well as *bar* in plant cells.

The next subject of the present invention are transformed lettuce, pea and tobacco cells and plants regenerated from them, as well as plants from subsequent generative and/or vegetative progeny characterized by the simultaneous expression of protein subunits of the surface antigen (S-HBsAg or M-HBsAg or L-HBsAg) or the HBcAg core antigen of the HBV virus of the strains *ayw4* and *adw4* as well as resistance to phosphinotricine herbicides. The resistance of the plants to herbicides makes it possible to use such a product as the raw material for the production of an oral vaccine or to culture the plants in field conditions according to pertinent requirements and guidelines.

The transformation of lettuce, pea and tobacco plants using the vectors described in the present invention as well as pea and tobacco plants transformed with the vector pKHB SBA are described in invention number P.382769 (Fig. 1) facilitates the production of

transgenic plant lines (Fig. 2), whose characteristic property is the resistance to phosphinotricine herbicides, i.e. Basta, as well as the expression of HBsAg proteins at the following levels: the small surface antigen S-HBsAg at a level of more than her equal to 5 μg/g fresh weight (FW) to 200 μg/g FW, the medium surface antigen M-HBsAg at a level greater than or equal to 4 μg/g FW to 35 g/g FW, the large surface antigen L-HBsAg a level greater than or equal to 2 μg/g FW up to 20 g/gs FW or the HBcAg core antigen at a level greater than or equal to 50 μg/g FW up to 500 μg/g FW (Figs. 3, 6) as well as up to a dozen or more micrograms of HBV proteins programs of seeds (Fig .8). These properties are maintained both in the first generative generation T1 as well as in the subsequent generative generations and in letteuce plant clones (Fig. 5, Tab. 1) vegetatively propagated according to the designed method (Fig. 4). HBV antigenic proteins produced in the plants are characterized by a native structure, the ability to be glycosylated as well as the retained capability of folding into highly immunogenic sub-viral particles or virus-like particles (VLPs) or smaller aggregates, which has been demonstrated by immunoenzymatic analyses (ELISA) including the use of a specialist kit from Abbott, directed against epitope "a" of HBsAg proteins folded into VLPs or aggregates. In turn, among the bands observed in western blotting which correspond to various forms of the HBsAg and HBcAg proteins there are also glycosylated forms or dimers, which are the first step in the assembly of VLPs from us-HBsAg or HBcAg (Figs. 7, 9). Such particles have also been observed directly and that tobacco leaf mesophyll cells (Fig. 9 B) using a transmission electron microscope, JEM 1200EXII (Jeol).

The transgenic plant lines have been analyzed using PCR in order to initially confirm the presence of the transgene sequence in the genomic DNA, as well as to determine/confirm the men dealing three: one mechanism of transgene inheritance in the transgenic plants (Fig. 2). Next, the genomic DNA of plants from the individual lines was hybridized with probes complementary to the sequence of the S-HBs, M-HBs, L-HBs, or HBc transgenes using the Southern blot method (Fig. 2) in order to determine the number of T-DNA integration sites containing the transgene. The regenerated plants of the T0 generation as well as plants of the T-1 generation and their vegetative clones were also examined for the expression of the protein subunits of the surface antigen, HBsAg, and core antigen, HBcAg, using western blotting (Fig. 7) as well as the quantitative and qualitative sandwich ELISA assay using specific antibodies from Biodesign (Figs. 3, 5, 6, 8). The presence of S-HBs and HBc antigens formed into the structure of virus-like particles in the mesophyll layers was visualized using a transmission electron microscope (JEM1200EXII, Jeol) (Fig. 9 B). Standard serial sections and contrasting techniques with slight modifications were

used to visualize the VLPs (Kocjan 1996).

The next aspect of the invention is a method of vegetative propagation (micropropagation) in an *in vitro* culture of transgenic lettuce plants, which facilitates the consistent and repeatable production of plants with a high efficiency, meaning 8 to 13 progeny plants obtained from one initial plant (Fig. 4). In the clones produced, the level of expression of the individual HBV proteins is comparable, the variance is insignificant, and furthermore in at least 64% of plans, the HBV protein level is comparable between the initial plant and plants obtained from seeds (Fig. 5, Tab. 1). For at least 75% of clones, the variance in HBV vaccinating protein content does not exceed 20% (Tab. 1). The method of micropropagation according to the present invention facilitates the industrial proliferation of plants characterized by a desirable, high level of expression, ensuring an at least several dozenfold increased level of material with a high, and at the same time statistically stable level of antigenic proteins as a raw material for the production of an oral vaccine against viral HepB.

The next subject of the present invention is the use of a transgenic lettuce and peas resistant to phosphinotricine, which produce the antigenic proteins S-HBsAg, M-HBsAg, L-HBsAg and HBcAg, in the production of a prophylactic and therapeutic, oral III-generation vaccine against viral hepatitis type B, meaning one containing the full set of HBV structural proteins. Lettuce (*Lactuca sativa* L.) as well as peas (*Pisum sativa* L.) are species which, in contrast to plant species used to date, are characterized by properties conducive to the production of a vaccine to be administered orally. In in contrast to the majority of cultivated plants, lettuce leaves and pea seeds are directly palatable without the need for preparation, particularly for thermal processing. They also lack any anti-nutritive substances or allergens, whose presence could construe a limiting factor. The characteristic property of transgenic lettuce and peas producing S-HBsAg, M-HBsAg, L-HBsAg and HBcAg and at the same time resistant to herbicides is their utility and conduciveness for use as a raw material for the production of an oral vaccine against viral HepB.

The next aspect of the present invention is lyophilised plant material, its use in the production of a III generation oral vaccine in the form of its derivatives: a suspension, a syrup, granulate, tablets or capsules containing the vaccinating proteins S-HBsAg, M-HBsAg, L-HBsAg and HBcAg, as well as the above-mentioned derivative forms of lyophilisate and a method of preparing them from plant material.

An effective oral III generation vaccine against viral HepB, in contrast to the solutions used thus far (see literature) comprises a condensed form of pulverized lyophilisate used in the form of a suspension, a syrup, granulate, tablets or capsules. The initial material for the

production of the condensate are leaves of the individual lettuce lines, possibly pea seeds, containing S-HBsAg, M-HBsAg, L-HBsAg and HBcAg, which are lyophilised. Material from the individual lines is ground to a powder and may then be composed into a preparation containing definite amounts of the HBV antigenic proteins of various subtypes, appropriate to the induction of the desired immunological response. Using physiological saline solution or a similar buffer, i.e. PBS, or following the addition of ancillary substances, this preparation can be transformed into a suspension or syrup, granulate, tablets or capsules (Fig. 9 A). The pulverized, lyophilized plant material and the suspension/syrup form or the granulate/tablet/capsule form of the III generation oral vaccine against viral HepB (Fig. 9 A), is characterized by characteristic properties (in contrast to the raw plant material) which make it possible to:

- preserve the plant material while maintaining the high content of the vaccinating proteins, S-HBsAg, M-HBsAg, L-HBsAg and HBcAg of various subtypes
- to concentrate the doses of the vaccinating factors, the S-HBsAg, M-HBsAg, L-HBsAg and HBcAg proteins of various subtypes to a level sufficient for oral administration
- to administer stable, standardized doses of S-HBsAg, M-HBsAg, L-HBsAg and HBcAg due to a) the regulation of the level of antigenic proteins at individual stages of preparation of components, lyophilisate's containing the individual antigens, B) the regulation of the level of antigenic proteins at the stage of preparation of the suspension or syrup or granulate, tablets or capsules, as well as C) the selection of appropriate raw materials and ancillary constituents
- to determine the appropriate ratios of S-HBsAg, M-HBsAg, L-HBsAg and HBcAg of various subtypes to obtain an appropriate level of protective immunological response, or to obtain the desired therapeutic effect against chronic viral HepB, most often caused by extant HBV strains
- to determine an effective vaccination protocol through the regulation of the dose and the chronological dosing regime of immunization
- to easily store the powdered lyophilisate and granulate, tablets or capsules at room temperature while maintaining the stability of S-HBsAg, M-HBsAg, L-HBsAg and HBcAg over a period of at least 12 months
- to easily vaccinate orally, through drinking or swallowing

The next subject of the present invention is the use of transgenic tobacco resistant to phosphinotrycine, which produces the antigenic proteins S-HBsAg, M-HBsAg, L-HBsAg and HBcAg for the production of a prophylactic or therapeutic parenteral or nasal III generation vaccine against viral hepatitis type B. Tobacco (*Nicotiana tabacum* L.) is a

plant species characterized by properties significant for protein production. The production of heterologous proteins in herbicide resistant tobacco is characterized by its economic efficiency, which is a product of low production costs both under greenhouse and field conditions, rapid growth, large biomass and the stability and efficiency of the expression of the heterologous proteins. The antigenic HBV proteins forming VLPs or aggregates in tobacco leaves (Fig. 9 B) may be extracted and purified from plant material using standard physical methods such as centrifugation, and physicochemical methods such as affinity chromatography. The purified S-HBsAg, M-HBsAg, L-HBsAg and HBcAg antigens of various subtypes can then be used to manufacture a preparation containing previously defined proportions of the individual antigens to produce the desired level of protective immunological response against viral HepB, or to elicit the desired therapeutic affect against chronic viral HepB. The vaccinating protein preparation, following the addition of adjuvants, stabilizers and other substances, can be used in vaccinations according to extant procedures via parenteral injections (intramuscular, intraperitoneal, or subcutaneous) or through mucous membranes in the form of an aerosol or droplets applied nasally (Fig. 9 B). The characteristic property of transgenic tobacco producing S-HBsAg, M-HBsAg, L-HBsAg and HBcAg and at the same time resistant to herbicides is its utility and amenability for use as the raw material for the production of prophylactic and therapeutic III generation vaccines against viral HepB, comprising purified antigens and applied parenterally or nasally.

III generation vaccines produced according to the present invention can be used in vaccination against viral HepB through oral application using a suspension, a syrup, a granulate, tablets or capsules produced from pulverized lyophilisate from lettuce or pea seeds containing the S-HBsAg, M-HBsAg, L-HBsAg and HBcAg antigens, or through parenteral application with the aid of injections or nasally with the aid of an aerosol or droplets containing the extracted and purified antigens S-HBsAg, M-HBsAg, L-HBsAg and HBcAg extracted and purified from tobacco tissues. The preparations produced can also be used in combined immunizations: oral-parenteral, oral-nasal or nasal-parenteral.

The surface antigens, HBsAg, and core antigen, HBcAg, of the HBV virus are strong immunogens which induce an immune response in a number of mammalian species, including mice, chimpanzees and humans. The immunological response may be a cellular response encompassing the production of specific cytotoxic lymphocytes, or of the humoral type, consisting of the production of specific antibodies. It is believed that in humans and chimpanzees, a humoral response induced at an appropriately high level is sufficient to protect against illness following exposure to the virus. Depending on the

method of administering the immunogen, the immunological reaction against HBV antigens encompasses the production of mainly IgG class antibodies as well as other classes through parenteral immunization (intramuscular, intraperitoneal or subcutaneous injections) as well as IgG and IgA during immunization through the mucous membranes of the alimentary tract, oral administration with the aid of a suspension, syrup, a granulate, tablets or capsules, and through the epithelium of the respiratory tract, through nasal administration with the aid of an aerosol or droplets, possibly also vaginally, rectally or through the skin.

A significant element of the method of ummunization using HBV antigens produced in plants according to the present invention is immunization through mucous membranes, of the intestine in the case of the oral III generation vaccine produced from lettuce or peas, or the nasal mucosa in the case of the nasal III generation vaccine made from antigens extracted and purified from tobacco.

According to extant knowledge (Mowat and Viney 1997, Langridge 2000, Mowat 2003, Poonam 2007), antigen proteins found in contact with mucous membranes induce locally associated immunological cells, including gut associated lymphoid tissue (GALT) in the gut, nasal associated lymphoid tissue (NALT) in the nasal mucosa and bronchus associated lymphoid tissue (BALT) of the lower respiratory tract, and others. The GALT, NALT and BALT structures contain specialized cells, i.e. M. cells in Peyer islets in the intestinal mucosa, which collect degraded or complete antigenic proteins and viruses, bacteria or protozoans from the external environment, meaning the lumen of the intestine, nasal cavity and lower respiratory pathways. The antigens or microbiota are then transported to immune system cells, including antigen presenting cells, dendrites and macrophages as well as T- and B-lymphocytes; both locally within the mucous membranes and to the peripheral lymphatic organs as a result of systemic circulation. Antigen presentation to Th lymphocytes present in the gastric or respiratory tract mucosa results in a local humoral immunological response through the activation of Th lymphocytes, cytokine release and the subsequent activation of B lymphocytes characterized by the secretion, through the mucous membranes, of antigen-specific IgA antibodies. During the subsequent stages, the Th lymphocytes migrate into the blood and, upon reaching the peripheral organs of the immune system, elicit a systemic humoral response with IgG-class antibodies, and antibodies of the remaining classes (IgM, IgA) via a similar activation pathway, and possibly a cellular response consisting of the production of a subpopulation of Tc cytotoxic lymphocytes activated by the antigen, as well as immunological memory.

As was shown before (patent application P.382769), the S-HBsAg antigen subtype *ayw4* is

immunogenic in mice following the preservation of plant material from lettuce containing S-HBsAg via lyophilisation and suspension thereof immediately prior to administration. The immune response to the S-HBsAg antigen occurs as a result of the priming with the antigen and then as a result of one or more repeated immunizations or boosting. It can be quite safely assumed that a similar immune response following oral administration will be induced by a III generation vaccine preparation, one containing the S-HBsAg antigen and the remaining HBV antigens, M-HBsAg, L-HBsAg and HBcAg of the *ayw4* and *adw4* subtypes. The reason for this assumption is the structural similarity of the HBsAg surface antigens, also characterized by their ability to form higher-order structures, namely VLPs consisting of S-HBsAg or aggregates formed by M-HBsAg and L-HBsAg. Likewise, HBcAg, although physicochemically it is different from the surface proteins, also has the capability of forming higher-order particles (pseudocapsids) and activates similar mechanisms of mucosal immunological response within the GALT and NALT/BALT systems (Brandtzaeg 2007, Poonam 2007) and enables one to assume that a nasally applied III generation vaccine preparation containing the purified HBV antigens will induce a local and systemic immunological response.

The antigens according to the present invention, produced in plants and purified, will also be susceptible of use as the components of vaccines applied in standard fashion, via a parenteral injection according to commonly used procedures. The alternative method of producing the vaccine antigens in plants is significant for this method of vaccination.

The production of the individual HBV antigens in separate transgenic plant lines according to the present invention makes it possible to optimally set and standardize the proportions of the individual immunogens in the vaccine for producing at highly effective prophylactic and therapeutic III generation vaccine against viral HepB, for oral, nasal as well as parenteral administration. The possibility of using the S-HBsAg, M-HBsAg, L-HBsAg and HBcAg antigens of the two most common HBV subtypes, *ayw4* and *adw4*, or other incident subtypes facilitates the design of optimal parameters for an effective immunization, meaning the number and size of antigen doses, the intervening period between individual immunizations, the use of adjuvants, etc. In the next stage, the vaccine will be standardized through the appropriate selection of the proportions of the antigens, S-HBsAg, M-HBsAg, L-HBsAg and HBcAg, purified in the case of the nasal or parenteral vaccine or, in the case of the oral vaccine, present in the preserved plant material (a powdered lyophilisate containing predetermined concentrations of the antigens).

The type of vaccine according to the present invention, an oral vaccine in the form of a suspension, syrup, a granulate, a tablet or capsule, or a nasal vaccine in the form of an

aerosol or droplets, or or a parenteral vaccine in the form of an injection facilitates the use of a wide spectrum of immunization methods, depending on the age of the immunized animals and people, their overall condition and immunocompetence, sex, body weight and other parameters which influence the immune response. The variability of the types of vaccines according to the present invention also enables one to use a single method or a combination of various methods of immunization, meaning oral-nasal, oral-parenteral, nasal-parenteral, etc. in order to produce the maximum level and scope of the humoral immune response through the induction of IgG class antibodies or IgG and IgA or a cellular response. Because it is assumed that the vertical transmission of HBV can encompass the mucous membranes as the site of an infection, a combination of the methods of immunization (combining the intestinal-mucosal, nasal-mucosal and parenteral pathways) using various forms of the prophylactic and therapeutic III generation vaccine according to the present invention will constitute a potentially more impervious immunological protection to the families of persons infected with HBV or chronic HBV carriers, where vertical transmission of the infection can occur.

To better illustrate the nature of the present invention, this description has been supplemented with a list of sequences and figures.

Sequence No. 1 (SEQ ID No. 1) represents the nucleotide sequence of the expression cassette P35S-SHBs*adw4*-NOSt located in the binary vector pKHBSadwBAR designed for transforming plants and described in the examples.

Sequence No. 2 (SEQ ID No. 2) represents the nucleotide sequence of the expression cassette PlegA-SHBs*ayw4*-NOSt located in the binary vector pPLAHBSBAR designed for transforming plants and described in the examples.

Sequence No. 3 (SEQ ID No. 3) represents the nucleotide sequence of the expression cassette PlegA-SHBs*adw4*-NOSt located in the binary vector pPLAHBSadwBAR designed for transforming plants and described in the examples.

Sequence No. 4 (SEQ ID No. 4) represents the nucleotide sequence of the expression cassette P35S-MHBs*ayw4*-NOSt located in the binary vector pKHBMBAR designed for transforming plants and described in the examples.

Sequence No. 5 (SEQ ID No. 5) represents the nucleotide sequence of the expression cassette P35S-MHBs*adw4*-NOSt located in the binary vector pKHBMadwBAR designed for transforming plants and described in the examples.

Sequence No. 6 (SEQ ID No. 6) represents the nucleotide sequence of the expression cassette PlegA-MHBs*ayw4*-NOSt located in the binary vector pPLAHBMBAR designed for transforming plants and described in the examples.

Sequence No. 7 (SEQ ID No. 7) represents the nucleotide sequence of the expression cassette PlegA-MHBs*adw4*-NOSt located in the binary vector pPLAHBMadwBAR designed for transforming plants and described in the examples.

Sequence No. 8 (SEQ ID No. 8) represents the nucleotide sequence of the expression cassette P35S-LHBs*ayw4*-NOSt located in the binary vector pKHBLBAR designed for transforming plants and described in the examples.

Sequence No. 9 (SEQ ID No. 9) represents the nucleotide sequence of the expression cassette P35S-LHBs*adw4*-NOSt located in the binary vector pKHBLadwBAR designed for transforming plants and described in the examples.

Sequence No. 10 (SEQ ID No. 10) represents the nucleotide sequence of the expression cassette PlegA-LHBs*ayw4*-NOSt located in the binary vector pPLAHBLBAR designed for transforming plants and described in the examples.

Sequence No. 11 (SEQ ID No. 11) represents the nucleotide sequence of the expression cassette PlegA-LHBs*adw4*-NOSt located in the binary vector pPLAHBLadwBAR designed for transforming plants and described in the examples.

Sequence No. 12 (SEQ ID No. 12) represents the nucleotide sequence of the expression cassette P35S-HBc*ayw4*-NOSt located in the binary vector pKHBCBAR designed for transforming plants and described in the examples.

Sequence No. 13 (SEQ ID No. 13) represents the nucleotide sequence of the expression cassette P35S-HBc*adw4*-NOSt located in the binary vector pKHBCadwBAR designed for transforming plants and described in the examples.

Sequence No. 14 (SEQ ID No. 14) represents the nucleotide sequence of the expression cassette PlegA-HBc*ayw4*-NOSt located in the binary vector pPLAHBCBAR designed for transforming plants and described in the examples.

Sequence No. 15 (SEQ ID No. 15) represents the nucleotide sequence of the expression cassette PlegA-HBc*adw4*-NOSt located in the binary vector pPLAHBCadwBAR designed for transforming plants and described in the examples.

Sequence No. 16 (SEQ ID No. 16) represents the nucleotide sequence of the expression cassette PNOS-bar-g7t located in the binary vectors pKHBSadwBAR, pPLAHBSBAR, pPLAHBSadwBAR, pKHBMBAR, pKHBMadwBAR, pPLAHBMBAR, pPLAHBMadwBAR, pKHBLBAR, pKHBLadwBAR, pPLAHBLBAR, pPLAHBLadwBAR, pKHBCBAR, pKHBCadwBAR, pPLAHBCBAR, pPLAHBCadwBAR designed for transforming plants.

Figure 1 represents a schematic of the construction of vectors pKHBSadwBAR, pPLAHBSBAR, pPLAHBSadwBAR, pKHBMBAR, pKHBMadwBAR, pPLAHBMBAR,

pPLAHBMadwBAR, pKHBLBAR, pKHBLadwBAR, pPLAHBLBAR, pPLAHBLadwBAR, pKHBCBAR, pKHBCadwBAR, pPLAHBCBAR, pPLAHBCadwBAR used in the transformation lettuce, peas and tobacco, and for comparison the vector pKHBSBAR according to application P.382769 in a novel use: the transformation of peas and tobacco, containing a sequence encoding the small, S-HBsAg, or medium, M-HBsAg, or large, L-HBsAg, surface antigen or HBc core antigen of the Hepatitis Type B virus (HBV) of the strains *ayw4* or *adw4*, under the control of the constitutive CaMV 35S RNA promoter of the (P35S) or the tissue-specific *legA* promoter (PlegA) and nopaline synthase terminator (NOSt) and the sequence of the *bar* gene encoding phosphinotricine acetyltransferase under the control of the nopaline synthase promoter (PNOS) and the g7t terminator, warranting transgenic plant resistance to phosphinotricine herbicides.

Legend: S-HBs – sequence encoding the small surface antigen of HBV *ayw4* or *adw4*, M-HBs – sequence encoding the medium surface antigen of HBV *ayw4* or *adw4*, L-HBs – sequence encoding the large surface antigen of HBV *ayw4* or *adw4*, HBc – sequence encoding the core antigen of HBV *ayw4* or *adw4*, P35S – cauliflower mosaic virus (CaMV) 35S RNA promoter, PlegA – legumine A promoter, NOSt – nopaline synthase terminator, *bar* – sequence encoding the *bar* gene-phosphinotricine acetyltransferase, PNOS – nopaline synthase promoter, g7t – g7 terminator, RB, LB – right and left border sequences of the T-DNA, NPT III – gen neomycine phosphotransferase, GUS – sequence encoding β-glucuronidase, GUS-INT – sequence encoding β-glucuronidase with an intron; (ayw/adw) – identyczna konstrukcja plazmidu z sequence encoding HBV antigen of the strain *ayw4* and *adw4*, binary vector name without an index – the vector contains a sequence encoding an HBV antigen of the *ayw4* strain, vector name with the index "adw"– the vector contains a sequence encoding an HBV antigen of the strain *adw4*.

Figure 2 represents the results of analyses to determine the presence of transgenes containing a sequence encoding an HBV antigen in the genomic DNA of transformant progeny of lettuce (generation T1) and tobacco using PCR and primers specific for a given coding sequence and Southern blotting using probes specific against individual coding sequences.

A - electrophoretic separation of the amplification of M-HBs transgenes in lettuce line LT9A-16B

Legend of electrophoretic lanes: M – DNA fragment mass marker (200 bp DNA Ladder, MBI Fermentas), 1-27 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBMBAR.

B - electrophoretic separation of the amplification products of L-HBs transgenes of lettuce line LT11-17A

Legend of electrophoretic lanes: M – DNA fragment mass marker (200 bp DNA Ladder, MBI Fermentas), 16-42 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBLBAR.

C - electrophoretic separation of the amplification products of S-HBs transgenes of tobacco line Tt13

Legend of electrophoretic lanes: M – DNA fragment mass marker (200 bp DNA Ladder, MBI Fermentas), 2-109 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBSBAR.

D - electrophoretic separation of the amplification products of L-HBs transgenes of tobacco line Tt14

Legend of electrophoretic lanes: M – DNA fragment mass marker (200 bp DNA Ladder, MBI Fermentas), 23A-54 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBLBAR.

E - Result of Southern blot analysis of lettuce line LT10 for the transgene S-HBs*adw4*

Legend of blot lanes: 4D/15-26G/22 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBsadwBAR digested with *Eco*R I as well as *Eco*R I and *Kpn* I

F - Result of Southern blot analysis of lettuce line LT12 for the transgene HBc

Legend of blot lanes: 2I/12-16A/27 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBcBAR digested with *Eco*R I as well as *Eco*R I and *Kpn* I

G - Result of Southern blot analysis of tobacco line Tt8 for the transgene M-HBs

Legend of blot lanes: 8-4/1 -8-9/15 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBMBAR digested with *Eco*R I as well as *Eco*R I and *Kpn* I

H - Result of Southern blot analysis of tobacco line Tt15 for the transgene HBc

Legend of blot lanes: 15-11 - 15-126 – analysed plants, N - negative control – DNA of a non-transgenic plant, P - positive control – plasmid pKHBCBAR digested with *Eco*R I as well as *Eco*R I and *Kpn* I

Figure 3 represents a graph of HBV antigenic protein content made using an immunoenzymatic ELISA test on the leaves of transformant progeny of lettuce (generation T1) containing a sequence encoding an HBV antigen under the control of the constitutive 35S promoter. The content of the individual proteins is given in μg/g fresh weight as an arythmetic mean with a standard deviation from 3 repeat measurements.

A – S-HBsAg content analysis *adw4* in plant line LT10-26G

B - M-HBsAg content analysis in plant line LT9A-16B

C - L-HBsAg content analysis in plant line LT11-4H

D - HBcAg content analysis in plant line LT12-4J

Legend: T0 – content of HBV antigen in a plant of the T0 generation (parental), T1 – arithmetic mean of the HBV antigen content in plants of the T1 generation (progeny)

Figure 4 represents the process and efficiency of vegetative propagation in an in vitro culture of transgenic lettuce producing HBsAg proteins for the industrial propagation of plants as a raw material vaccines.

A – vegetative propagation of transgenic lettuce in an in vitro culture of: on the left - culture on LRM2 medium, in the middle - single multiplantlet, on the right - rooted vegetative clones

B - the efficiency of vegetative propagation in an in vitro culture of a transgenic lettuce line producing S-HBsAg

C - the efficiency of vegetative propagation in an in vitro culture of a transgenic lettuce line producing M-HBsAg

D - the efficiency of vegetative propagation in an in vitro culture of a transgenic lettuce line producing L-HBsAg

Legend for B-D: GM – geometric mean for all the passages of a culture, control - geometric mean for non-transgenic lettuce

Figure 5 represents a graph of HBsAg antigenic protein content made using an immunoenzymatic ELISA test on the leaves of vegetatively propagated progeny of lettuce (generation T1) containing a sequence encoding an HSsAg antigen under the control of the constitutive 35S promoter. The content of the individual proteins is given in μg/g fresh weight as an arythmetic mean with a standard deviation from 3 repeat measurements.

A – S-HBsAg content analysis in vegetative clones of plant line LT10-4D

B - M-HBsAg content analysis in vegetative clones of plant line LT9A-15E

C - L-HBsAg content analysis in vegetative clones of plant line LT11-6D

Table 1 represents analysis results (χ^2 test, F test) of the significance of changes in the levels of HBsAg proteins in vegetative clone plants obtained through propagation

(micropropagation) as well as results of the analysis of the variance of HBsAg protein content in clones and control plants with a natural development cycle. The results were analysed statistically (following logarithmic transformation of each individual parental plant separately) through an analysis of variance using a mixed model, taking into account the constant effects of propagated plant progeny (generation T1) as well as random effects of vegetative clones and allowing for heterogenous variance between clones.

Figure 6 represents a graph of HBV antigenic protein content made using an immunoenzymatic ELISA test on the leaves of tobacco transformants containing a sequence encoding an HSsAg antigen under the control of the constitutive 35S promoter. The content of the individual proteins is given in μg/g fresh weight as an arithmetic mean with a standard deviation from 3 repeat measurements.

A – S-HBsAg content analysis in plant line Tt13

B - M-HBsAg content analysis in plant line Tt8

C - L-HBsAg content analysis in plant line Tt14

D - HBcAg content analysis in plant line Tt15

Figure 7 represents an analysis of the expression and forms of HBV antigenic proteins in the leaves of transformant progeny of lettuce and tobacco using western blotting and specific monoclonal antibodies or rabbit polyclonal antibodies.

A – western blot analysis of S-HBsAg*adw4* protein in leaf extracts of lettuce progeny using mouse monoclonal antibody Mab C86600 (Biodesign), specific against S domain of HBsAg proteins

Legend of blot lanes: M – protein mass marker (MBI Fermentas) 4D/4 – 26G/6 – analysed plants, N-non-transgenic plant-negative control, P – S-HBsAg antigen obtained from Prof. R. Schirmbeck of the University of Ulm, Germany - positive control

The following forms of the S-HBsAg protein were determined:

p24 - monomer of non-glycosylated S-HBsAg protein, 24 kDa

gp27 - monomer of glycosylated S-HBsAg protein, 27 kDa

gp30 – putative characteristic glycosylated monomer of the S-HBsAg protein, 30 kDa

p48 - dimer of non-glycosylated S-HBsAg p24, 48 kDa

gp54 - dimer of glycosylated S-HBsAg gp27, 54 kDa

B - western blot analysis of M-HBsAg protein in leaf extracts of lettuce progeny using mouse monoclonal antibody Mab C86600 (Biodesign), specific against S domain of HBsAg proteins.

Legend of blot lanes: M – protein mass marker (MBI Fermentas), 1E/4 – 18A/8 – analysed plants, N - non-transgenic plant-negative control, P – M/L-HBsAg antigen obtained from Prof. R. Schirmbeck of the University of Ulm, Germany - positive control

The following forms of the M-HBsAg protein were determined:

p31 - monomer of non-glycosylated M-HBsAg protein, 31 kDa

gp33 - monomer of glycosylated M-HBsAg protein, 33 kDa

C - western blot analysis L-HBsAg in leaf extracts of lettuce progeny using rabbit polyclonal antibody Pab B65811R (Biodesign), specific against S domain of HBsAg proteins.

Legend of blot lanes: M – protein mass marker (MBI Fermentas), 4H/8 – 18C/34 – analysed plants, N-extract from a non-transgenic plant-negative control, P – M/L-HBsAg antigen obtained from Prof. R. Schirmbeck of the University of Ulm, Germany - positive control

The following forms of the L-HBsAg protein were determined:

p39 - monomer of non-glycosylated L-HBsAg protein, 39 kDa

gp42 - monomer of glycosylated L-HBsAg protein, 42 kDa

D - western blot analysis of protein HBcAg of leaf extracts of lettuce progeny using specific mouse monoclonal antibody Mab C86340M (Biodesign).

Legend of blot lanes: M – protein mass marker (MBI Fermentas), 16A/2 – 2I/6 – analysed plants, N - extract from a non-transgenic plant - negative control, P – antigen HBcAg Cat. No. R8A120 (Biodesign) - positive control

The following forms of HBcAg proteins were determined:

p18 –likely a characteristic, partially degraded monomer of HBcAg, 18 kDa

p20 - likely a characteristic, partially degraded monomer of HBcAg, 20 kDa

p22 - monomer of HBcAg, 22 kDa

p25 - monomer HBcAg, 25 kDa

p26 - likely a characteristic, partially glycosylated monomer of HBcAg, 26 kDa

p36 - likely a dimer of HBcAg p18 monomer, 36 kDa

p40 - likely a dimer of HBcAg p20 monomer, 40 kDa

p44 - dimer of HBcAg p22 monomer, 44 kDa

E – western blot analysis of protein S-HBsAg in tobacco leaf extracts using mouse monoclonal antibody Mab C86600 (Biodesign), specific against S domain of HBsAg proteins.

Legend of blot lanes: M – protein mass marker (MBI Fermentas) 13-1 –13-176 – analysed plants, N - non-transgenic plant-negative control, P – S-HBsAg antigen obtained from Prof. R. Schirmbeck of the University of Ulm, Germany - positive control

The following forms of S-HBsAg proteins were determined:

p24 - monomer of non-glycosylated S-HBsAg protein, 24 kDa

gp27 - monomer of glycosylated S-HBsAg protein, 27 kDa

gp30 - likely a characteristic glycosylated monomer of the S-HBsAg protein, 30 kDa

p48 - dimer of non-glycosylated S-HBsAg p24 protein , 48 kDa

gp54 - dimer of glycosylated S-HBsAg gp27 protein, 54 kDa

F - western blot analysis of M-HBsAg protein in tobacco leaf extracts from rabbit polyclonal antibody Pab B65811R (Biodesign), specific against S domain of HBsAg proteins.

Legend of blot lanes: M – protein mass marker (MBI Fermentas), 8-4/1 – 8-9/19 – analysed plants, N - non-transgenic plant-negative control, P – antigen M/L-HBsAg obtained from Prof. R. Schirmbeck of the University of Ulm, Germany - positive control

The following forms of M-HBsAg proteins were determined:

p31 - monomer of non-glycosylated M-HBsAg protein, 31 kDa

gp33 - monomer of glycosylated M-HBsAg protein, 33 kDa

G - western blot analysis of protein L-HBsAg in leaf extracts of lettuce progeny using rabbit polyclonal antibody Pab B65811R (Biodesign), specific against S domain of HBsAg proteins.

Legend of blot lanes: M – protein mass marker (MBI Fermentas), 14-2 – 14-28 – analysed plants, N - extract from a non-transgenic plant – negative control, P – antigen M/L-HBsAg obtained from Prof. R. Schirmbeck of the University of Ulm, Germany - positive control

The following forms of L proteins were determined:

p39 - monomer of non-glycosylated L-HBs protein, 39 kDa

gp42 - monomer of glycosylated L-HBs protein, 42 kDa

H - western blot analysis of HBcAg proteins in tobacco leaf extracts using specific mouse monoclonal antibody Mab C86340M (Biodesign).

Legend of blot lanes: M – protein mass marker (MBI Fermentas), 16A/2 – 2I/6 – analysed plants, N-extract from a non-transgenic plant-negative control, P – antigen HBcAg Cat. No. R8A120 (Biodesign)-positive control

The following forms of HBcAg proteins were determined:

p18 - likely a characteristic, partially degraded monomer of HBcAg, 18 kDa

p20 - likely a characteristic, partially degraded monomer of HBcAg, 20 kDa

p22 - monomer of HBcAg, 22 kDa

p25 - monomer of HBcAg, 25 kDa

p26 - likely a characteristic, partially glycosylated monomer of HBcAg, 26 kDa

p40 - likely a dimer of the HBcAg p20 monomer, 40 kDa

p44 - dimer of the HBcAg p22 monomer, 44 kDa

Figure 8 represents a graph of HBV antigenic protein content made using an immunoenzymatic ELISA test on pea and tobacco seeds containing a sequence encoding an a HBV antigen under the control of the tissue specific *legA* promoter. The content of the individual proteins is given in μg/g fresh weight as an arythmetic mean with a standard deviation from 3 repeat measurements.

A – S-HBsAg content analysis in plant line Tt9

B - M-HBsAg content analysis in plant line Tt2

C - L-HBsAg content analysis in plant line Tt10

D - HBcAg content analysis in plant line Tt11

E - M-HBsAg content analysis pea lines IM and AM

Legend: KN – negative control – non-transgenic plant

Figure 9 illustrates the use of transgenic plants in the production of derivative prophylactic and therapeutic III generation vaccines against viral HepB

A – the use of transgenic lettuce producing the individual HBV antigenic proteins in the production of powdered lyophilisate as a prefabricated component for the production of an oral vaccine in the form of a suspension or syrup, or shaped into a granulate, tablets or capsules.

B – the use of subviral particles (VLPs) produced in tobacco leaf mesophyll, which may be used in the form of a parenteral injection or as a nasal preparation administered in the form of an aerosol or droplets, following extraction, purification and the addition of ancillary substances. In the plant cells, the subviral particles accumulate in vesicles which are then locked inside of the endoplasmic reticulum, ER. The microphotographs were made using a transmission electron microscope, JEM1200 EXII (Jeol).

The following example embodiments of the present invention have only been given to better illustrate the idividual aspects of the present invention and should not be understood to be its entire scope, which is defined in the attached claims.

Example 1. Construction of the vectors pKHBSadwBAR, pPLAHBSBAR, pPLAHBSadwBAR, pKHBMBAR, pKHBMadwBAR, pPLAHBMBAR, pPLAHBMadwBAR, pKHBLBAR, pKHBLadwBAR, pPLAHBLBAR,

pPLAHBLadwBAR, pKHBCBAR, pKHBCadwBAR, pPLAHBCBAR, pPLAHBCadwBAR for the transformation of lettuce, tobacco and peas.

The preparation of a vector containing a sequence encoding the individual antigenic proteins: S-HBsAg, M-HBsAg, L-HBsAg and HBcAg of the HBV strains *ayw4* and *adw4* under the control of the 35S or *legA* promoters encompassed the following stages:

PCR using whole DNA of*Agrobacterium tumefaciens* of the strain C58 as a template was used to amplify the nopaline synthase terminator (NOSt) (Croy 1993). At the same time, the NOSt sequence was supplemented with recognition sites for the restrictases *Pst* I, *Xho* I at the 5' end as well as *Hin*d III at the 3' end. The terminator was cloed into plasmid pUC18 (MBI Fermentas, Yanisch-Perron 1985, Genebank L09136) yielding p18PNOSt, which was then sequenced.

The previously cloned CaMV 35S promoter (P35S) was adapted from vector p35SGUS-INT (Vannaceyt 1990) into plasmid pBluescript KS (Stratagene, Alting-Mees and Short 1989, Genebank X52327) removing the *Pst* I restriction site at the 5' end of the promoter through digestion with *Pst* I, degradation of 3' sticky ends using T4 DNA polymerase, and then re-ligation yielding the plasmid plasmid pKSP35SGI.

PCR using whole DNA of peas (*Pisum sativum* L.) as a template was used to amplify the promoter of legumine A (PlegA), one of the seed storge proteins (Lycett 1984, Genebank X02982). The PlegA sequence was at that time supplemented with recognitions sequences for the restrictases *Eco*R I at the 5' end and *Bam*H I at the 3' end. The promoter was then cloned into plasmid pUC18 (MBI Fermentas, Yanisch- 1985, Genebank L09136) yielding p18PlegA, and then sequenced.

The 35S promoter from plasmid pKSP35SGI was cloned into p18PNOSt yielding the vector pMG2A

The legA promoter from plasmid p18legA was cloned into p18PNOSt yielding the vector pMG5A

PCR was used to amplify the sequences encoding M-HBs (pos. 3173 – 837), L-HBs (pos. 2849-837) and HBc (pos. 1992-2454) on the basis of the plasmid pHBV312 produced earlier, which contains the complete genome of the Polish isolate of HBV *ayw4* (Płucienniczak 1994a). Similarly, the sequence encoding the HBV antigen of the strain *adw4* (Płucienniczak 1994b), S-HBsadw (pos. 156-837), M-HBsadw (pos. 3212-837), L-HBsadw (pos. 2855-837) and HBcadw (pos. 1902-2460) were amplified. Appropriate nucleotide substitutions were made in the sequences of the individual oligonucleotides so as to remove the recognition sites for the restrictases *Eco*R I, *Bam*H I as well as *Pst* I contained therein, while maintaining the original amino-acid codon sequence. At the same

time, recognition sites were introduced into the sequences encoding the individual antigens for *Bam*H I at the 5' end and *Pst* I at the 3' end. The modified sequence was cloned into pGEM-T (Promega, Marcus 1996), yielding pGTHBSadw, pGTHBM, pGTHBMadw, pGTHBL, pGTHBLadw, pGTHBC, pGTHBCadw, which were then sequenced.

The sequences encoding the individual antigens from pGTHBSadw, pGTHBM, pGTHBMadw, pGTHBL, pGTHBLadw, pGTHBC and pGTHBCadw were transcloned into the vector pMG2A, respectively yielding pMG2AHBSadw, pMG2AHBM, pMG2AHBMadw, pMG2AHBL, pMG2AHBLadw, pMG2AHBC, and pMG2AHBCadw.

The sequences of the individual antigens from pGTHBS (according to invention P.382769), pGTHBSadw, pGTHBM, pGTHBMadw, pGTHBL, pGTHBLadw, pGTHBC and pGTHBCadw were transcloned into vector pMG5A, respectively yielding pMG5AHBS, pMG5AHBSadw, pMG5AHBM, pMG5AHBMadw, pMG5AHBL, pMG5AHBLadw, pMG5AHBC, pMG5AHBCadw.

The complete expresion cassettes: P35S-SHBsadw-NOSt, PlegA-SHBsayw-NOSt, PlegA-SHBsadw-NOSt, P35S-MHBsayw-NOSt, P35S-MHBsadw-NOSt, PlegA-MHBsayw-NOSt, PlegA-MHBsadw-NOSt, P35S-LHBsayw-NOSt, P35S-LHBsadw-NOSt, PlegA-LHBsayw-NOSt, PlegA-LHBsadw-NOSt, P35S-HBcayw-NOSt, P35S-HBcadw-NOSt, PlegA-HBcayw-NOSt and PlegA-HBcadw-NOSt were transferred into vector pGPTV-BAR (Becker 1992) at the same time removing the fragment GUS-NOSt, yielding the vectors pKHBSadwBAR, pPLAHBSBAR, pPLAHBSadwBAR, pKHBMBAR, pKHBMadwBAR, pPLAHBMBAR, pPLAHBMadwBAR, pKHBLBAR, pKHBLadwBAR, pPLAHBLBAR, pPLAHBLadwBAR, pKHBCBAR, pKHBCadwBAR, pPLAHBCBAR and pPLAHBCadwBAR used to transform plants.

The vectors used to transform plants were prepared using restrictases, Taq DNA polymerase and other reagents from MBI Fermentas. A schematic of the construction of a binary vector according to the present invention as well as, for comparison, the vector pKHBsBAR according to invention P.382769 is shown in detail in a schematic diagram (Fig. 1).

The prepared binary vectors were introduced intoAg*robacterium tumefaciens* cells of the strains LBA4404, EHA105, AgL0 and AgL1. Plasmid presence inAg*robacterium* clones was determined using PCR and primers specific for the individual HBV antigenic proteins.

Example 2. Propagation of transgenic lettuce producing HBV antigenic proteins.

Lettuce lines were selected for the production of the initial vaccinating plant material which efficiently produce the surface antigens of HBV (S-HBsAg, M-HBsAg, L-

HBsAg) using vegetative propagation in an *in vitro* culture, and the clones thus produced were cultured *ex vitro*. material for further work was selected after analysing the HBsAg protein content.

Those lettuce lines were selected for vegetative propagation (micropropagation, propagation) which were characterised by the presence of transgenes in the genomic DNA, as confirmed by PCR and Southern blotting (Fig. 2) as well as those efficiently expressing the HBV antigenic proteins (Fig. 3). In order to germinate the seeds of the lettuce, cultivar Syrena, of the selected transgenic lines producing the individual HBsAg, the seeds were sterilised for 12 min. in 20% Clorox® bleach with 0.01% Tween® 20, and then rinsed 5-6 times in sterile deionised water in order to remove the steriliser solution. The seeds were germinated in semi-darkness, in a 16/8h light/dark fotoperiod. The initial micropropagation material consisted of 2 – 3 day-old sprouts, which were placed on LRM1 selective medium containing: micro- and macroelements according to SH medium (Schenk and Hildebrandt 1972), vitamins according to B5 medium (Gamborg 1968), saccharose 3%, kinetine – 5 mgl^{-1}, pH 5,75, agar – 0,8%, with phosphinotricine (Riedel de Haën), the active substance of the Basta herbicide at 2.5 mgl^{-1}. The sprouts and the multiplants developing therein were maintained during the micropropagation at ca. 24°C with a day/night fotoperiod of 16/8 h and alight level of 3000-4000 lx. During weeks 4-6 of culturing on LRM1 medium, we observed the initiation of lateral meristem activity, and then the development of lettuce plants, vegetative clones. The multiplants produced were then transferred onto LRM2 selective medium containing: macro- and microelements according to SH medium, B5 vitamins, saccharose-3%, kinetine – 0.5 mgl^{-1}, pH 5.75, agar – 0.8%, and 2,5 mgl^{-1} phosphinotricine. A number of the developing plants were cleaved off and retransferred onto LRM2 medium for further propagation, and a portion were transferred onto 1/2SH selective medium containing half of the macroelements according to SH and a full dose of microelements according to SH, B5 vitamins, saccharose – 3%, , pH 5.75, agar – 0.8% with phosphinotricine as above. Transgenic plants which had rooted in 1/2SH selective medium were transferred into *ex vitro* conditions in a greenhouse and adapted to soil conditions, garden soil in a phytotron (day/night foroperiod of 16/8h and a temperature of 22°C, 15-20 klx lighting intensity). The micropropagation procedure according to the present invention, the vegetative propagation of lettuce in an *in vitro* culture, which is a subject of the present invention makes it possible to produce at least several dozen vegetative clones for each plant of a transgenic lettuce line, which stems from the high effciciency of proliferatio, some 8-13 clones per passage with at least 8 passages (Fig. 4). An ELISA test was used to ascertain the content of HBsAg proteins in plants (See

Example 3). The variance of HBsAg levels was examined in vegetative clones of individual plants, between the clones of consecutive plants of a given line as well as the significance of the differences in HBsAg levels between clone plants and control plants, meaning ones not propagated vegetatively. The above parameters were analyzed statistically χ^2 test, F test) (following logarithmic transformation of each individual parental line) through analysis of variance taking into account the constant effects of proliferated plant progeny (T1 generation) as well as random effects of the vegetative clones and allowing for heterogenous variance between clones. The calculations were performed using GenStat 10® software (VSN International Ltd.). The plant clones produced were characterised by a high level of HBsAg proteins, which in 64% of the clones did not differ from control plants grown from seeds and not passaged through the *in vitro* culture and micropropagation. Moreover, the HBsAg protein content in the clones of a given plant was similar, and the observed deviations in a majority of cases were statistically insignificant. For 75% of the clones, the differences in the HBV vaccinating protein content did not exceed 20% (Fig. 5, Tab. 1). The described procedure of propagating lettuce turned out to be equally efficient for plants producing different HBsAg antigens. It can be stated with a high degree of certainty that HBcAg level in plants propagated as described above would not exceed the 20% variance threshold in at least 75% of the vegetative clones.

The procedure of micropropagating lettuce, its vegetative propagation in an *in vitro* culture, being the subject of the invention facilitates the propagation of plants on a semi-industrial and industrial scale, ensuring the production of large quantities of initial vaccinating material with desirable properties: a high and stable level of a given HBV antigenic protein produced.

The salts, growth and development regulators as well as the remaining medium reagents were purchased from POCh and Sigma.

Example 3. Determination of HBV antigens in transgenic plants of lettuce, tobacco and peas using ELISA

The content of the HBs and HBc antigens in plant tissues was determined using ELISA via designed tests using: primary monoclonal antibodies (Mab): anti-SHBs C86132M and C86123M (Biodesign), anti-preS2 C8A031M (Biodesign), anti-preS1 5a-19 (obtained from Prof. A Budkowska, Instytut Pasteur'a) as well as anti-HBc C31190M (Biodesign); primary polyclonal antibodies (Pab) anti-HBs B65811R (Biodesign) as well polyclonal anti-HBc antibodies from rabbit serum, obtained from Prof. B Szewczyk (University of Gdańsk) following a triple immunisation of a New Zealand Large rabbit

with the antigen antigen HBc R8A120 (Biodesign). The specially designed tests make it possible to determine the total level of HBsAg and HBcAg proteins in the tested material, ones formed into VLPs, pseudocapsids, aggregates of several dozen molecules as well as single molecules.

In order to determine HBsAg and HBcAg in transgenic plants, we prepared extracts through grinding seed or leaf samples samples in a buffer gradually added up to a volue equal to the mass of the sample multiplied fiftyfold (1 mg = 50 µl buffer). The extraction was berformed using the following buffer: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 10.3 mM Na_2SO_3, 2% PVP40000, 0.2% BSA, 1% Tween® 20, pH = 7.4. The ground up samples were centrifuged twice for 5 minutes at 10000 RPM at room temperature. 20 µl extract aliquots were collected from the supernatant for determination. The ELISA tests were performed on Nunc-Immuno Plate F96 Maxisorb plates from NUNC™ using a Model 1575 rinser from Bio-Rad. The plate was coated overnight at 4°C with a monoclonal antibody against a given antigen in carbonate buffer, at a concentration of 0.5 µg/ml. The plate was rinsed 3 times in PBST, and then blocked for 60-90 minutes at 25°C with 5% skim milk in PBS. The sample was rinsed as above and plant extracts were loaded, which were then diluted with PBS 1/1 to obtain 50-, 100-, 200- and 400-fold dilutions. plate was incubated for 45 min. at room temperature on a shaker set at 400 RPM Following reagent removal and a triple rinse, as above, Pab B65811R anti-HBs or rabbit anti-HBc serum diluted 5000× in PBS and were loaded and incubated for 45 min. at room temp and 400 RPM. Following reagent removal and a triple rinse as above, the plate was loaded with biotinylated monoclonal antibody anti-Fc rabbit (Sigma) diluted 20000× in PBS and incubated for 45 min. at room temp and 400 RPM. Following reagent removal and triple rinsing as above, the plate was loaded with streptavidin conjugated with alkaline phosphatase (Sigma) diluted 4000× in PBS and incubated for 45 min. at room temperature and 400 RPM. Following reagent removal and triple rinsing the substrate alkaline phosphatase was loaded, p-nitrophenyl phosphate (pNPP, Sigma). Most of the reagents were loaded at 100 µl/well, except for the blocker and rinsing solution, which were loaded at 400 µl. After 1 hour and stopping the reaction, the absorbance was read at 405 nm using a Model 680 microplate reader from Bio-Rad. Absorbance was recalculated in terms of the content of a given protein according to a calibration curve preformed for each reading using the appropriate standards: R86800 and R86872 (Biodesign) for S-HBsAg, M/L-HBsAg preparation obtained from Prof. R. Schirmbeck of the University of Ulm, Germany as well as R8A120 (Biodesign) for HBcAg.

The antigenic protein content was determined at least three times during the development of the plants *ex vitro*. The average level of a given protein was calculated as an arythmetic mean with a standard deviation of partial determinations (Figs. 3, 5, 6, 8).

The analysis reagents, excluding the antibodies were purchased from POCh and Sigma.

Example 4. Detection of the HBV antigen in transgenic lettuce and tobacco using western blotting.

The individual HBV antigenic proteins were detected in transgenic plants using western blotting using specific antibodies. S-HBsAg was detected using monoclonal antibody (Mab) C86600M (Biodesign), M-HBsAg was detected using Mab C86600M as well as a polyclonal antibody (Pab) B65811R (Biodesign). L-HBsAg was detected using Pab B65811R, whereas HBcAg was analysed using Mab C86340M (Biodesign) as well as polyclonal antibodies present in rabbit serum obtained from Prof. B Szewczyk (University of Gdańsk) following the triple immunization of a New Zealand White rabbit with HBc R8A120 (Biodesign).

In order to perform the western blots, plant extracts were made through grinding the samples of lettuce leaves in five volumes (1 mg = 5 µl) PBS buffer with 0.5% Tween® 20. The ground up tissues were supplemented with denaturing sample buffer (Laemmli 1970) with 50 mM DTT and incubated for 15 min. at 65°C. The extract samples, protein markers (S-HBsAg and M/L-HBsAg obtained from Prof. R. Schirmbeck, University of Ulm, Germany; HBcAg – R8A120 Biodesign) as well as molecular mass marker (MBI Fermentas) were loaded onto a 12.5% denaturing gel and electrophoresis was performed in the Laemmli buffer. The protein was transferred from the gel onto a nitrocellulose membrane via a "wet" electrotransfer in a Bio-Rad cell. After washing 3x in TBST (TBS with 0.05% Tween® 20), the membrane with transferred proteins was blocked with 3% BSA in TBS. Following 3x rinsing in TBST, the membrane was incubated in an antibody solution in TBS: 1 µg/ml of a given Mab or 0.5 µg/ml of a given PaB Following 3x rinsing in TBST, the membrane was incubated in solutions of secondary antibody conjugated with antibodies in TBS: 10000× dilution of mouse monoclonal antibody anti-rabbit Fc (Sigma) or 10000× dilution of goat polyclonal anti-rabbit antibodies of the "whole molecule" type (Sigma) or 10000× diluted goat polyclonal "anti-mouse" antibodies of the "whole molecule" type (Sigma). Following 3x rinses in TBST, the membrane was incubated in a 4000× solution of extravidin tagged with horseradish peroxidase or alkaline phosphatase (Sigma). Following 5x rinses in TBST, the membrane was incubated in diaminobenzidine (DAB, Sigma) as a substrate for the peroxidase or 5-bromo-4-chloro-3-indolilo-phosphate/

nitro blue tetrazolium (BCIP/NBT, Sigma) as a substrate for the alkaline phosphatase. All of the membrane incubations and rinses were performed withAgitation at ca. 100 rpm.

The observed western blot bandings (Fig. 7) correspond to various forms of HBsAg and HBcAg proteins: glycosylated and unmodified monomers. Dimers were observed for S-HBsAg and HBcAg (Fig. 7), which are the first stage of assembly of VLPs from S-HBsAg or pseudocapsids from HBcAg (Fig. 9B).

Salts for the buffers, BSA etc. were purchased from POCh and Sigma.

Example 5. Preparation of lyophilisates containing HBV vaccinating antigens from transgenic lettuce leaves.

Plants of a selected transgenic lettuce line, characterised by relatively high levels of HBsAg or HBcAg, above 15 µg/ g FW were cultured in a hothouse under a natural fotoperiod and 20-22°C during the day and 14-16°C at night. Leaves from well-growing heads were collected, frozen and partially disrupted in liquid nitrogen, and then stored at –80°C. The frozen material was placed in a BETA 1-16 lyophiliser from CHRIST® and lyophilised for 24-36 h in a 0.2 mbar vacuum at a freezing temperature of –80°C and a shelf temperature of 55°C, on which the material was placed on metal trays. The llyophilised material was pulverised and stored in sealed containers in the presence of a dessicator at room temperature, to be used as the raw materiel in the manufacture of various forms of oral vaccine (Fig. 9A). The procedure of lyophilising plant material was controlled through the determination of the HBs and HBc antigens according to the procedure described in Example 3.

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tacgcgatag aagacaaaat atagcgcgca aactaggata aattatcgcg cgcggtgtca 1980
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ttgcgttgat gaagctt 2417

<210> 6
<211> 2801
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette PlegA-MHBs*ayw4*-NOSt in the binary vector pPLAHBMBAR
<400> 6

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gcaattaagt agagaaaaca aaaagaataa atggaagaag ttgaggaaat ctatatttac 180
acgatcaatt agtatgtgtt aagagtcatg tatcatgatc aattagtatg tgttaaagtc 240
ttgtatcaga taatatataa tccaaatata tttttctaaa tgaggacaaa tctaacctta 300
caaataagtt ttttagagtt aaattagatt caatcacatt ttatttttta ttttttgaac 360
agtaagaaat aagatctata ttttcttctc tatttgttta cgtccataca aaaaatgtgc 420
aatgattgtg aaagatgtca tgcatatgca gtcaccatat attatttaca taaaaagaac 480
tacttattct ttcggcctca aattttacct aggaattatg tatgcaaata tgaaatattc 540
atggactttt ttcgtccatt ctttctctgg aaattactcc ctatgtttat agaatttgat 600
ttcttttgag taaattagca ctttaaatgt aaaagtatgg catcttatca aacaaccggt 660
tgatgaaaat ttcacatttt caggtagtaa tatgaaattg ataatggaaa gatgatatag 720
tattaataat aaatatattt gaaaagataa caataaatgt attatatcta taaatttaca 780
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ggctattccc actaattcgt cctgctggtt tgcgccaaga taaatcagtg catctcctta 2580
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cctgagaaat aggcctacct caagagcaag tcctttctgt gctcgtcgga aattcctctc 2760
ctgtcagacg gtcgtgcgca tgtcttgcgt tgatgaagct t 2801

<210> 7
<211> 2801
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette PlegA-MHBsa*dw4*-NOSt in the binary vector pPLAHBMadwBAR
<400> 7

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gcaattaagt agagaaaaca aaaagaataa atggaagaag ttgaggaaat ctatatttac 180
acgatcaatt agtatgtgtt aagagtcatg tatcatgatc aattagtatg tgttaaagtc 240
ttgtatcaga taatatataa tccaaatata tttttctaaa tgaggacaaa tctaacctta 300
caaataagtt ttttagagtt aaattagatt caatcacatt ttatttttta ttttttgaac 360
agtaagaaat aagatctata ttttcttctc tatttgttta cgtccataca aaaaatgtgc 420
aatgattgtg aaagatgtca tgcatatgca gtcaccatat attatttaca taaaaagaac 480
tacttattct ttcggcctca aattttacct aggaattatg tatgcaaata tgaaatattc 540
atggactttt ttcgtccatt ctttctctgg aaattactcc ctatgtttat agaatttgat 600
ttcttttgag taaattagca ctttaaatgt aaaagtatgg catcttatca aacaaccggt 660
tgatgaaaat ttcacatttt caggtagtaa tatgaaattg ataatggaaa gatgatatag 720
tattaataat aaatatattt gaaaagataa caataaatgt attatatcta taaatttaca 780
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cctgagaaat aggcctacct caagagcaag tcctttctgt gctcgtcgga aattcctctc 2760
ctgtcagacg gtcgtgcgca tgtcttgcgt tgatgaagct t 2801

<210> 8
<211> 2741
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette P35S-LHBs*ayw4*-NOSt in the binary vector pKHBLBAR
<400> 8

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aggcttacgc agcaggtctc atcaagacga tctacccgag caataatctc caggaaatca 120
aataccttcc caagaaggtt aaagatgcag tcaaaagatt caggactaac tgcatcaaga 180
acacagagaa agatatattt ctcaagatca gaagtactat tccagtatgg acgattcaag 240
gcttgcttca caaaccaagg caagtaatag agattggagt ctctaaaaag gtagttccca 300
ctgaatcaaa ggccatggag tcaaagattc aaatagagga cctaacagaa ctcgccgtaa 360
agactggcga acagttcata cagagtctct tacgactcaa tgacaagaag aaaatcttcg 420
tcaacatggt ggagcacgac acacttgtct actccaaaaa tatcaaagat acagtctcag 480
aagaccaaag ggcaattgag acttttcaac aaagggtaat atccggaaac ctcctcggat 540
tccattgccc agctatctgt cactttattg tgaagatagt ggaaaaggaa ggtggctcct 600
acaaatgcca tcattgcgat aaaggaaagg ccatcgttga agatgcctct gccgacagtg 660
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cgtcttcaaa gcaagtggat tgatgtgata tctccactga cgtaagggat gacgcacaat 780
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cctgagaaat aggcctacct caagagcaag tcctttctgt gctcgtcgga aattcctctc 2700
ctgtcagacg gtcgtgcgca tgtcttgcgt tgatgaagct t 2741

<210> 9
<211> 2774
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette P35S-LHBs*adw4*-NOSt in the binary vector pKHBLadwBAR
<400> 9

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aggcttacgc agcaggtctc atcaagacga tctacccgag caataatctc caggaaatca 120
aataccttcc caagaaggtt aaagatgcag tcaaaagatt caggactaac tgcatcaaga 180
acacagagaa agatatattt ctcaagatca gaagtactat tccagtatgg acgattcaag 240
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ctgaatcaaa ggccatggag tcaaagattc aaatagagga cctaacagaa ctcgccgtaa 360
agactggcga acagttcata cagagtctct tacgactcaa tgacaagaag aaaatcttcg 420
tcaacatggt ggagcacgac acacttgtct actccaaaaa tatcaaagat acagtctcag 480
aagaccaaag ggcaattgag acttttcaac aaagggtaat atccggaaac ctcctcggat 540
tccattgccc agctatctgt cactttattg tgaagatagt ggaaaaggaa ggtggctcct 600
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cgttgatgaa gctt 2774

<210> 10
<211> 3125
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette PlegA-LHBs*ayw4*-NOSt in the binary vector pPLAHBLBAR
<400> 10

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acgatcaatt agtatgtgtt aagagtcatg tatcatgatc aattagtatg tgttaaagtc 240
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caaataagtt ttttagagtt aaattagatt caatcacatt ttatttttta ttttttgaac 360
agtaagaaat aagatctata ttttcttctc tatttgttta cgtccataca aaaaatgtgc 420
aatgattgtg aaagatgtca tgcatatgca gtcaccatat attatttaca taaaaagaac 480
tacttattct ttcggcctca aattttacct aggaattatg tatgcaaata tgaaatattc 540
atggactttt ttcgtccatt ctttctctgg aaattactcc ctatgtttat agaatttgat 600
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aaacacagca aatccagatt gggattacaa tcccaacaag gacacctggc cagacgccaa 1380
caaggtagga gctggagcat tcgggctggg attcacccca ccgcacggag gccttttggg 1440
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taccaatcgc cagtcaggaa ggcagcctac cccgctgtct ccacctttga gaaacactca 1560
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gacaagaatc ctcacaatac cgcagagtct agactcgtgg tggacttctc tcaatttttct 1860
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acgtccgaac acttgataca tgtgcctgag aaataggcct acctcaagag caagtccttt 3060
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agctt 3125

<210> 11
<211> 3158
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette PlegA-LHBsa*dw4*-NOSt in the binary vector pPLAHBLadwBAR
<400> 11

gaattcttta gaattatttt tttaggtctc aatagattaa gaagttggcg tctcattgat 60
tgaccatgga caatttgaaa gaaaaaaaag atcacctttg ttttttagag gaaaaaggaa 120
gcaattaagt agagaaaaca aaaagaataa atggaagaag ttgaggaaat ctatatttac 180
acgatcaatt agtatgtgtt aagagtcatg tatcatgatc aattagtatg tgttaaagtc 240
ttgtatcaga taatatataa tccaaatata tttttctaaa tgaggacaaa tctaacctta 300
caaataagtt ttttagagtt aaattagatt caatcacatt ttatttttta ttttttgaac 360
agtaagaaat aagatctata ttttcttctc tatttgttta cgtccataca aaaaatgtgc 420
aatgattgtg aaagatgtca tgcatatgca gtcaccatat attatttaca taaaaagaac 480
tacttattct ttcggcctca aattttacct aggaattatg tatgcaaata tgaaatattc 540
atggactttt ttcgtccatt ctttctctgg aaattactcc ctatgtttat agaatttgat 600
ttcttttgag taaattagca ctttaaatgt aaaagtatgg catcttatca aacaaccggt 660
tgatgaaaat ttcacatttt caggtagtaa tatgaaattg ataatggaaa gatgatatag 720
tattaataat aaatatattt gaaaagataa caataaatgt attatatcta taaatttaca 780
aggttcttat atttacatac aaacaaatgc agtaatgttt caaacaatat gcagtaagta 840
attaacactt taatttgaag gattaatcaa tttggtaact gaagtagcta attgaaagtt 900
tattctttat aaatctttgt aatgcagaat atgtaagaaa gaaacatgga gtataagaag 960
taaagccatg gtcccctgcc accgatttca gctataagaa ttgcaagtat gctctttgtc 1020
tggtaatgga gatgatgaag ccattagcca cctcctctat cagacatagg tgtaaagcat 1080
tatgcttcca tagccatgca agctgcagaa tgtccaattc tcaacatccc actttcaatg 1140
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ccgcatcaca accaacattc tcttagtatc tctcttcatg ggatccatgg gaggttggtc 1260
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tcccgatcat cagttggacc ctgcattcgg agccaactca aacaatccag attgggactt 1380
caaccccatc aaggaccact ggccagcagc caaccaggta ggagtgggag cattcgggcc 1440
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gaccacagtg tcaacaattc ctcctcctgc ctacaccaat cggcagtcag gaaggcagcc 1560
tactcccatc tctccacctc taagagacag tcatcctcag gccatgcagt ggaactccac 1620
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ctcgaggatt ggggaccctg cgctgaacat ggagaacatc acatcaggat tcctaggacc 1800
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gttcctctgt cttgtgaaat gaactgctga ctgcccccca agaaagcctc ctcatctccc 3000
agttggcggc ggctgataca ccatcgaaaa cccacgtccg aacacttgat acatgtgcct 3060
gagaaatagg cctacctcaa gagcaagtcc tttctgtgct cgtcggaaat tcctctcctg 3120
tcagacggtc gtgcgcatgt cttgcgttga tgaagctt 3158

<210> 12
<211> 2123
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette P35S-HBc*ayw4*-NOSt in the binary vector pKHBCBAR
<400> 12

gaattcccca gattagcctt ttcaatttca gaaagaatgc taacccacag atggttagag 60
aggcttacgc agcaggtctc atcaagacga tctacccgag caataatctc caggaaatca 120
aataccttcc caagaaggtt aaagatgcag tcaaaagatt caggactaac tgcatcaaga 180
acacagagaa agatatattt ctcaagatca gaagtactat tccagtatgg acgattcaag 240
gcttgcttca caaaccaagg caagtaatag agattggagt ctctaaaaag gtagttccca 300
ctgaatcaaa ggccatggag tcaaagattc aaatagagga cctaacagaa ctcgccgtaa 360
agactggcga acagttcata cagagtctct tacgactcaa tgacaagaag aaaatcttcg 420
tcaacatggt ggagcacgac acacttgtct actccaaaaa tatcaaagat acagtctcag 480
aagaccaaag ggcaattgag acttttcaac aaagggtaat atccggaaac ctcctcggat 540
tccattgccc agctatctgt cactttattg tgaagatagt ggaaaaggaa ggtggctcct 600
acaaatgcca tcattgcgat aaaggaaagg ccatcgttga agatgcctct gccgacagtg 660
gtcccaaaga tggaccccca cccacgagga gcatcgtgga aaaagaagac gttccaacca 720
cgtcttcaaa gcaagtggat tgatgtgata tctccactga cgtaagggat gacgcacaat 780
cccactatcc ttcgcaagac ccttcctcta tataaggaag ttcatttcat ttggagagaa 840
cacgggggac tctagaggat ccatggacat cgacccttat aaagaatttg gagctactgt 900
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aacggtcata gagtatttgg tgtctttcgg agtgtggatt cgcactcctc cagcttatag 1260
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caggtccccc agaagaagaa ctccctcgcc tcgcagacga agatctcaat cgccgcgtcg 1380
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aagtttctgc tcatgccgac aggcataact tagatattcg cgggctattc ccactaattc 1860
gtcctgctgg tttgcgccaa gataaatcag tgcatctcct tacaagttcc tctgtcttgt 1920
gaaatgaact gctgactgcc ccccaagaaa gcctcctcat ctcccagttg gcggcggctg 1980
atacaccatc gaaaacccac gtccgaacac ttgatacatg tgcctgagaa ataggcctac 2040
ctcaagagca agtcctttct gtgctcgtcg gaaattcctc tcctgtcaga cggtcgtgcg 2100
catgtcttgc gttgatgaag ctt 2123

<210> 13
<211> 2129
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette P35S-HBc*adw4*-NOSt in the binary vector pKHBCadwBAR
<400> 13

gaattcccca gattagcctt ttcaatttca gaaagaatgc taacccacag atggttagag 60
aggcttacgc agcaggtctc atcaagacga tctacccgag caataatctc caggaaatca 120
aataccttcc caagaaggtt aaagatgcag tcaaaagatt caggactaac tgcatcaaga 180
acacagagaa agatatattt ctcaagatca gaagtactat tccagtatgg acgattcaag 240
gcttgcttca caaaccaagg caagtaatag agattggagt ctctaaaaag gtagttccca 300
ctgaatcaaa ggccatggag tcaaagattc aaatagagga cctaacagaa ctcgccgtaa 360
agactggcga acagttcata cagagtctct tacgactcaa tgacaagaag aaaatcttcg 420
tcaacatggt ggagcacgac acacttgtct actccaaaaa tatcaaagat acagtctcag 480
aagaccaaag ggcaattgag acttttcaac aaagggtaat atccggaaac ctcctcggat 540
tccattgccc agctatctgt cactttattg tgaagatagt ggaaaaggaa ggtggctcct 600
acaaatgcca tcattgcgat aaaggaaagg ccatcgttga agatgcctct gccgacagtg 660
gtcccaaaga tggaccccca cccacgagga gcatcgtgga aaaagaagac gttccaacca 720
cgtcttcaaa gcaagtggat tgatgtgata tctccactga cgtaagggat gacgcacaat 780
cccactatcc ttcgcaagac ccttcctcta tataaggaag ttcatttcat ttggagagaa 840
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taataatttg gaagatccag catccaggga tctagtagtc aattatgtta ataataacat 1140
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gttgccggtc ttgcgatgat tatcatataa tttctgttga attacgttaa gcatgtaata 1560
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cgcgcggtgt catctatgtt actagatcga tcaaacttcg gcactgtgta atgacgatga 1740
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taattcgtcc tgctggtttg cgccaagata aatcagtgca tctccttaca agttcctctg 1920
tcttgtgaaa tgaactgctg actgcccccc aagaaagcct cctcatctcc cagttggcgg 1980
cggctgatac accatcgaaa acccacgtcc gaacacttga tacatgtgcc tgagaaatag 2040
gcctacctca agagcaagtc ctttctgtgc tcgtcggaaa ttcctctcct gtcagacggt 2100
cgtgcgcatg tcttgcgttg atgaagctt 2129

<210> 14
<211> 2507
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette PlegA-HBc*ayw4*-NOSt in the binary vector pPLAHBCBAR
<400> 14

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tgaccatgga caatttgaaa gaaaaaaaag atcacctttg ttttttagag gaaaaaggaa 120
gcaattaagt agagaaaaca aaaagaataa atggaagaag ttgaggaaat ctatatttac 180
acgatcaatt agtatgtgtt aagagtcatg tatcatgatc aattagtatg tgttaaagtc 240
ttgtatcaga taatatataa tccaaatata tttttctaaa tgaggacaaa tctaacctta 300
caaataagtt ttttagagtt aaattagatt caatcacatt ttatttttta ttttttgaac 360
agtaagaaat aagatctata ttttcttctc tatttgttta cgtccataca aaaaatgtgc 420
aatgattgtg aaagatgtca tgcatatgca gtcaccatat attatttaca taaaaagaac 480
tacttattct ttcggcctca aattttacct aggaattatg tatgcaaata tgaaatattc 540
atggactttt ttcgtccatt ctttctctgg aaattactcc ctatgtttat agaatttgat 600
ttcttttgag taaattagca ctttaaatgt aaaagtatgg catcttatca aacaaccggt 660
tgatgaaaat ttcacatttt caggtagtaa tatgaaattg ataatggaaa gatgatatag 720
tattaataat aaatatattt gaaaagataa caataaatgt attatatcta taaatttaca 780
aggttcttat atttacatac aaacaaatgc agtaatgttt caaacaatat gcagtaagta 840
attaacactt taatttgaag gattaatcaa tttggtaact gaagtagcta attgaaagtt 900
tattctttat aaatctttgt aatgcagaat atgtaagaaa gaaacatgga gtataagaag 960
taaagccatg gtcccctgcc accgatttca gctataagaa ttgcaagtat gctctttgtc 1020
tggtaatgga gatgatgaag ccattagcca cctcctctat cagacatagg tgtaaagcat 1080
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actgtgtaat gacgatgagc aatcgagagg ctgactaaca aaaggtatgc ccaaaaacaa 2160
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catgtgcctg agaaataggc ctacctcaag agcaagtcct ttctgtgctc gtcggaaatt 2460
cctctcctgt cagacggtcg tgcgcatgtc ttgcgttgat gaagctt 2507

<210> 15
<211> 2513
<212> DNA
<213> Artificial
<220>
<223> Sequence of the expression cassette PlegA-HBc*adw4*-NOSt in the binary vector pPLAHBCadwBAR
<400> 15

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tgaccatgga caatttgaaa gaaaaaaaag atcacctttg ttttttagag gaaaaaggaa 120
gcaattaagt agagaaaaca aaaagaataa atggaagaag ttgaggaaat ctatatttac 180
acgatcaatt agtatgtgtt aagagtcatg tatcatgatc aattagtatg tgttaaagtc 240
ttgtatcaga taatatataa tccaaatata tttttctaaa tgaggacaaa tctaacctta 300
caaataagtt ttttagagtt aaattagatt caatcacatt ttatttttta ttttttgaac 360
agtaagaaat aagatctata ttttcttctc tatttgttta cgtccataca aaaaatgtgc 420
aatgattgtg aaagatgtca tgcatatgca gtcaccatat attatttaca taaaaagaac 480
tacttattct ttcggcctca aattttacct aggaattatg tatgcaaata tgaaatattc 540
atggactttt ttcgtccatt ctttctctgg aaattactcc ctatgtttat agaatttgat 600
ttcttttgag taaattagca ctttaaatgt aaaagtatgg catcttatca aacaaccggt 660
tgatgaaaat ttcacatttt caggtagtaa tatgaaattg ataatggaaa gatgatatag 720
tattaataat aaatatattt gaaaagataa caataaatgt attatatcta taaatttaca 780
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attaacactt taatttgaag gattaatcaa tttggtaact gaagtagcta attgaaagtt 900
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ccgcatcaca accaacattc tcttagtatc tctcttcatg ggatccatgg acattgaccc 1260
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agaaatattt caataactga ttatatcagc tggtacattg ccgtagatga aagactgagt 1080
gcgatattat gtgtaataca taaattgatg atatagctag ctta 1124

Claims

1. An expression cassette comprising the sequence encoding the small (S-HBsAg), medium (M-HBsAg) or large (L-HBsAg) subunits of the surface antigen or core antigen (HBcAg) of HBV of the strain *ayw4* or *adw4*, as well as regulatory sequences controlling its expression, preferably Cauliflower Mosaic Virus (CaMV) 35S RNA promoter or the *legA* promoter as well as the sequence of the nopaline synthase terminator (NOSt).
2. An expression cassette according to Claim 1, characterised in that contains a sequence represented as SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14 or SEQ ID No. 15.
3. A T-DNA molecule comprising T-DNA flanking sequences as well as two xpression cassettes located between them:

- of the expression cassette comprising a sequence encoding the S-HBsAg or M-HBsAg or L-HBsAg or HBcAg protein of the HBV strain *ayw4* or *adw4* as well as regulatory sequences controlling its expression, preferably the CaMV 35S RNA promoter with a single enhancer and nopaline synthase transcription terminator (NOSt) or of the expression cassette comprising a sequence encoding the S-HBsAg, M-HBsAg or L-HBsAg or HBcAg protein as well as regulatory sequences controlling its expression, preferably the sequence of the *legA* promoter and nopaline synthase transcription terminator (NOSt), as well as
- of the expression cassette comprising a sequence encoding a herbicide resistance gene, perferably the *bar* gene, as well as regulatory sequences controlling its expression, preferably the sequence of the nopaline synthase promoter (PNOS) and g7t transcription terminator.

4. A T-DNA molecule according to Claim 3,, characterised in that it contains at least one of the following sequences: SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16.
5. An expression vector containing an expression cassette according to Claim 1-2, preferably a T-DNA molecule according to Claim 3-4.
6. An *E. coli* cell containing an expression vector according to Claim 5.
7. An Ag*robacterium tumefaciens* cell containing an expression vector according to Claim 5.

8. The use of a plant expression vector according to Claim 5 in the production of transgenic plants, preferably lettuce, tobacco and peas as well as the use of a vector according to Claim 5 of invention P.382769 in the production of tobacco and peas.
9. A transgenic plant cell comprising a T-DNA molecule according to Claim 4, which possesses resistance to phosphinotricine herbicides and and is capable of expressing HBV proteins of the strains *ayw4* or *adw4*: the small surface antigen S-HBsAg at a level ≥ 5 μg/g FW or the medium surface antigen M-HBsAg at a level ≥ 4 μg/g FW or the large surface antigen L-HBsAg at a level ≥ 2 μg/g FW or HBcAg core antigen at a level ≥ 50 μg/g FW.
10. A cell according to Claim 9, characterised in that it is a lettuce cell.
11. A cell according to Claim 9, characterised in that t is a pea cell.
12. A cell according to Claim 9, characterised in that it is a tobacco cell.
13. A method of vegetatively propagating lettuce plants regenerated from cells according Claims 9 – 10, characterised in that: a) it has an efficiency ≥ 8 progeny plants per initial plant/propagation cycle; b) at least 60% of the plant clones produced are characterised by a content of the small S-HBsAg or medium M-HBsAg or large L-HBsAg surface antigen or HBcAg core antigen proteins of HBV at a level not statistically significantly different; c) at least 75% of the plant clones produced are characterised by a statistically even to ±20%, content of the small S-HBsAg or medium M-HBsAg or large L-HBsAg surface antigen or HBcAg core antigen proteins of HBV; d) it can be used for the industrial propagation of plants producing S-HBsAg or M-HBsAg or L-HBsAg or HBcAg as raw materials for a vaccines.
14. A use of a plant cell according to Claims 9-11 and 13 in the production of an oral vaccine against viral Hepatitis type B
15. A use according to Claim 14, characterised in that the plant cells used are in the form of plant biomass, particularly a lyophilised plant material, preferably lettuce or peas.
16. A use according to Claim 14, characterised in that vaccine produced is in the form of a suspension, syrup, granulate, tablets or capsules.
17. The use of a plant cell according to Claim 9 and 12 in the production of a parenterallly or nasally administered vaccine against viral Hepatitis type B
18. A use according to Claim 17, characterised in that the plant cells used, preferably tobacco, produce the small S-HBsAg or medium M-HBsAg or large L-HBsAg surface antigen or HBcAg core antigen proteins of HBV, which may be extravted and purified.
19. A use according to Claim 17, characterised in that the vaccine produced is in the form of an injection administered parenterally, or nasally as an aerosol or droplets.

Claims

1. An expression cassette comprising the sequence encoding the small (S-HBsAg), medium (M-HBsAg) or large (L-HBsAg) subunits of the surface antigen or core antigen (HBcAg) of HBV of the strain *ayw4* or *adw4*, as well as regulatory sequences controlling its expression, preferably Cauliflower Mosaic Virus (CaMV) 35S RNA promoter or the *legA* promoter as well as the sequence of the nopaline synthase terminator (NOSt).
2. An expression cassette according to Claim 1, characterised in that contains a sequence represented as SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14 or SEQ ID No. 15.
3. A T-DNA molecule comprising T-DNA flanking sequences as well as two xpression cassettes located between them:

- of the expression cassette comprising a sequence encoding the S-HBsAg or M-HBsAg or L-HBsAg or HBcAg protein of the HBV strain *ayw4* or *adw4* as well as regulatory sequences controlling its expression, preferably the CaMV 35S RNA promoter with a single enhancer and nopaline synthase transcription terminator (NOSt) or of the expression cassette comprising a sequence encoding the S-HBsAg, M-HBsAg or L-HBsAg or HBcAg protein as well as regulatory sequences controlling its expression, preferably the sequence of the *legA* promoter and nopaline synthase transcription terminator (NOSt), as well as
- of the expression cassette comprising a sequence encoding a herbicide resistance gene, perferably the *bar* gene, as well as regulatory sequences controlling its expression, preferably the sequence of the nopaline synthase promoter (PNOS) and g7t transcription terminator.

4. A T-DNA molecule according to Claim 3,, characterised in that it contains at least one of the following sequences: SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16.
5. An expression vector containing an expression cassette according to Claim 1-2, preferably a T-DNA molecule according to Claim 3-4.
6. An *E. coli* cell containing an expression vector according to Claim 5.
7. An Ag*robacterium tumefaciens* cell containing an expression vector according to Claim 5.

8. The use of a plant expression vector according to Claim 5 in the production of transgenic plants, preferably lettuce, tobacco and peas as well as the use of a vector according to Claim 5 of invention P.382769 in the production of tobacco and peas.
9. A transgenic plant cell comprising a T-DNA molecule according to Claim 4, which possesses resistance to phosphinotricine herbicides and and is capable of expressing HBV proteins of the strains *ayw4* or *adw4*: the small surface antigen S-HBsAg at a level ≥ 5 μg/g FW or the medium surface antigen M-HBsAg at a level ≥ 4 μg/g FW or the large surface antigen L-HBsAg at a level ≥ 2 μg/g FW or HBcAg core antigen at a level ≥ 50 μg/g FW.
10. A cell according to Claim 9, characterised in that it is a lettuce cell.
11. A cell according to Claim 9, characterised in that t is a pea cell.
12. A cell according to Claim 9, characterised in that it is a tobacco cell.
13. A method of vegetatively propagating lettuce plants regenerated from cells according Claims 9 – 10, characterised in that: a) it has an efficiency ≥ 8 progeny plants per initial plant/propagation cycle; b) at least 60% of the plant clones produced are characterised by a content of the small S-HBsAg or medium M-HBsAg or large L-HBsAg surface antigen or HBcAg core antigen proteins of HBV at a level not statistically significantly different; c) at least 75% of the plant clones produced are characterised by a statistically even to ±20%, content of the small S-HBsAg or medium M-HBsAg or large L-HBsAg surface antigen or HBcAg core antigen proteins of HBV; d) it can be used for the industrial propagation of plants producing S-HBsAg or M-HBsAg or L-HBsAg or HBcAg as raw materials for a vaccines.
14. A use of a plant cell according to Claims 9-11 and 13 in the production of an oral vaccine against viral Hepatitis type B
15. A use according to Claim 14, characterised in that the plant cells used are in the form of plant biomass, particularly a lyophilised plant material, preferably lettuce or peas.
16. A use according to Claim 14, characterised in that vaccine produced is in the form of a suspension, syrup, granulate, tablets or capsules.
17. The use of a plant cell according to Claim 9 and 12 in the production of a parenterallly or nasally administered vaccine against viral Hepatitis type B
18. A use according to Claim 17, characterised in that the plant cells used, preferably tobacco, produce the small S-HBsAg or medium M-HBsAg or large L-HBsAg surface antigen or HBcAg core antigen proteins of HBV, which may be extravted and purified.
19. A use according to Claim 17, characterised in that the vaccine produced is in the form of an injection administered parenterally, or nasally as an aerosol or droplets.

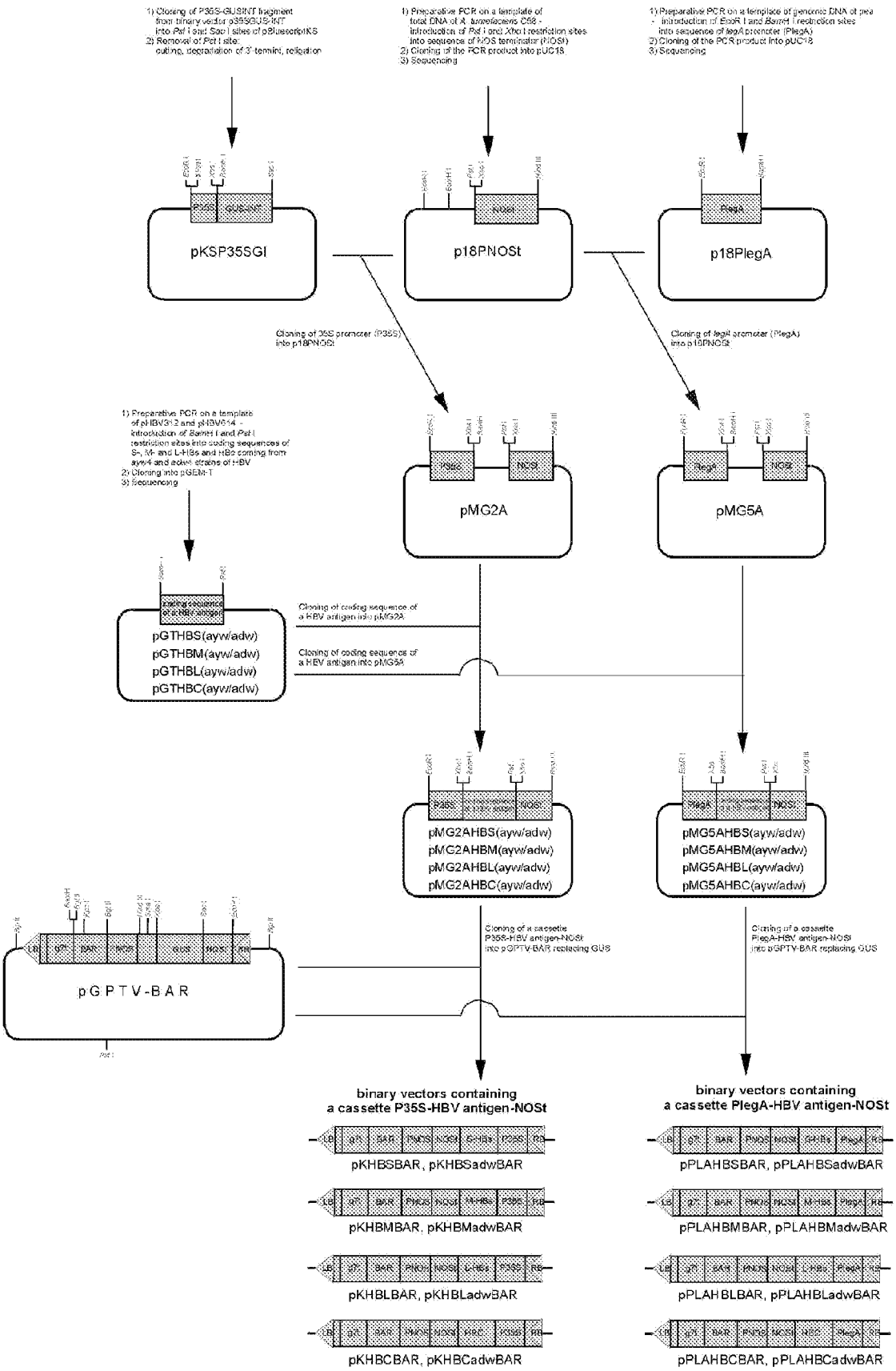

1) Cloning of P35S-GUSINT fragment from binary vector p35SGUS-INT into Pst I and Sac I sites of pBluescriptKS
2) Removal of Pst I site: cutting, degradation of 3' termini, religation
1) Preparative PCR on a template of total DNA of A. tumefaciens C58 - introduction of Pst I and Xho I restriction sites into sequence of NOS terminator (NOSt)
2) Cloning of the PCR product into pUC18
3) Sequencing
1) Preparative PCR on a template of genomic DNA of pea - introduction of EcoR I and BamH I restriction sites into sequence of legA promoter (PlegA)
2) Cloning of the PCR product into pUC18
3) Sequencing
pKSP35SGI
p18PNOSt
p18PlegA
Cloning of 35S promoter (P35S) into p18PNOSt
Cloning of legA promoter (PlegA) into p18PNOSt
1) Preparative PCR on a template of pHBV312 and pHBV914 - introduction of BamH I and Pst I restriction sites into coding sequences of S-, M- and L-HBs and HBc coming from ayw4 and adw4 strains of HBV
2) Cloning into pGEM-T
3) Sequencing
pMG2A
pMG5A
pGTHBS(ayw/adw)
pGTHBM(ayw/adw)
pGTHBL(ayw/adw)
pGTHBC(ayw/adw)
Cloning of coding sequence of a HBV antigen into pMG2A
Cloning of coding sequence of a HBV antigen into pMG5A
pMG2AHBS(ayw/adw)
pMG2AHBM(ayw/adw)
pMG2AHBL(ayw/adw)
pMG2AHBC(ayw/adw)
pMG5AHBS(ayw/adw)
pMG5AHBM(ayw/adw)
pMG5AHBL(ayw/adw)
pMG5AHBC(ayw/adw)
pGPTV-BAR
Cloning of a cassette P35S-HBV antigen-NOSt into pGPTV-BAR replacing GUS
Cloning of a cassette PlegA-HBV antigen-NOSt into pGPTV-BAR replacing GUS
binary vectors containing a cassette P35S-HBV antigen-NOSt
binary vectors containing a cassette PlegA-HBV antigen-NOSt
pKHBSBAR, pKHBSadwBAR
pKHBMBAR, pKHBMadwBAR
pKHBLBAR, pKHBLadwBAR
pKHBCBAR, pKHBCadwBAR
pPLAHBSBAR, pPLAHBSadwBAR
pPLAHBMBAR, pPLAHBMadwBAR
pPLAHBLBAR, pPLAHBLadwBAR
pPLAHBCBAR, pPLAHBCadwBAR

Figure 1

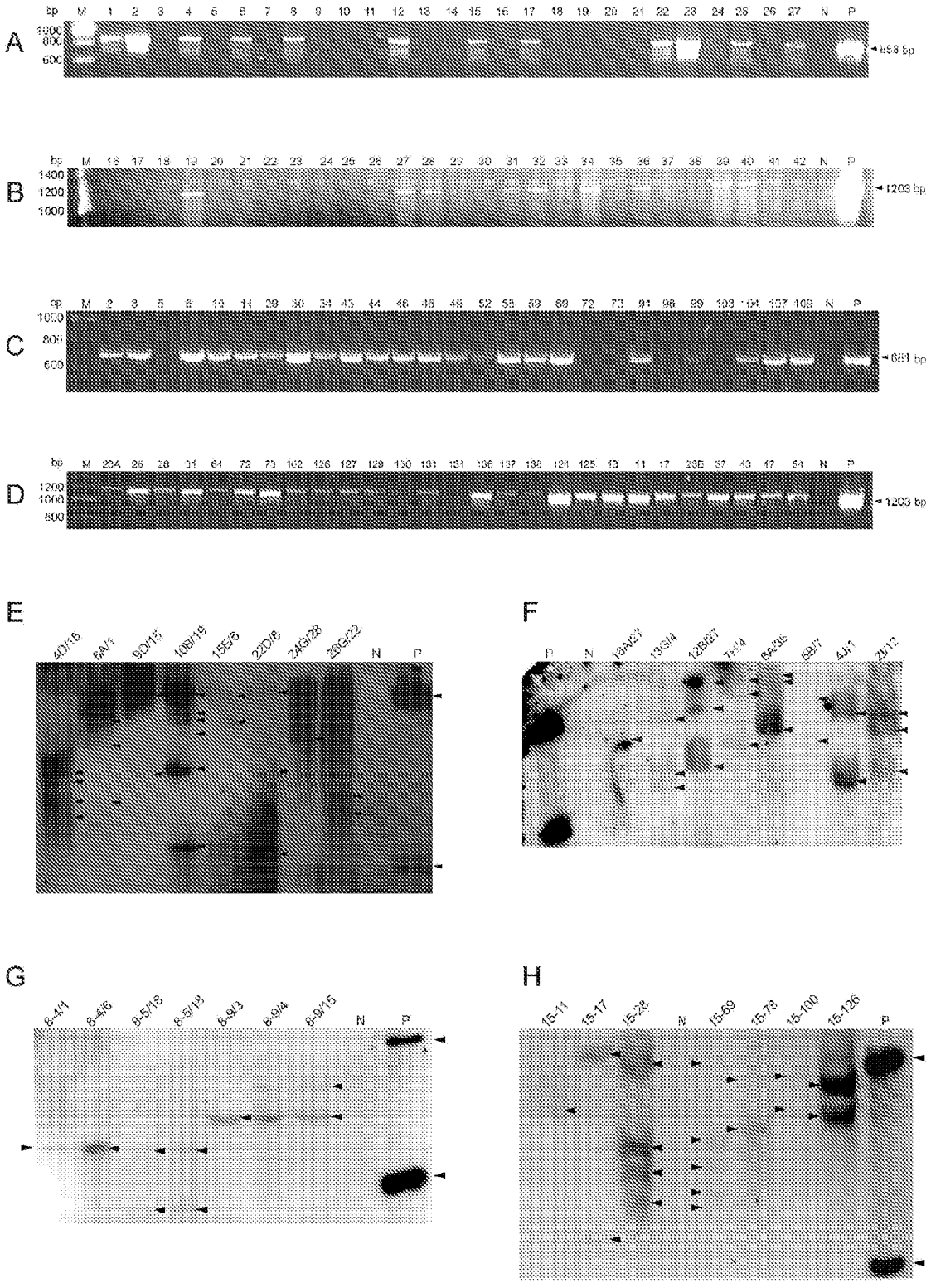


Figure 2

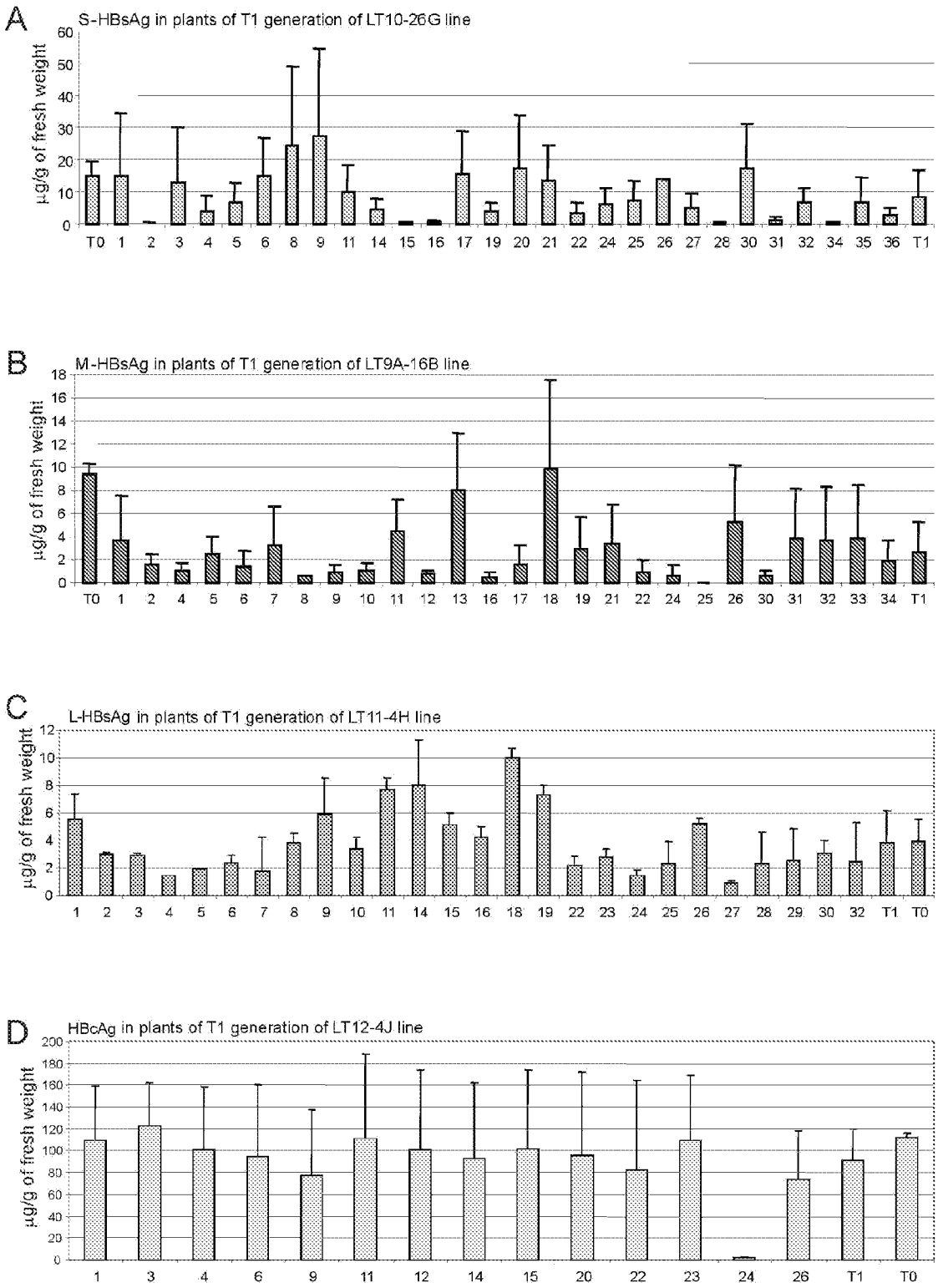
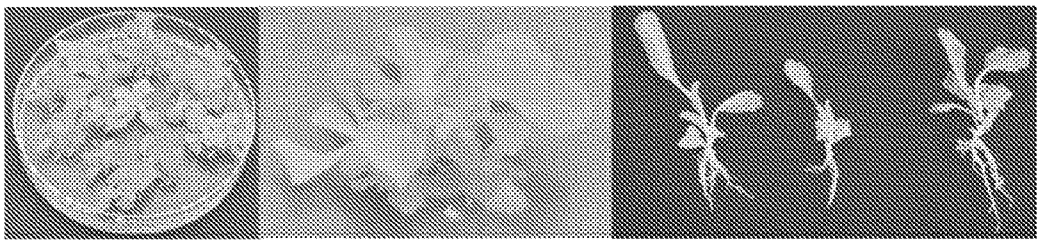
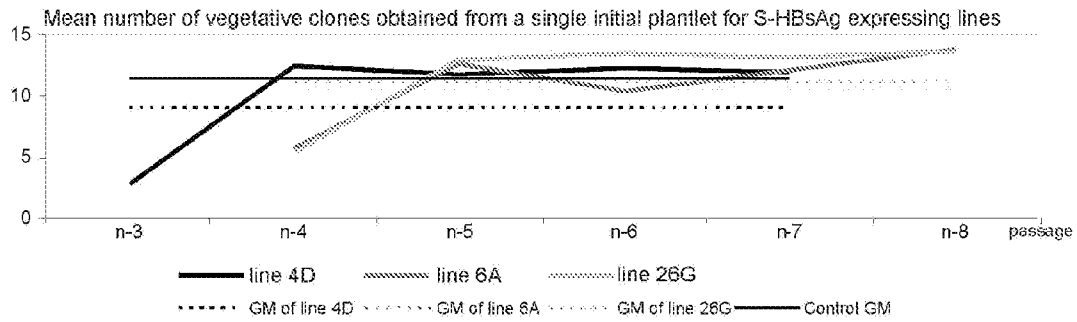

A
S-HBsAg in plants of T1 generation of LT10-26G line
µg/g of fresh weight
0 10 20 30 40 50 60
T0 1 2 3 4 5 6 8 9 11 14 15 16 17 19 20 21 22 24 25 26 27 28 30 31 32 34 35 36 T1
B
M-HBsAg in plants of T1 generation of LT9A-16B line
µg/g of fresh weight
0 2 4 6 8 10 12 14 16 18
T0 1 2 4 5 6 7 8 9 10 11 12 13 16 17 18 19 21 22 24 25 26 30 31 32 33 34 T1
C
L-HBsAg in plants of T1 generation of LT11-4H line
µg/g of fresh weight
0 2 4 6 8 10 12
1 2 3 4 5 6 7 8 9 10 11 14 15 16 18 19 22 23 24 25 26 27 28 29 30 32 T1 T0
D
HBcAg in plants of T1 generation of LT12-4J line
µg/g of fresh weight
0 20 40 60 80 100 120 140 160 180 200
1 3 4 6 9 11 12 14 15 20 22 23 24 26 T1 T0

Figure 3

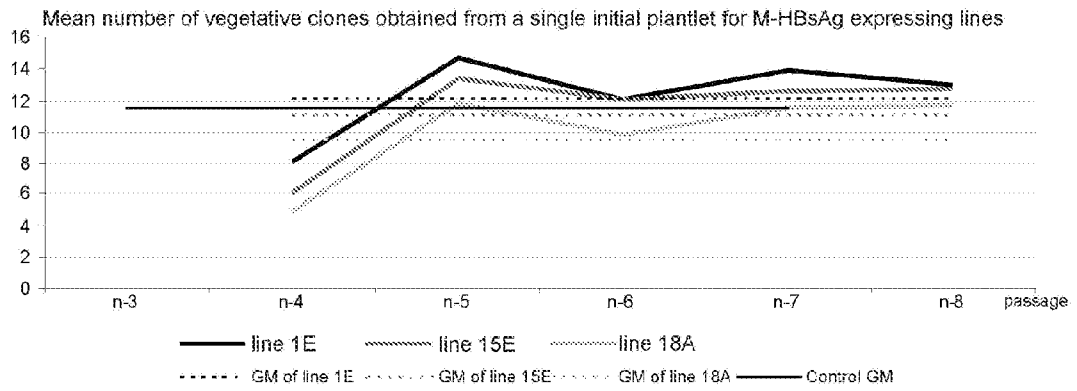
A



B



C



D

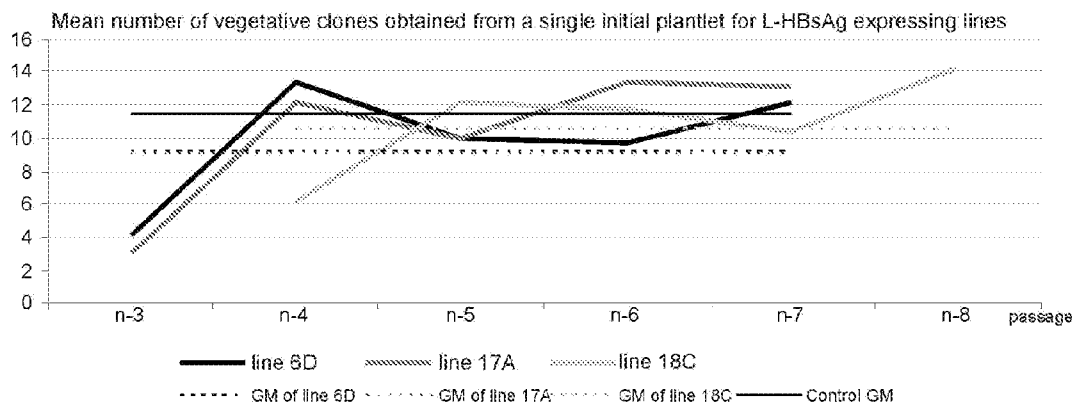
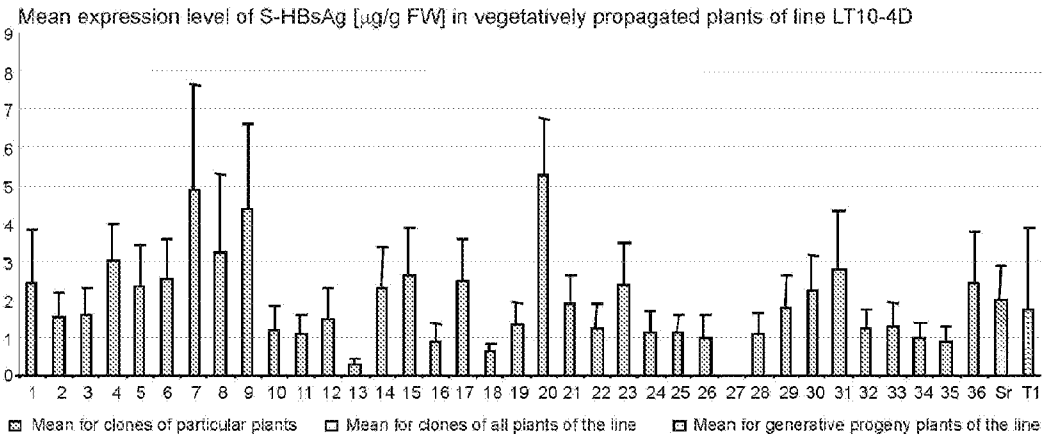
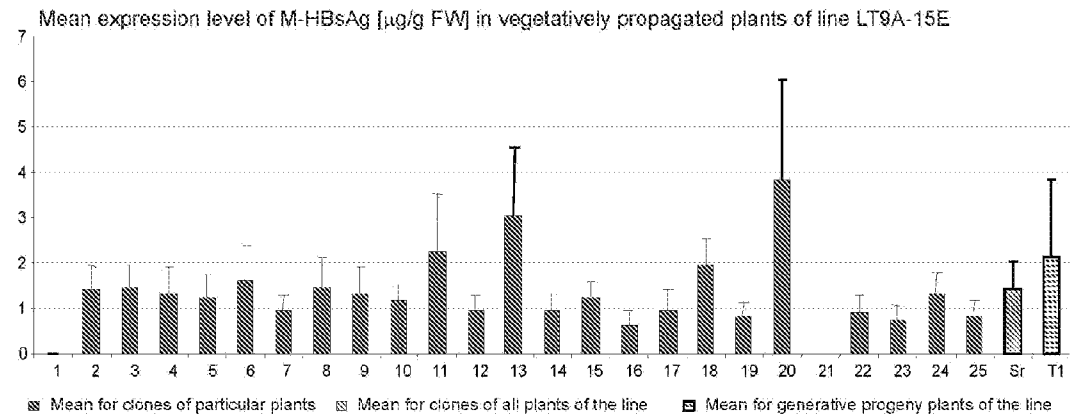


Figure 4

A


Mean expression level of S-HBsAg [µg/g FW] in vegetatively propagated plants of line LT10-4D
0 1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 Sr T1
Mean for clones of particular plants
Mean for clones of all plants of the line
Mean for generative progeny plants of the line

B


Mean expression level of M-HBsAg [µg/g FW] in vegetatively propagated plants of line LT9A-15E
0 1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 Sr T1
Mean for clones of particular plants
Mean for clones of all plants of the line
Mean for generative progeny plants of the line

C

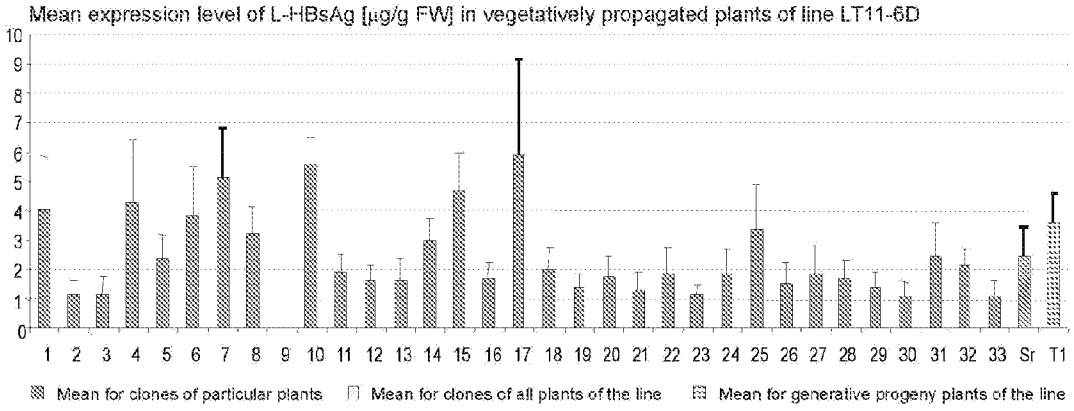

Mean expression level of L-HBsAg [µg/g FW] in vegetatively propagated plants of line LT11-6D
0 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 Sr T1
Mean for clones of particular plants
Mean for clones of all plants of the line
Mean for generative progeny plants of the line

Figure 5

The data were analysed statistically (after logarithmic transformation, for each parental plant separately) by analysis of variance in a mixed model involving fixed effects of propagated plants of T1 generation and random effects of clones and allowing for heterogeneous variance between clones.

Tab. 1. Statistical analysis of micropropagation results of progeny lettuce plants of T1 generation expressing surface antigens of HBV (HBsAg).

Expressed antigen	Parental plant	Number of propagated plants of T1 generation	Number of clones derived from T1 plants	Mean number of clones per T1 plant	Mean value of HBsAg expression level in clones (for all derived clones) ± std. error	Mean value of HBsAg expression level in control[1] plants ± std. error (number of plants)		Percentage of derived clones with mean HBsAg values not significantly different from the mean for control plants	Percentage of derived plants with the coefficient of variability of HBsAg level between clones in the range:			P-value for testing equality of variance between clones for derived plants
									less than 10%	10-20%	more than 20%	
S-HBsAg	LT10-4D	35	250	7,1 ± 2,3	1154,4 ± 60.9	1335,0 ± 219,0	(21)	94,44	19,4	75,0	5,6	0,477
	LT10-6A	26	157	6,0 ± 2,7	1630,0 ± 135,0	1895,0 ± 353,0	(22)	92,31	42,3	38,5	19,2	0,015
	LT10-26G	25	143	5,7 ± 2,1	4391,0 ± 341,0	6585,0 ± 1123,0	(28)	68,00	65,4	30,8	3,8	0,097
M-HBsAg	LT9A-1E	22	179	8,1 ± 1,7	1731,4 ± 97,4	806,6 ± 84,6	(31)	63,64	43,5	56,5	0,0	0,506
	LT9A-15E	23	141	6,1 ± 2,3	895,0 ± 50,7	1053,0 ± 102,0	(30)	100,00	62,5	37,5	0,0	1,000
	LT9A-18A	13	83	6,4 ± 1,4	1120,4 ± 94,2	718,9 ± 87,4	(34)	84,62	78,6	21,4	0,0	1,000
L-HBsAg	LT11-6D	32	208	6,3 ± 2,1	1224,1 ± 84,2	1617,0 ± 317,0	(31)	100,00	21,2	57,6	21,2	0,994
	LT11-17A	33	212	6,4 ± 2,2	875,3 ± 62,2	781,0 ± 133,0	(42)	100,00	14,7	58,8	26,5	1,000
	LT11-18C	23	104	3,9 ± 2,0	1515,0 ± 139,0	806,0 ± 133,0	(35)	100,00	50,0	45,8	4.2	0,997
	Total	232	1477	6,2 ± 2,4								

[1]) Control plants – plants of T1 generation of natural life cycle i.e. not vegetatively propagated

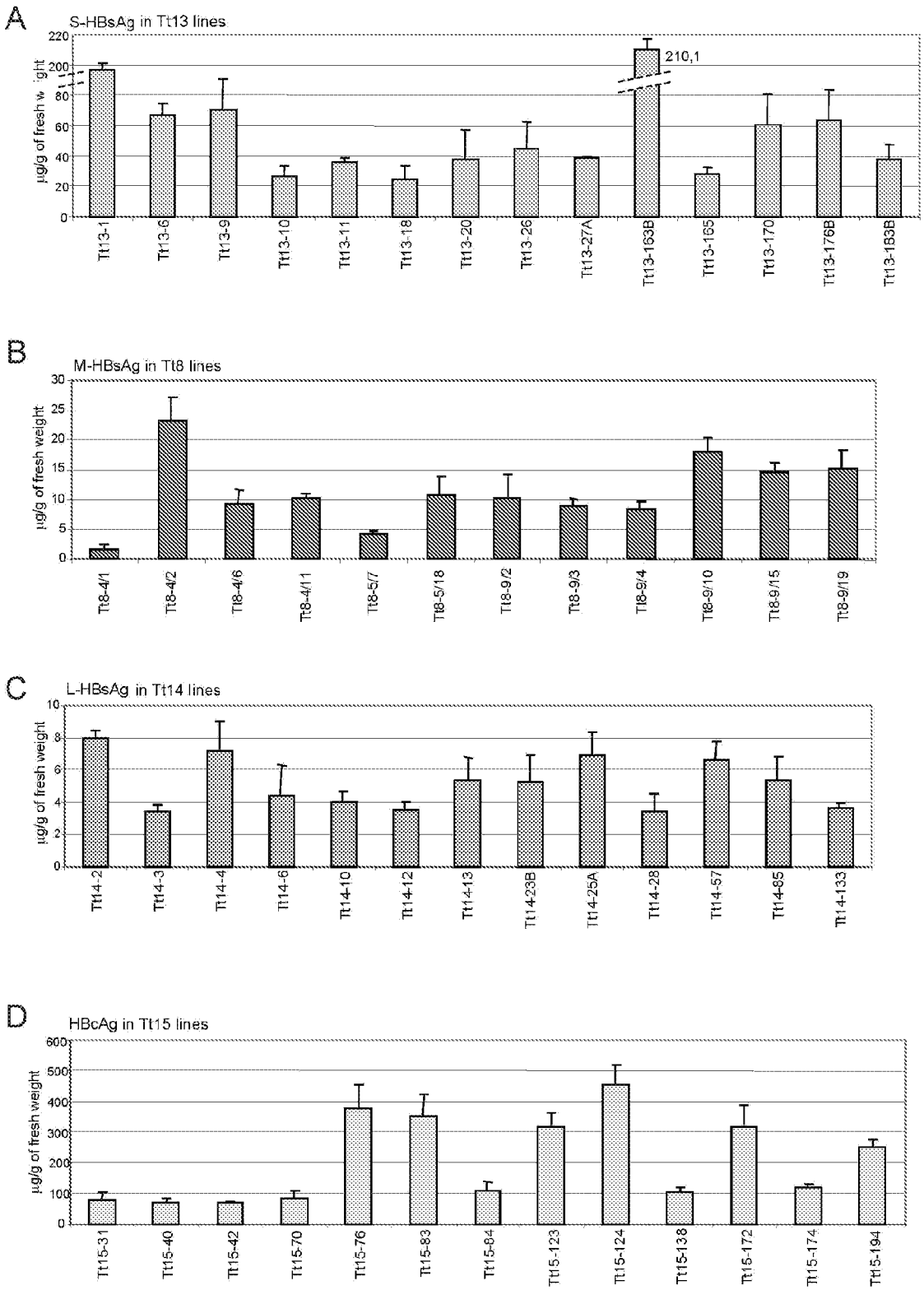

A
S-HBsAg in Tt13 lines
µg/g of fresh weight
210,1
Tt13-1
Tt13-6
Tt13-9
Tt13-10
Tt13-11
Tt13-18
Tt13-20
Tt13-26
Tt13-27A
Tt13-163B
Tt13-165
Tt13-170
Tt13-176B
Tt13-183B
B
M-HBsAg in Tt8 lines
µg/g of fresh weight
Tt8-4/1
Tt8-4/2
Tt8-4/6
Tt8-4/11
Tt8-5/7
Tt8-5/18
Tt8-9/2
Tt8-9/3
Tt8-9/4
Tt8-9/10
Tt8-9/15
Tt8-9/19
C
L-HBsAg in Tt14 lines
µg/g of fresh weight
Tt14-2
Tt14-3
Tt14-4
Tt14-6
Tt14-10
Tt14-12
Tt14-13
Tt14-23B
Tt14-25A
Tt14-28
Tt14-57
Tt14-85
Tt14-133
D
HBcAg in Tt15 lines
µg/g of fresh weight
Tt15-31
Tt15-40
Tt15-42
Tt15-70
Tt15-76
Tt15-83
Tt15-84
Tt15-123
Tt15-124
Tt15-138
Tt15-172
Tt15-174
Tt15-194

Figure 6

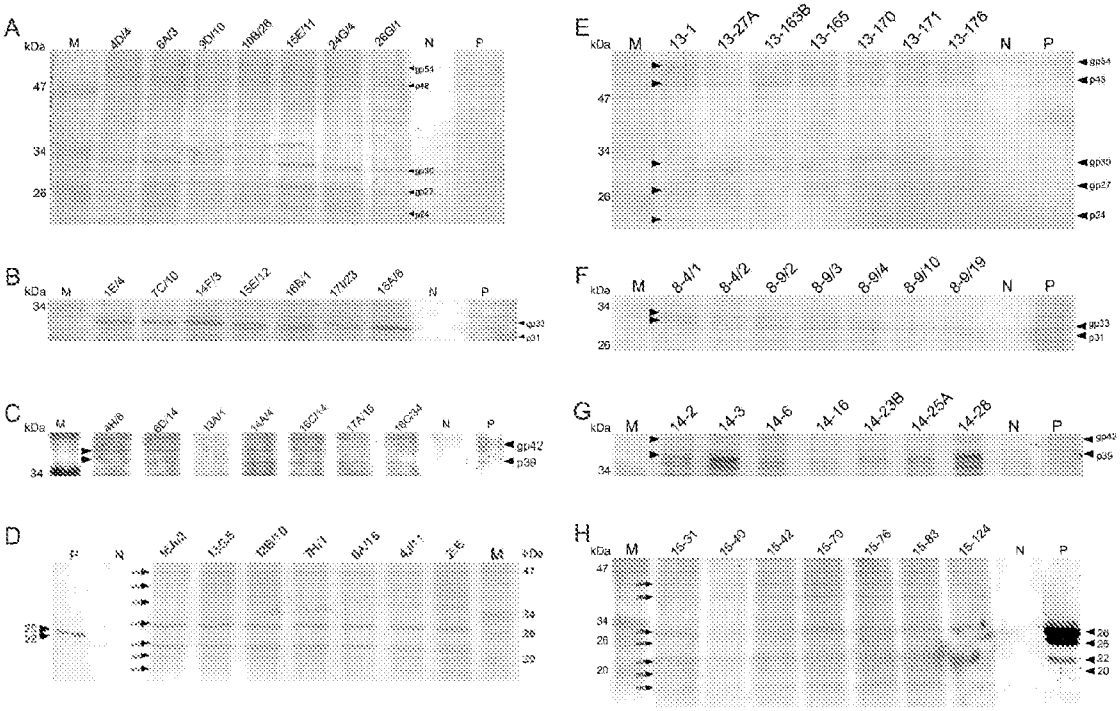

A
kDa
M
4D/4
8A/3
9D/10
10B/26
15E/11
24G/4
26G/1
N
P
47
34
28
gp54
p48
gp39
gp27
p24
B
kDa
M
1E/4
7C/10
14F/3
15E/12
16B/1
17D/23
18A/8
N
P
34
gp33
p31
C
kDa
M
4A/8
6D/14
10A/1
14A/4
16C/14
17A/16
18C/24
N
P
34
gp42
p39
D
N
P
4c4/3
13C/5
14B/10
7H/1
8A/16
4J/1
2/E
M
kDa
47
34
28
20
E
kDa
M
13-1
13-27A
13-163B
13-165
13-170
13-171
13-176
N
P
47
34
28
gp54
p48
gp39
gp27
p24
F
kDa
M
8-4/1
8-4/2
8-9/2
8-9/3
8-9/4
8-9/10
8-9/19
N
P
34
26
gp33
p31
G
kDa
M
14-2
14-3
14-6
14-16
14-23B
14-25A
14-26
N
P
34
gp42
p39
H
kDa
M
15-31
15-40
15-42
15-70
15-76
15-83
15-124
N
P
47
34
28
20
28
25
22
20

Figure 7

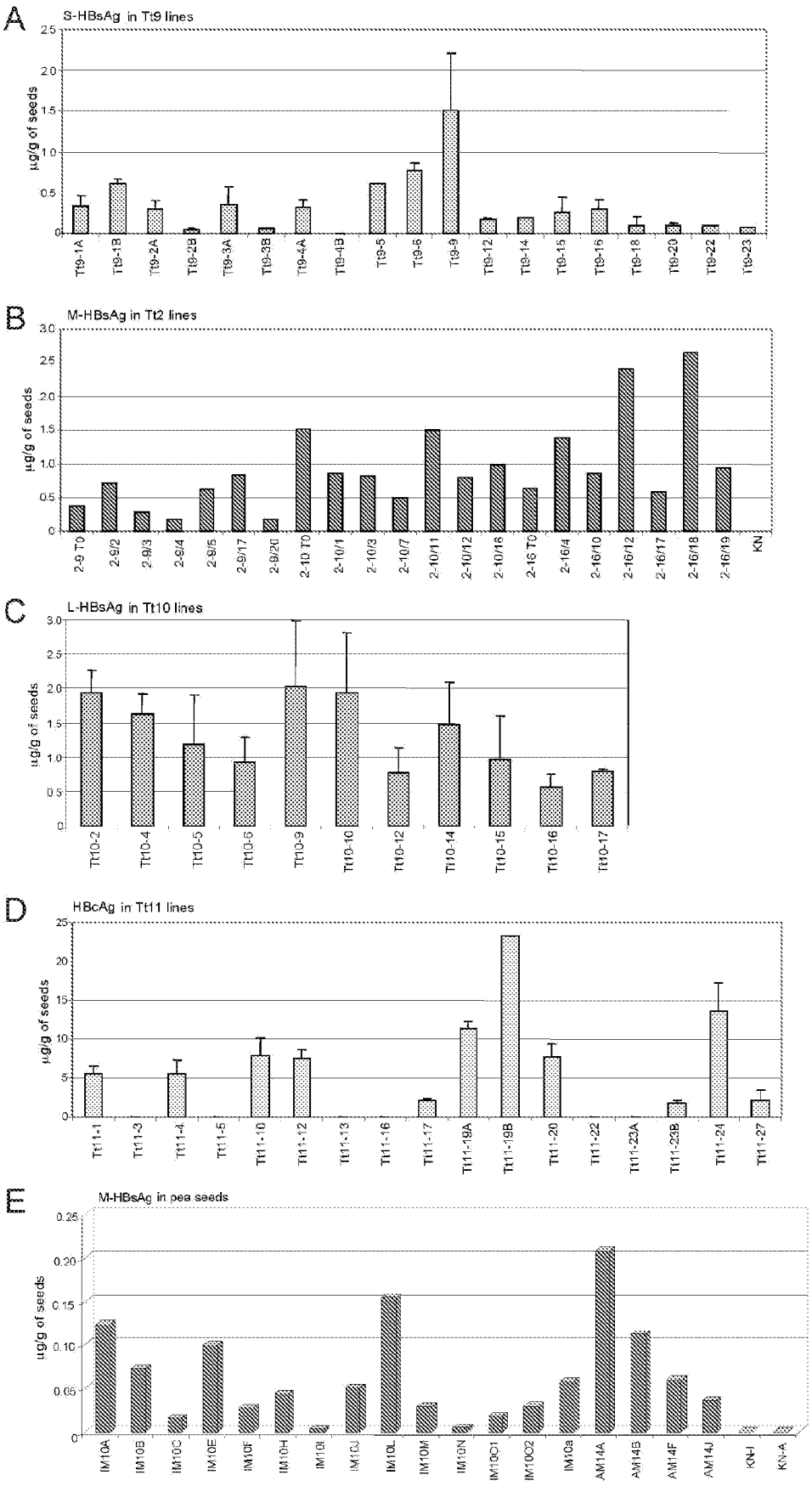

A
S-HBsAg in Tt9 lines
µg/g of seeds
Tt9-1A
Tt9-1B
Tt9-2A
Tt9-2B
Tt9-3A
Tt9-3B
Tt9-4A
Tt9-4B
Tt9-5
Tt9-6
Tt9-9
Tt9-12
Tt9-14
Tt9-15
Tt9-16
Tt9-18
Tt9-20
Tt9-22
Tt9-23
B
M-HBsAg in Tt2 lines
µg/g of seeds
2-9 T0
2-9/2
2-9/3
2-9/4
2-9/5
2-9/17
2-9/20
2-10 T0
2-10/1
2-10/3
2-10/7
2-10/11
2-10/12
2-10/16
2-16 T0
2-16/4
2-16/10
2-16/12
2-16/17
2-16/18
2-16/19
KN
C
L-HBsAg in Tt10 lines
µg/g of seeds
Tt10-2
Tt10-4
Tt10-5
Tt10-6
Tt10-9
Tt10-10
Tt10-12
Tt10-14
Tt10-15
Tt10-16
Tt10-17
D
HBcAg in Tt11 lines
µg/g of seeds
Tt11-1
Tt11-3
Tt11-4
Tt11-5
Tt11-10
Tt11-12
Tt11-13
Tt11-16
Tt11-17
Tt11-19A
Tt11-19B
Tt11-20
Tt11-22
Tt11-23A
Tt11-23B
Tt11-24
Tt11-27
E
M-HBsAg in pea seeds
µg/g of seeds
IM10A
IM10B
IM10C
IM10E
IM10F
IM10H
IM10I
IM10J
IM10L
IM10M
IM10N
IM10O1
IM10O2
IM10a
AM14A
AM14B
AM14F
AM14J
KN-I
KN-A

Figure 8

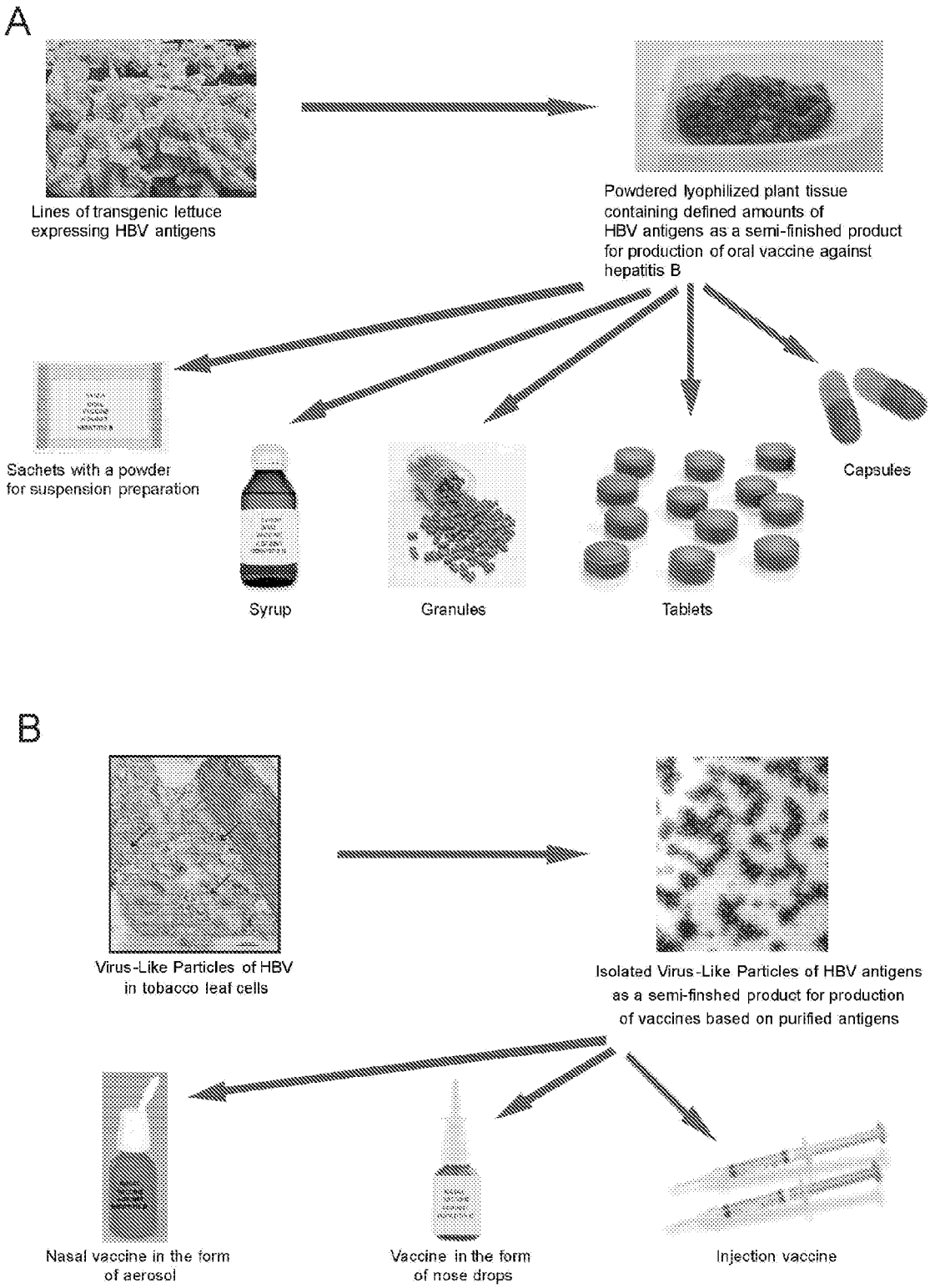

A
Lines of transgenic lettuce expressing HBV antigens
Powdered lyophilized plant tissue containing defined amounts of HBV antigens as a semi-finished product for production of oral vaccine against hepatitis B
Sachets with a powder for suspension preparation
Syrup
Granules
Tablets
Capsules
B
Virus-Like Particles of HBV in tobacco leaf cells
Isolated Virus-Like Particles of HBV antigens as a semi-finshed product for production of vaccines based on purified antigens
Nasal vaccine in the form of aerosol
Vaccine in the form of nose drops
Injection vaccine

Figure 9