ISSN 1021-4437, Russian Journal of Plant Physiology, 2007, Vol. 54, No. 6, pp. 770–775. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © E.B. Rukavtsova, T.V. Abramikhina, N.Ya. Shulga, V.A. Bykov, Ya.I. Bur'yanov, 2007, published in Fiziologiya Rastenii, 2007, Vol. 54, No. 6, pp. 864–869.

RESEARCH PAPERS

Tissue Specific Expression of Hepatitis B Virus Surface Antigen in Transgenic Plant Cells and Tissue Culture

E. B. Rukavtsova[a], T. V. Abramikhina[a], N. Ya. Shulga[b], V. A. Bykov[b], and Ya. I. Bur'yanov[a]

[a] *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Pushchino Branch, Russian Academy of Sciences, pr. Nauki 6, Pushchino, Moscow oblast, 142290 Russia;*
fax: 7 (4967) 33-0527; e-mail: ruk@fibkh.serpukhov.su

[b] *All-Russian Institute of Medicinal and Aromatic Plants, ul. Grina 7, Moscow, 113628 Russia*

Received February 7, 2007

Abstract—The tobacco plants (*Nicotiana tabacum* L.) carrying the *HBsAg* gene controlled by $(Aocs)_3$AmasPmas, the hybrid promoter that includes regulatory elements of the agrobacterial octopine and mannopine synthase genes, as well as plants controlled by the same promoter and *adh1*, maize alcohol dehydrogenase gene intron were obtained. The presence of the *adh1* gene intron did not significantly change the level of expression of the *HBsAg* gene in plants. The analysis of expression of hepatitis B virus surface antigen (HBs-antigen) in transformed plants expressing the *HBsAg* under the control of different promoters was made. The level of HBs-antigen in plants carrying the *HBsAg* gene controlled by $(Aocs)_3$AmasPmas, the hybrid agrobacterium-derived promoter, was the highest in roots and made up to 0.01% of total amount of soluble protein. The level of HBs-antigen in plants carrying the *HBsAg* gene controlled by the dual 35S RNA cauliflower mosaic virus promoter was the same in all organs of the plant and made up to 0.06% of the total amount of soluble protein. Hairy root and callus cultures of plants carrying the *HBsAg* gene and expressing the HBs-antigen were obtained.

DOI: 10.1134/S1021443707060088

Key words: Nicotiana tabacum - transformation - promoters - HBsAg

INTRODUCTION

The transgenic plant-based technologies for production of subunit vaccines of a new generation, which may become cheaper and safer alternatives to traditionally obtained vaccine preparations, are now being developed. Plant cells contain enzymatic systems of post-translational modification necessary for the assembling of monomer vaccine proteins they synthesize into the immunogenic multimer forms. Plants are fully able to synthesize target antigens that can cause an active immune response [1] of the host organism. The viral and bacterial antigens were shown to stimulate the production of immunoglobulines against the corresponding pathogens [2–7]. Various transgenic plants are now being produced and tested in research centers worldwide as potential producers of vaccines against infection agents causing various human diseases, including the hepatitis B virus [3, 8, 9]. However, the transgenic plant-derived vaccine preparations are not yet commercially available.

Earlier we have obtained the tobacco plants expressing the synthetic gene of the hepatitis B surface antigen (*HBsAg*) controlled by single and dual 35S RNA cauliflower mosaic virus promoters (CaMV 35S and CaMV 35SS, respectively) [10, 11]. The presence of the dual 35S promoter increased expression of the antigen to the level of 0.05% of total amount of soluble protein. The transgenic potato plants expressing the *HBsAg* gene controlled by the same promoter and also by the patatin promoter of potato tubers were also produced. The amount of the HBs-antigen in potato tubers exceeded 1 μg/g of tuber mass and was the highest in plants expressing the *HBsAg* gene controlled by the dual CaMV 35SS promoter.

To obtain plants with tissue-specific expression of the hepatitis B vaccine gene in tissue cultures and whole plants (as potential producers of the vaccine), tissue-specific promoters, especially of the hybrid agrobacterium-derived promoters (Aocs)3AmasPmas, prove to be effective. These promoters consist of the regulatory elements of the octopine synthase (*ocs*) and mannopine syntase (*mas*) genes of *Agrobacterium tumefaciens*. As was shown earlier, these promoters strongly induced the expression of the β-glucoronidase (GUS) gene in plants up to the level greatly exceeding that in plants controlled by the dual 35S RNA cauliflower mosaic virus promoter [12].

The objective of this study was to obtain transgenic tobacco plants synthesizing the hepatitis B surface anti-

Abbreviations: BA—benzyladenine; CaMV 35SS—dual 35S RNA cauliflower mosaic virus promoter; HAsAg, HBs-antigen—hepatitis B virus surface antigen; Km—kanamycin, MS—Murashige and Skoog nutrient medium.

gen controlled by $(Aocs)_3$AmasPmas promoters and regulated by the elements of agrobacterial octopine synthase and mannopine synthase genes and also to analyze the expression profile of the *HBsAg* gene in different cells of the whole plant as well as that in callus and hairy root tissue cultures.

MATERIALS AND METHODS

Construction of plasmids for plant transformation. pSS-HBsAg, the recombinant plasmid, carrying a synthetic gene encoding the HBsAg/mayw polypeptide [13], was used as a gene source. The two vectors both containing the $(Aocs)_3$AmasPmas promoter were used for cloning and plant transformation: pE1802 and pE1945 (courtesy of Dr Gelvin, Purdue University, United States) [12]. The extension of the *HBsAg* gene with specific sequences necessary for molecular cloning was done by means of PCR with two primers containing the *Kpn*I and *Sal*I restriction sites: 5'-CGGGTACCATGGAAAACATTACTT and 5'-CGGTCGACCTATCATTAAATGTAAAC, respectively. The reaction mixture contained 0.1 μg of pDES20 plasmid DNA as a template, 25 mM KCl, 60 mM Tris–HCl, pH 8.5, at 25°C, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 0.2 mM dNTPs mixture (USB, United States), 0.25 μM of each primer and 2.5 units of Taq DNA polymerase (SibEnzim, Russia).

The reaction volume was 50 μl. The reaction started with 5 min at 94°C and was followed by 30 cycles: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and ended with 7 min at 72°C. The Gene Amp PCR System 2400 (Perkin-Elmer, United States) was used for experiments. The amplified gene was cloned into a binary pE1802 vector for transformation of plants between the restriction sites *Kpn*I and *Sal*I. For cloning purposes, the *HBsAg* gene was cut out of the pSS-HBsAg plasmid between the *Xho*I and *Cfr*9I restriction sites and incorporated into the pE1945 vector between the same sites. The plasmid constructions obtained were used for transformation of *Escherichia coli* strain HB101. The constructions containing the *HBsAg* gene downstream the promoter were transferred into strain LBA4404 (pAL4404) of *A. tumefaciens* [14] by means of direct transformation [15]. The analysis of DNA of the obtained clones was done by Southern hybridization with the ^{32}P-labeled amplification product of the *HBsAg* gene [16].

Transgenic plants. The *Nicotiana tabacum* L., cv. Samsun plants were cultivated in vitro in 0.5- to 1-l cultivation containers on the agar-solidified phytohormone-free MS medium [17] at 24 to 26°C, 2 klx, and 65% relative humidity. The resulting agrobacterial strain culture was used for the infection of leaf disks according to a standard protocol [18]. The leaf disks were co-cultivated with the overnight agrobacterial cultures for two days and then transferred onto the selection MS medium containing hormones (1 mg/l of BA and 0.1 mg/l of NAA), 50 mg/l of kanamycin sulfate (Km), and 500 mg/l of cefotaxime. The regenerated shoots were passed onto the selection MS medium. Calli were obtained from leaf explants of transformed plants on the MS medium containing 3% sucrose, 0.5 mg/l BA, 2 mg/l NAA, and 50 mg/l Km. The hairy root culture was grown on the hormone-free MS medium from disk leaves of transformed plants infected with strain A4 of *A. rhizogenes* [19].

Extraction of DNA from tobacco leaves. The DNA from tobacco leaves was extracted as described in [20]. Leaves were crushed in 1.5 ml Eppendorf tubes, 0.4 ml of the extraction buffer was added (containing 0.2 M Tris–HCl, pH 7.5, 0.25 M NaCl, 25 mM EDTA, and 0.5% SDS); the mixture was incubated for 1 h at room temperature and then centrifuged at 12000 rpm for clarification. An equal volume of isopropanol was added, and the DNA precipitate was dissolved in 100 μl of the TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). The plant DNA obtained was used as a template for PCR.

PCR analysis of the *HBsAg* gene. For the PCR analysis of *HBsAg* gene, 0.5 to 1 μg of plant genomic DNA was used. The reaction mixture and cycle conditions were the same as above.

Immunoassay of the surface antigen level. The immunoassay of the hepatitis B virus surface antigen level in transformed plants was done as earlier described by us [12] with minor modifications. Leaves, roots, and calli of tested plants were ground in liquid nitrogen, and the extraction buffer (0.05 M sodium phosphate buffer, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.3% Tween 20, 0.4 mM phenylmethanesulfonyl fluoride, and 0.5% sodium ascorbate). The extract obtained was centrifuged for 20 min at 3500 rpm, the supernatant was transferred to 1.5-ml Eppendorf tubes and recentrifuged for 10 min at 12000 rpm. The "VektoHep B-HBs-antigen" test systems (JSC Vektor-Best, Russia) were used for measuring the HBs-antigen level in the supernatant. The recombinant yeast cell-derived HBs-antigen [21] was used as a positive control. The assay was carried out according to the manufacturer's instructions. The total amount of soluble protein was measured according to Bradford [22].

Mechanical leaf wounding for induction of *HBsAg* gene expression. Leaves were immersed into the liquid MS medium, cut with a scalpel into stripes 1 to 2 mm wide, and incubated at 24 to 26 °C for 24 h. The injured leaves were used for immunoassay.

RESULTS AND DISCUSSION

The recombinant plasmids used for transformation of plants are shown in Fig. 1. These plasmids are based on the pE1802 and pE1945 vectors containing the $(Aocs)_3$AmasPmas promoter [12]. Both plasmids are carrying *HBsAg*, a synthetic gene [10]. The difference between the two vectors is that the pE1945 is carrying the *adh1* the maize alcohol dehydrogenase gene intron,

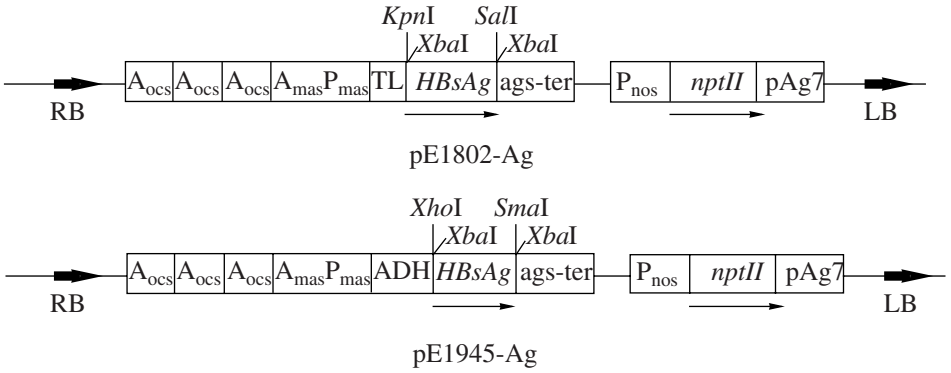

Fig. 1. The constructs of recombinant plasmids carrying *HBsAg* incorporated in two binary vectors for plant transformation: pE1802 and pE1945.

A_{ocs} gene activator; A_{mas}—*mas* gene activator; TL—enhancer; P_{mas}—*mas* gene promoter; P_{nos}—nopaline synthase gene promoter; ags-ter and pAg7—polyadenylation and termination signals for agropine synthase and Ag7 genes, respectively; ADH– maize alcohol dehydrogenase gene intron; *HBsAg*—hepatitis B virus surface antigen; *nptII*—neomycin phosphotransferase II gene; RB, LB—right and left ends of O-DNA, respectively.

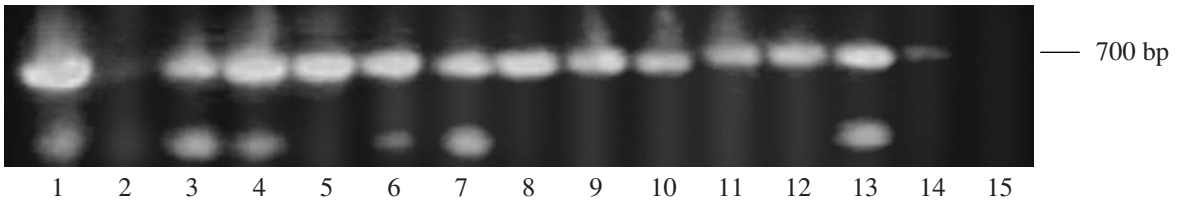

Fig. 2. PCR analysis of DNA of the transformed tobacco plants carrying the hepatitis B virus surface antigen.

(1) *HBsAg* gene carrying plasmid DNA; (2) control PCR with no DNA; (3–8) DNA of plants transformed by pE1802-Ag; (9–14) DNA of plants transformed by pE1945-Ag; (15) untransformed plant DNA.

but is lacking the TL enhancer. The obtained plasmid constructs carrying the *HBsAg* gene under the $(Aocs)_3$AmasPmas promoter were used for the direct transformation of strain LBA4404 (pAL4404) of *A. tumefaciens*. Separate agrobacterial colonies were collected for extraction of plasmid DNA and its visualization in agarose gel electrophoresis. The transformed agrobacterial strains were used for the infection of tobacco leaf disks. 15 to 20 lines of transformed plants of each type were selected for subsequent molecular, genetic, and biochemical analysis.

The *HBsAg* gene presence in transformed plants was confirmed by means of PCR. The target DNA, which corresponds to the hepatitis B virus surface antigen, was found in all transgenic plants tested (Fig. 2).

The immunoassay was carried out to study the expression profile of the surface antigen HBsAg in obtained plants. The HBs-antigen was found in different amounts in plants of all transgenic tobacco lines tested (see the table). The expression level of this antigen in the in vitro grown transgenic tobacco plants was up to 0.01% of total amount of soluble protein. Maximum expression of the surface HBsAg antigen was observed in roots. The genetic construct pE1945-Ag used for some of our experiments contained the alcohol dehydrogenase gene intron. The inclusion of introns into plant cells may enhance the expression of foreign genes [23]. However, in our case the presence of the *adh1* gene intron did not increase the expression of the target gene. The enhancement effect seems to be dependent on the location of the intron in the genetic construct carrying the target gene.

The table shows the expression levels of the HBs-antigen in plants controlled by different promoters. Thus, the expression level of the HBs-antigen in plants controlled by $(Aocs)_3$AmasPmas, the hybrid agrobacterial promoter, reached the maximum of 0.01% of total amount of soluble protein in roots. The expression level of the HBs-antigen in plants controlled by the dual 35S RNA cauliflower mosaic virus promoter was the same in all organs of the plant, accounting for up to 0.06% of total amount of soluble protein. Therefore, $(Aocs)_3$AmasPmas, the hybrid agrobacterial promoter, can be regarded as a tissue-specific element of the *HBsAg* gene expression, maximum expression being induced in roots.

Level of the HBs-antigen in leaves and roots of the transformed tobacco plants controlled by different promoters

Line	The level of HBs-antigen, % of total soluble protein	
	in leaves	in roots
	Untransformed plants	
	0	0
	pE1802-Ag (with $(Aocs)_3$AmasPmas promoter)	
1	0	0.001
8	0.002	0.01
11	0	0.004
12	0	0.01
13	0	0.01
16	0	0.005
21	0.0008	0.005
	pE1945-Ag (with $(Aocs)_3$AmasPmas promoter and adh1 gene intron)	
1	0.004	0.005
2	0	0.004
3	0.0007	0.004
4	0	0.004
5	0	0.001
6	0.0006	0.01
7	0	0.0002
8	0	0.0003
9	0	0.001
10	0	0.002
11	0	0.001
	*pSS-Ag (with CaMV 35SS promoter)**	
1-1	0.01	0.01
1-6	0.03	0.04
17-5	0.05	0.06

Notes: Average values are presented for three independent experiments; standard error in each of them did not exceed 0.0001%.

* The transformation technology is described in [11].

The activity of the agrobacterial mannopine synthase gene can be very different in different tissues and organs of the plant, maximum expression being observed in roots and calli [24, 25]. The expression level of the HBs-antigen in leaves of transgenic plants controlled by the $(Aocs)_3$AmasPmas promoter substantially increased after wounding and reached to 0.01% of total amount of soluble protein. These results correspond with the available data on the induction of foreign gene expression in leaves of transgenic plants controlled by the mannopine synthase promoter under similar wounding conditions [24, 26].

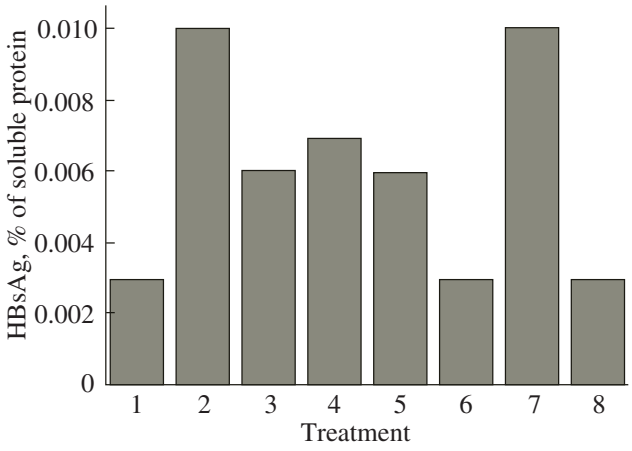

Fig. 3. Level of the HBs-antigen in hairy root cultures originating from selected lines of transformed tobacco plants. (1–4) Tobacco plants transformed with pE1802-Ag (lines 1, 8, 11, 16); (5–8) tobacco plants transformed with pE1945-Ag (lines 1, 5, 6, 10). Average values are presented for three independent experiments; standard error in each of them did not exceed 0.0001%.

Eight lines of transgenic plants with the highest level of expression of the HBs-antigen were selected to obtain so-called hairy root tissue cultures by means of infection of leaf disks with strain A4 of *A. rhizogenes* culture. Hairy roots, being effectively plant tumors transformed by *A. rhizogenes* [19], are a convenient system for production of secondary metabolites and recombinant proteins due to their genetic stability and fast growth in the hormone-free medium. As a result of retransformation of plants carrying the *HBsAg* gene with strain A4 of *A. rhizogenes*, hairy root cultures were obtained also carrying the HBs-antigen (Fig. 3). The level of expression of the HBs-antigen in different lines of hairy root cultures remained the same as that in parent transgenic plants making up to 0.01% of total soluble protein.

Leaf explants of the transformed plants expressing the HBs-antigen controlled by the $(Aocs)_3$AmasPmas promoter were used to obtain callus cultures. The HBs-antigen expression in these cultures was also observed (Fig. 4). This could be explained by the induction of activity of the mannopine synthase and octopine synthase promoters by a higher level of auxins in callus culture cultivated in the auxin-containing medium [24].

It should be noted that the results of our study do not confirm the existing data on more efficient expression of foreign genes controlled by the $(Aocs)_3$AmasPmas hybrid promoters that those controlled by the dual CaMV 35SS promoter [12]. In those study, the activity of the $(Aocs)_3$AmasPmas promoter was judged on the basis of GUS staining assay. Another data, however, are available [27] when the researchers did not notice a considerable difference between the *Bt-cry1Ia1*, an

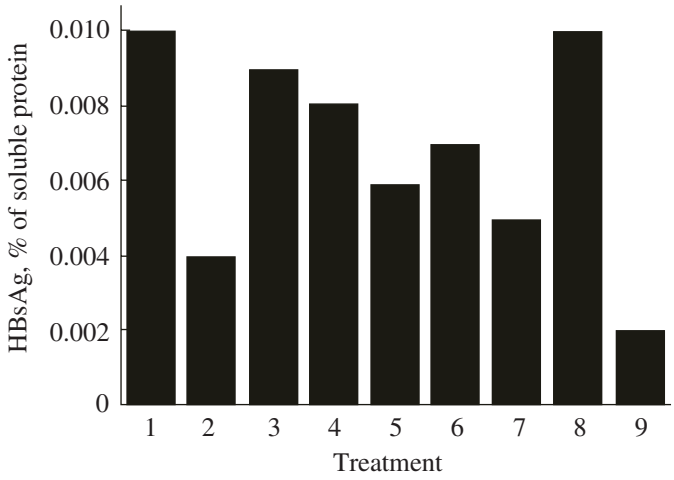

Fig. 4. Level of the HBs-antigen in callus cultures originating from selected lines of transformed tobacco plants. (1–5) Tobacco plants transformed with pE1802-Ag (lines 8, 11, 12, 13, 21); (6–9) tobacco plants transformed with pE1945-Ag (lines 1, 4, 6, 9). Average values are presented for three independent experiments; standard error in each of them did not exceed 0.0001%.

entomotoxine gene expression under the control of CaMV 35S or $(Aocs)_3$AmasPmas promoters. The attempts have also been made to use the $(Aocs)_3$AmasPmas hybrid promoter for transformation of plants that could potentially produce the vaccine against SARS (Severe Acute Respiratory Syndrome) [28]. The expression of SARS-CoV, the target S protein, was the highest in roots of transformed tobacco and immature fruits of the transformed tomato plants.

$(Aocs)_3$AmasPmas, the hybrid promoter, can be considered as a potential tool for tissue specific expression of various pharmaceutically important proteins in transformed plants and their cell cultures.

ACKNOWLEDGMENTS

The authors thank S.V. Chernyshov for assistance in the development of the *pE1945-Ag* genetic construct.

This study was supported by the Presidium of Russian Academy of Sciences, the program "Dynamics of Plant, Animal, and Human Genofonds" and also by Russian Foundation for Basic Research (projects nos. 05-08-01473 and 06-04-81022).

REFERENCES

1. Mason, H.S. and Arntzen, C.J., Transgenic Plants as Vaccine Production Systems, *Trends Biotechnol.*, 1995, vol. 13, pp. 388–392.
2. Mason, H.S., Lam, D.M.K., and Arntzen, C.J., Expression of Hepatitis B Surface Antigen in Transgenic Plants, *Proc. Natl. Acad. Sci. USA*, 1992, vol. 89, pp. 11745–11749.
3. Arntzen, C.J., Edible Vaccines, *Publ. Health Rep.*, 1997, vol. 112, pp. 190–197.
4. Gomez, N., Carillo, C., Salinas, J., Parra, F., Borca, M.V., and Escribano, J.M., Expression of Immunogenic Glycoprotein S Polypeptides from Transmissible Gastroenteritis Coronavirus in Transgenic Plants, *Virology*, 1998, vol. 249, pp. 352–358.
5. Ma, J.K. and Vine, N.D., Plant Expression Systems for the Production of Vaccines, *Curr. Top. Microbiol. Immunol.*, 1999, vol. 236, pp. 275–292.
6. Richter, L. and Kipp, P.B., Transgenic Plants as Edible Vaccines, *Curr. Top. Microbiol. Immunol.*, 1999, vol. 240, pp. 159–176.
7. Tuboly, T., Yu, W., Bailey, S., Degrandis, S., Du, S., Erickson, L., and Nagy, E., Immunogenicity of Porcine Transmissible Gastroenteritis Virus Spike Protein Expressed in Plants, *Vaccine*, 2000, vol. 18, pp. 2023–2028.
8. Liu, Y.L., Wang, J.F., Qiu, B.S., Zhao, S.Z., and Tian, B., Expression of Human Hepatitis B Virus Surface Antigen Gene in Transgenic Tobacco, *Sci. China (Ser. B)*, 1994, vol. 37, pp. 37–41.
9. Ehsani, P., Khabiri, A., and Domansky, N.N., Polypeptides of Hepatitis B Surface Antigen Produced in Transgenic Potato, *Gene*, 1997, vol. 190, pp. 107–111.
10. Zolova, O.E., Rukavtsova, E.B., Bur'yanov, Ya.I., Borisova, V.N., and Mel'nikov, V.A., Production of Transgenic Tobacco Plants Expressing the Gene Encoding of Hepatitis B Virus Surface Antigen, *Biotekhnologiya*, 1999, no. 6, pp. 29–34.
11. Rukavtsova, E.B., Zolova, O.E., Bur'yanova, N.Ya., Borisova, V.N., Bykov, V.A., and Bur'yanov, Ya.I., Analysis of Transgenic Tobacco Plants Carring the Gene for Hepatitis B Virus Surface Antigen, *Genetika*, 2003, vol. 39, pp. 51–56.
12. Ni, M., Cui, D., Einstein, J., Narasimhulu, S., Vergara, C.E., and Gelvin, S.B., Strength and Tissue Specificity of Chimeric Promoters Derived from the Octopine and Mannopine Synthase Genes, *Plant J.*, 1995, vol. 7, pp. 661–676.

13. Shul'ga, N.Ya., Rukavtsova, E.B., Krymskii, M.A., Borisova, V.N., Mel'nikov, V.A., Bykov, V.A., and Bur'yanov, Ya.I., Expression of Hepatitis B Virus Surface Antigen in Transgenic Potato Plants and Its Characteristics, *Biokhimiya*, 2004, vol. 69, pp. 1422–1430.
14. Hoekema, A., Hooykaas, P.J.J., and Schilperoort, R.A., Transfer of Octopine T-DNA Segment to Plant Cells Mediated by Different Types of Agrobacterium Tumour- or Root-Inducing Plasmids: Generality of Virulence Systems, *J. Bacteriol.*, 1984, vol. 158, pp. 383–385.
15. An, G., Ebert, P.R., Mitra, A., and Ha, S.B., Binary Vectors, *Plant Molecular Biology Manual*, Gelvin, S.B., Schilperoort, R.A., and Verma, D.P.S., Eds., Dordrecht: Kluwer, 1988, pp. 1–19.
16. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor: Cold Spring Harbor Lab., 1989.
17. Murasige, T. and Skoog, F.A., Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures, *Physiol. Plant.*, 1962, vol. 15, pp. 473–497.
18. Draper, J., Scott, R., and Hamil, J., Transformation of Dicotyledonous Plant Cells Using the Ni Plasmid of *Agrobacterium tumefaciens* and Ri Plasmid of *A. rhizogenes, Plant Genetic Transformation and Gene Expression. A Laboratory Manual*, Draper, J., Scott, R., Armitage, P., and Walden, R., Eds., Oxford: Blackwell Sci., 1988, pp. 69–160.
19. Tepfer, D., Transformation of Several Species of Higher Plants by *Agrobacterium rhizogenes.* Sexual Transmission of the Transformed Genotype and Phenotype, *Cell*, 1984, vol. 37, pp. 959–967.
20. Edwards, K., Johnstone, C., and Thomson, C.A., A Simple and Rapid Method for the Preparation of Plant Genomic DNA for PCR Analysis, *Nucleic Acids Res.*, 1991, vol. 19, p. 1349.
21. Drutsa, V.L., Budanov, M.V., Borisova, V.N., and Yakovleva, I.M., Recombinant Plasmid DNA pDES20 Codes Hepatitis B Virus Surface Antigen (HBsAg/mayw), Yeast Strain *S. cerevisiae* DAN-041/pDTS20 – Producer of Hepatitis B Virus Antigen and Vaccine on Its Base: RF Patent no. 2 088 664, *Byull. Izobret.*, 1997, no. 24.
22. Bradford, M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein–Dye Binding, *Anal. Biochem.*, 1976, vol. 72, pp. 248–254.
23. Rose, A.B., The Effect of Intron Location on Intron-Mediated Enhancement of Gene Expression in Arabidopsis, *Plant J.,* 2004, vol. 40, pp. 744–751.
24. Langridge, W.H.R., Fitzgerald, K.J., Koncz, C., Schell, J., and Szalay, A.A., Dual Promoter of *Agrobacterium tumefaciens* Mannopine Synthase Genes Is Regulated by Plant Growth Hormones, *Proc. Natl. Acad. Sci. USA*, 1989, vol. 86, pp. 3219–3223.
25. Rukavtsova, E.B., Alekseeva, V.V., Zolova, O.E., Kalyaeva, M.A., Murenets, L.Yu., and Bur'yanov, Ya.I., Combined Usage of Regulated Promoters and Grafting Technique in Plant Gene Engineering Biotechnology, *Biotekhnologiya*, 1996, no. 9, pp. 24–28.
26. Teeri, T.H., Lehvaslaiho, H., Franck, M., Uotila, J., Heino, P., Palva, E.T., van Montagu, M., and Herrera-Estrella, L., Gene Fusions to *lacZ* Reveal New Expression Patterns of Chimeric Genes in Transgenic Plants, *EMBO J.*, 1989, vol. 8, pp. 343–350.
27. Lagnaoui, A., Canedo, V., and Douches, D.S., Evaluation of *Bt-cry1Ia1* (*cryV*) Transgenic Potatoes on Two Species of Potato Tuber Moth, *Phthorimaea operculella* and *Symmetrischema tangolias* (Lepidoptera: Gelechiidae) in Peru, *CIP Progr. Rep.*, 1999-2000, pp. 117–121.
28. Pogrebnyak, N., Golovkin, M., Andrianov, V., Spitsin, S., Smirnov, Y., Egolf, R., and Koprowski, H., Severe Acute Respiratory Syndrome (SARS) S Protein Production in Plants: Development of Recombinant Vaccine, *Proc. Natl. Acad. Sci. USA*, 2005, vol. 102, pp. 9062–9067.