PROCEEDINGS OF THE BALKAN SCIENTIFIC CONFERENCE OF BIOLOGY IN PLOVDIV (BULGARIA) FROM 19TH TILL 21ST OF MAY 2005 (EDS B. GRUEV, M. NIKOLOVA AND A. DONEV), 2005 (P. 329–338)

MOLECULAR FARMING – THE PRODUCTION OF RECOMBINANT SUBUNIT HEPATITIS B VACCINE IN TOBACCO PLANTS

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ABSTRACT. Molecular farming in plants may provide a tempting, safe and inexpensive alternative to conventional protein production. Transgenic plants can produce unlimited amounts of recombinant proteins – subunit vaccines, human pharmaceutical protein, recombinant antibodies and industrial enzymes. Plants have many advantages over the other production systems – scalability, safety and economy. Here we present data showing the production of hepatitis B nucleocapsid antigen (HBcAg) in transgenic tobacco. Enzyme linked immunosorbent assay (ELISA) confirmed the presence of the viral core antigen in the total protein extracts from tobacco leaves.

KEY WORDS. hepatitis B virus, core protein, molecular farming, multivalent vaccines, transgenic tobacco plants

INTRODUCTION

Molecular Farming is the growing and harvesting of genetically engineered crops of transgenic plants, to produce biopharmaceuticals. The idea is to use these genetically modified crops as biological factories to generate valuable medical products difficult or expensive to produce in any other way. Combining advance in molecular biology, gene engineering and immunology, the scientists take genes from

other sources, such as microorganisms, and splice them into the plant's genome. These genetically engineered plants synthesize recombinant proteins which can be vaccines, blood substitutes, enzymes or diagnostic reagents which are then extracted from the crop. Therapeutic proteins, edible vaccines are already in clinical trials. Several antigens from different infectious agents have been successfully produced in plants: Hepatitis B surface antigen [1], *E.coli* heat-labile enterotoxin B subunit [2], Norwalk virus capsid protein [3], Human cytomegalovirus (HCMV) immunodominant glycoprotein B (gB) complex [4], HIV–1 p24 protein [5], Rabies virus glycoprotein [6], Hepatitis B virus core antigen [4].

Host species

The tobacco plant is most suited to large-scale production of active agents. The continuing popularity of tobacco reflects its status as well established expression host for facile transformation procedure and well characterized regulatory elements for the control of transgene expression are available. The major advantages of tobacco include high biomass yield, seed production and the existence of a large scale processing infrastructure. There is a little risk that tobacco material may contaminate the food and feed chains. Tobacco has been used as molecular factory by several biotech companies, including Planet Biotechnology Inc. (http://www.planetbiotechnology.com) and Meristem Therapeutics companies ((http://www.meristem-therapeutics.com/) [7]. One of the disadvantages of leafy crops is that recombinant proteins are synthesized in an aqueous environment and are often unstable. The leaves must be frozen or processed soon after harvest [8].

Costs of production

A great advantage of transgenic plant for molecular farming is the low cost of large scale production of recombinant proteins. It is estimated that recombinant protein can be produced in plant at 2-10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cell culture [9].

Development timescale

The preparation of expression constructs, transformation, regeneration and testing several transgenic plants are time consuming process. The testing phase includes verification of expression stability, biochemical activity of the product, and absence of phenotypic changes in the host plant. These phase take up to two years depending on the plant species [10].

Vaccines

Different pathogenic microorganisms invade humans and animals and cause disease. It has been well determined that inoculation with attenuated bacteria or viruses or heat-killed bacteria and viruses can induce an immune response in mammals without causing the disease. This process, referred to as ‘vaccination”, is a main method to protect mammals against several disease [11]. The new vaccines based on the subunit principles – subunits from pathogens could provide full protection against disease,

e.g. the hepatitis B surface protein vaccine. This offered a new approach in vaccine development using small and safe molecules for vaccination.
The firs step in developing vaccines is to identify the antigen that is capable to induce protective immune response. The gene encoding the protein is isolated and used to constructed plant transformation vector. The next step is transformation of appropriate crop and regeneration of transgenic plants and evaluated antigenicity, assembly and accumulation of the recombinant Ag, select plant whit highest expressional level of the foreign protein.

Hepatitis B virus

Hepatitis B virus is a very common infection of the liver. The infections agent that causes hepatitis B is hepatitis B virus (HBV). More than a third of the world's population has been infected with HBV, and the World Health Organization estimates that it results in 1–2 million deaths every year. Persistent carriage of HBV, defined by the presence of surface antigen (HbsAg) in the serum for more than 6 month has been estimated to affect about 350 million people worldwide [12]. Many persons who are infected with hepatitis B virus have no symptoms. Others become ill with these symptoms: loss of appetite, tiredness, pain in muscles, joints, diarrhea or vomiting, jaundice (yellowing of the skin and whites of the eyes). In some people, especially infants and children, hepatitis B virus can cause chronic (lifelong) liver infection. Chronic infection can lead to liver damage (cirrhosis), liver cancer, and death. The best way of protecting from HBV is to be vaccinated. The existing vaccine to prevent HBV infection is a biotechnology product that falls in category of "subunite vaccines". This vaccine is highly effective and safe but injectable and expensive. This vaccine is out of reach for the poor national of the world. Research underway is dedicated to solving these limitations by finding ways to produce accessible vaccines in transgenic plants [13].

HBV nucleocapsid expression

The hepatitis B core protein (HBc) is the major component of the nucleocapsid shell packaging the HBV genome. These 185 amino acids (predominantly hydrophilic and charged amino acids) are expressed in cytoplasm of infected cells. The nucleocapsid of hepatitis B virus, or HBcAg, is a highly symmetric structure formed by multiples dimmers of a single core protein that contains potent T helper epitopes in its 183-aa sequence [14]. The particulate HBcAg is extremely immunogenic. It can function as both a T-cell-independent and a T-cell-dependent antigen [14] The dual functions of HBcAg as a T-cell-independent and a T-cell-dependent antigen may explain its enhanced immunogenicity. HBcAg can bind and activate B cells in a T-cell-independent way. Immunization with HBcAg preferentially primes Th1-type cellular immune responses [14]. HBcAg is an effective carrier of heterologous epitopes, and HBcAg-specific T cells support anti-envelope (anti-HBs), as well as anti-HBc, antibody production [15, 16, 17]. During chronic HBV infection, HBcAg is the only antigen that elicits a prominent immune response [18] .

The capsid proteins of hepatitis B virus can self assemble into naturally shaped virus-like particles (VLP), which are non-infectious and can be used as universal carriers for vaccine production. The process of correct self-assembly tolerates short insertions of foreign antigens into different regions of these capsid proteins. Our experimental approach is to use chimeric HBV capsid and carrying well-defined immunogenic epitopes from hepatitis B virus to produce single vaccines in plants.

MATERIAL AND METODS

Plant material

Tobacco plants *Nicotiana tabacum* cv Petit Havana SR1 were used for *Agrobacterium*-mediated gene transfer method through regeneration of the leaves explants. Tobacco seeds were surface sterilized for 5 min. in 5% NaOCl followed by three times washing in sterile water before transferring them to agar-solidified MS30 medium with 30g/l sucrose for germination. For transformation we used sterile young leaves from four weeks grown tobacco plants.

Constructs

We constructed synthetic HBV core gene in conformance with the plant codon, and improved the protein expression pattern at the molecular level by modifying the DNA to avoid ATTTA sequences and potential polyadenylation signal sites while maintaining the original amino acid sequence. We used two expression constructs. The difference between them was only promoter signal. The expression construct HBVCO/Tcup consists of Tcup promoter, the C terminal truncated codon optimized HBV sequence, an endoplasmic retention sequences encoding the tetrapeptide KDEL and the termination signal NOS-T. The expression construct HBVCO/35S consists of 35S cauliflower mosaic virus (CaMV) promoter. These constructs were inserted into a plant transformation binary vector pRD 400 that was mobilized into *Agrobacterim tumefaciens* strain EHA105 and GV1301 respectively.

Transformation of tobacco plants

Agrobacterim tumefaciens strain EHA105 and GV1301 were grown in liquid YEP medium until late exponential phase, then bacteria were pellet and resuspended in liquid MS medium [19], thus preparing the infection solution. Tobacco leaves segment explants were then immersed for 5 minutes in the *Agrobacterium* suspension and then the excess liquid blotted with filter paper and explants placed on solid MS medium with BAP 1mg/l and NAA 0, 1 mg/L for 1 day. After that explants were transferred on selective MS medium supplemented with BAP 1mg/l and NAA 0, 1 mg/L, kanamycin (Km) 100 mg/l, cefotaxime (Cx) 500 mg/l. Regenerated shoots were tested for rooting on MS medium containing Km 100 mg/l and Cx 500 mg/l and 0,1 mg/l NAA.

Protein extraction and expression analysis of HBc gene in transgenic plants by ELISA. Tissue culture leaves were homogenized with cold extraction buffer (1ml/g fresh wt.) containing phosphate buffer saline (PBS), 2 mM EDTA, 1mM

EGTA, 0,1% Triton X-100, 0,05 mg/ml sodium ascorbate, 1mM phenylmethylsulfonyl fluoride. The extract was centrifuged 20 *min* at 12000 g [20]. Total soluble protein was quantified using the BioRad assay (Bio-Rad Laboratories) with bovine serum albumin as a standard protocol.

ELISA protocol

1) The necessary number of wells in microplates were coated with 50 µl per well MAB 2C7-F8-D12 (for optimal dilution 1:400 in PBS ph 7.2) overnight at +4C.
2) The MAB was aspirated and blocked with 5% Non-fat dry milk in PBS then Incubated for 1 hour at room t C by use of 100 µl volume.
3) Washing was performed with PBS containing 0.05% Tween 20 3 x 1 min each using 200 µl volume.
4) Plant extracts were incubated (50ul per well) for 1h at 37C.
5) Wash was performed with PBS 0.05% Tween 20 3x 1 min each using 200 µl vol.)
6) 50 µl of the conjugate (MAB 12F4-B11-H8 1:800) was added in PBS Tween 20, 0.05% plus the blocking material (5%NFM) and incubated for 1h at 37^{o}C.
7) Wash was performed as before and a substrate added. The OPD-ortphenylenediamine substrate for 20-30 min in the dark was used.
8) The reaction was stopped with 1M H2SO4 (50 µl). The measurement was done at wavelength 492 nm.

RESULTS AND DISCUSSIONS

Agrobacterium transformation of tobacco plants

HBV CO/35S construct

Through *Agrobacterium*-mediated transformation thirty independent clones were generated with HBV CO/ 35S construct. First we observed solid dedifferential tissue. First shoots appear 30 days and the vast majority between 30^{th} - 50^{th} days.

When shoots have 2 leaves, they were excised and rooted on MS medium supplemented with Km and Cx and NAA. Fourteen shoots with HBV CO/35S construct were rooted. Kanamycin is a potent inhibitor of root formation - only transgenic plants that contained neomycin phosfotranferase gene were rooted. The present of rooting is 46%.

HBV CO/Tcup constructs

Thirty two independent clones were generated with HBVCO/ Tcup construct using *Agrobacterium* gene transfer. Sixteen shoots were rooted on selective medium. The present of rooting is 50%.

Total soluble protein extracts (quantified by BioRad assay) from the transgenic plants were tested for expression of HBcAg. Enzyme-linked immunosorbent assay was used to determine the level of expression of core gene in transgenic tobacco plants. Fifteen transgenic plants with HBV/CO-35S construct have been tested for expression of

HBcAg. Fourteen were positive in ELISA. The ratio of expression of core gene in transgenic plants is very similar.
Nineteen transgenic plants with HBV/CO Tcup construct have been tested for expression of HBcAg. Thirteen transgenic plants were positive in ELISA.

DISCUTION

Codon optimization and signal sequence lead to significantly higher expression in plant expression systems [20]. Using the plant signal sequence, extension, a plant codon optimized coding sequence and endoplasmic retention sequence we have high levels of expression of the HBV nucleocapsid protein. There is no considerable difference in expression levels between HBV CO/Tcup transgenic plant and HBVCO 35S transgenic plats.

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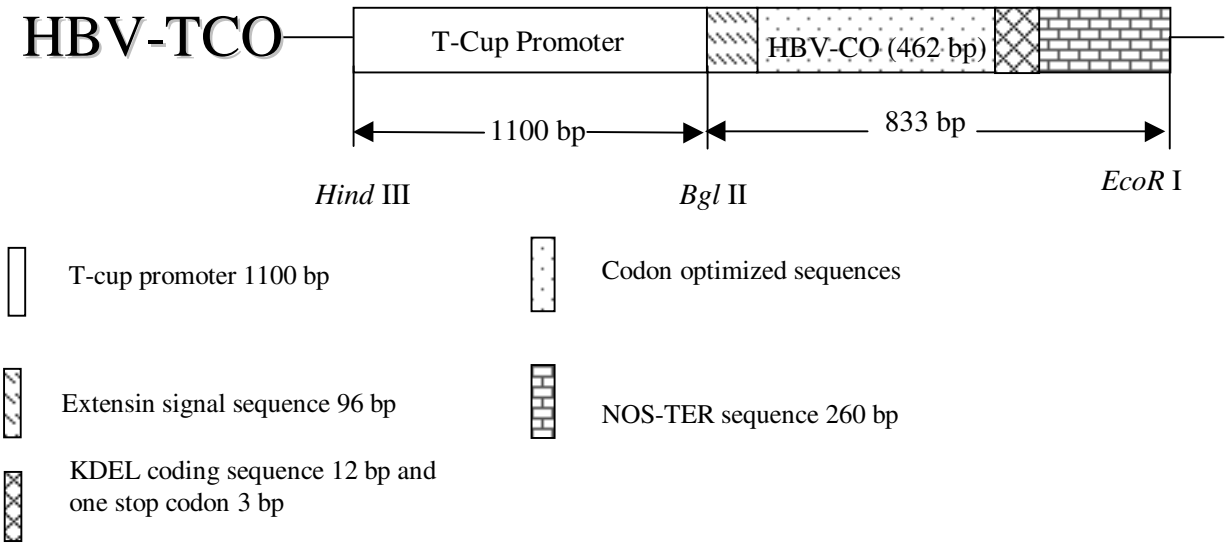

Fig. 1. First set of codon optimized expression constructs HBV-CO. The construct was digested with *Hind* III and *Eco*R I for subcloning into the pRD400 vector. The pRD400 backbone vectors were used to transform *Agrobacterium tumefaciens* strain EHA105 for subsequent transformation of both carrot and tobacco.

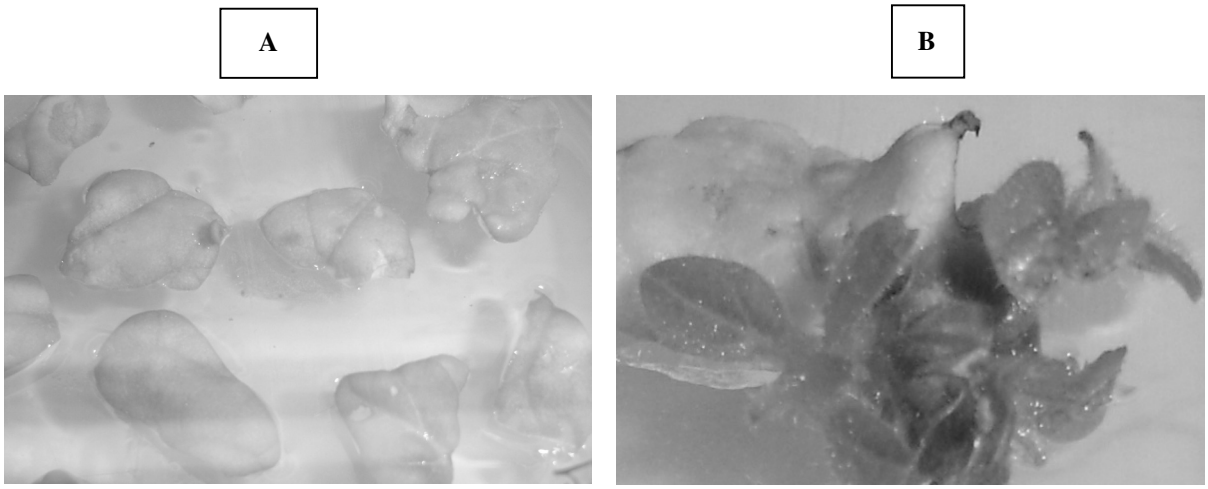

Fig. 2. Continuous selection for transformed tobacco leaf tissues. The photograph shows leaf sections after one month of selection on antibiotics. Leaf sections in control Petri dish A were not transformed and therefore necrotized. Plate B shows leaf sections that were transformed with HBVCo/35 expression construction and hence morphogenesis into plantlets was observed. .

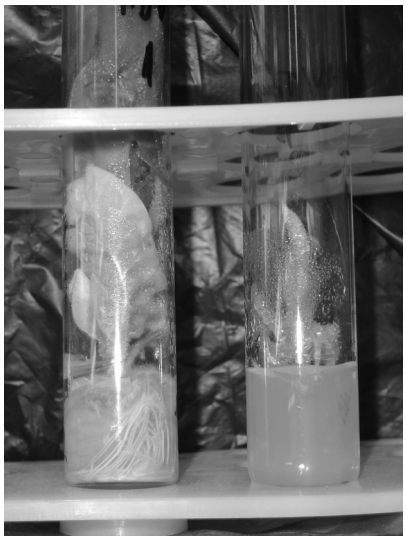

Fig. 2. A - HBV transgenic plant rooting on selective MS medim,
B- control non transgenic plant

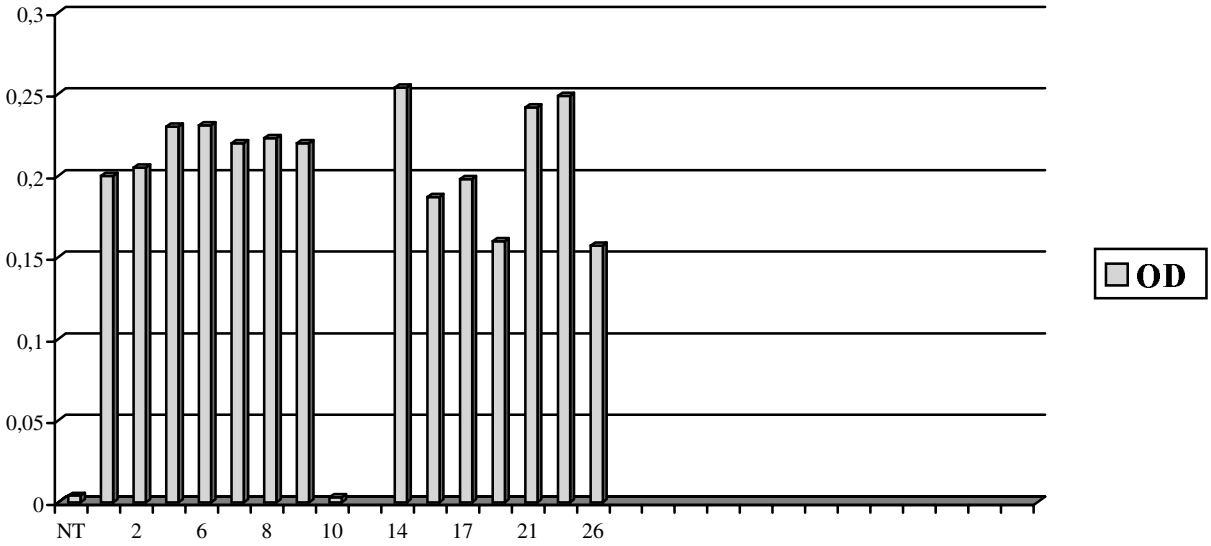

Fig 5 .ELISA HBVCO/35S with MAB

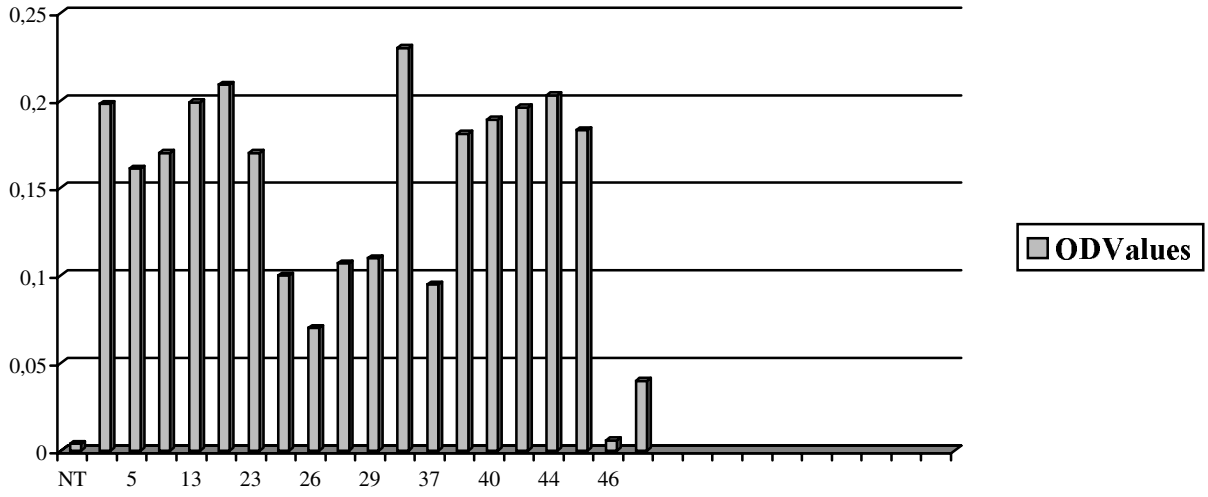

Fig 6. ELISA HBVCO Tcup with MAB