UDK 575.222.7:581.1 **doi: 10.15407/biotech8.05.039**

ACCUMULATION OF RECOMBINANT FUSION PROTEIN – SECRETORY ANALOG OF Ag85B AND ESAT6 *Mycobacterium tuberculosis* PROTEINS – IN TRANSGENIC *Lemna minor* L. PLANTS

A. A. Peterson
M. Yu. Vasylenko
N. A. Matvieieva
M. V. Kuchuk

Institute of Cell Biology and Genetic Engineering
of the National Academy of Sciences of Ukraine, Kyiv

E-mail: joyna56@gmail.com

Received 10.08.2015

Determination of the presence of the recombinant fusion protein (ESAT6-Ag85B(ΔTMD)-6His) and its accumulation level in duckweed plants (*Lemna minor* L.) was the aim of the research. ESAT6 and Ag85B are secretory proteins of *Mycobacterium tuberculosis* and are considered as potential candidates for development of new vaccine against tuberculosis. Transgenic duckweed plants were obtained previously by *Agrobacterium rhizogenes*-mediated transformation and possessed fusion gene sequence *esxA-fbpB*$^{\Delta TMD}$. Specific polyclonal antibodies were produced in immunized mice to identify levels of accumulation of tuberculosis antigens in plants. Recombinant antigen used for mice immunization was obtained in our laboratory by expression in *E. coli*. Western blot analysis revealed the recombinant tuberculosis antigen ESAT6-Ag85B(ΔTMD)-6His in extracts from transgenic *L. minor* plants. The level of accumulation of the protein corresponds to 0.4–0.5 μg protein per 1 g of fresh weight of plant. Additionally, the ability to save of recombinant protein was investigated in lyophilized transgenic plants after 1.5 year storage. Constructed duckweed plants accumulating a recombinant analogue of *M. tuberculosis* secretory proteins can be used for development of plant-based edible vaccines.

Key words: *Lemna minor*, ESAT6, Ag85B(ΔTMD), genetic transformation, *Agrobacterium rhizogenes*.

Achievements in the development of molecular-biological methods became the basis for new strategies in the synthesis of functionally active proteins. Using of plants as expression systems for production of medical proteins including antigens in recent decades is one of the main directions of biotechnological investigations [1–3]. Plant-derived recombinant proteins can be used for immunization of human and animals [4]. The synthesis of such products in plant cells has several advantages compared with the one in microbial or animal cells [5].

Different plant species were used as objects of genetic transformation in order to obtain antigen-produced plants. Thus, gene of hepatitis B virus surface antigen was transferred to genome of banana, potatoes, physalis, tobacco, tomato, peanut plants [6-10]. Plants produced a functional heat labile enterotoxin B subunit of *Escherichia coli* [11], native cholera toxin B subunit [12], Norwalk virus capsid protein [13, 14], human papillomavirus 16 E7 oncoprotein [15] have been obtained. The safety of the recombinant proteins use and a high level of immune response were determined by clinical trials of vaccines of plant origin [16].

Tuberculosis is a disease characterized by high level of morbidity and mortality. Vaccination including using of vaccine developed in 1919 by A. Calmette and C. Guérin (Bacillus Calmette-Guérin, BCG) is the only way to prevent the progress of the infection process. However, the use of this vaccine is not always effective. New approaches to create effective vaccines against tuberculosis are developing now [17–20]. 30-kD-secretory protein, ESAT6, Ag85A, Ag85B, CFP10 *Mycobacterium tuberculosis* proteins [21–23] can be used for these purposes. It was determined that the proteins are immunogenic and demonstrate an immunity effect. For example, using Ag85B and Ag85A

M. tuberculosis antigens has led to increasing of concentrations of IL-2 and γ-interferon in immunized mice [24]. The Ag85B-ESAT6 fusion protein showed immunogenicity and protective properties in case of *M. tuberculosis* infection [25, 26]. Ag85B and MPT64 proteins were tested in a mouse tuberculosis model to confirm their immunotherapeutic effect [27]. The medicine based on *Mycobacterium smegmatis* fusion protein ESAT6-CFP10 caused a greater immune response in mice compared to the BCG vaccine [28].

It was found that plants are able to synthesize mycobacterial recombinant proteins [29] and plant-derived *M. tuberculosis* antigens demonstrate biological activity and induce specific response [30]. In particular, the fusion protein CFP10-ESAT6-dIFN expressed in the roots of transgenic *Daucus carota* plants caused the immune response tested orally and after injection on mice [31]. Choice of the plant species for effective recombinant proteins synthesis is an important step in genetic transformation experiments. Plants using in biotechnological manipulations must meet a number of requirements to improve transformation efficiency and level of synthesis of recombinant proteins, reduced the cost of the final product. Effective methods of micropropagation and genetic transformation should be developed for these purposes. Plants should differ by rapid growth and high level of total protein accumulation because an amount of recombinant proteins is usually quite small. For example cholera toxin B subunit, chimeric protein CTB-P4 and CTB-P6 accumulated in amount 0.096 — 0.9% total soluble protein [32, 33].

Duckweed plants (*Lemna minor* L.) belonging to *Lemnaceae* family can be used for synthesis of recombinant proteins [34]. The rate of growth of these plants is high, since every 3-4 days plants number doubles. Duckweed is known as a natural source of protein which accumulates in cells up to 40% of the dry weight [35]. *L. minor* plants can also be grown in bioreactors under controlled conditions. This method allows safely for the environment cultivation of transgenic plants. In particular, duckweed plants were used to produce recombinant interferon [36]. Dried duckweed plants as a source of protein are used to feed domestic animals. So, duckweed plants can be used to immunize animals on the assumption of sustainable storage of biologically active recombinant antigens in dried biomass. But the possibility of long-term storage of dried recombinant proteins still not studied in duckweed plants. Thus, the aim of our work was to determine the presence and accumulation level of recombinant ESAT6-Ag85B (ΔTMD)-6His protein of *M. tuberculosis* in duckweed plants obtained after *Agrobacterium rhizogenes*-mediated transformation and also the content of this protein in lyophilized transgenic plants after prolonged storage.

Materials and Methods

Plant materials. Transgenic and wild type plants were being cultivated using modified Murashige-Skoog agar medium (twice lowered macrosalts concentration). The temperature of growing was +24 °C and light/dark period was 16/8 h. Before lyophilization plant materials was frizzed in liquid nitrogen. Lyophilization process was performed using Freezer dryer 4.5 (Labconco) according manufacturer manual. Freeze-dried transgenic and wild type plant materials were being preserved at room temperature during 1.5 years in tightly sealed flasks.

Protein extraction. To obtain total protein extracts, plant materials had been placed in cold (+4 °C) extraction solution (urea — 480 g; Tris-base — 6.06 g; HCl — to pH 8.0; deionized water — to 1 l). Grinding of fresh and freeze-dried plant materials in extraction solution and simultaneous extraction of protein was performed using dispersers Ultra Turrax tube drive with DT-20 tubes (IKA). Rotation speed was 6000 rpm and duration of grinding/extraction cycle was 5 min at room temperature. Immediately after grinding, obtained suspensions were clarified by centrifugation at 10000 g during 10 min, at +4 °C. Supernatants were mixed with loading buffer solution (Tris-base — 6.06 g; sodium chloride — 40 g; glycerol — 500 ml; bromphenol blue — 0.1 g; HCl — to pH 6.8; mercaptoethanol — 28 ml; deionized water — to 1 l) in 3:1 ratio. Obtained mixes were heated during 5 min at 95 °C.

To obtain positive control samples, the plant materials with predefined by Western blotting ESAT6-Ag85B(ΔTMD)-6His accumulation were used. Also, purified from *E. coli* recombinant ESAT6-Ag85B(ΔTMD)-6His protein was added to wild type plant total protein extract to obtain final loadings 0.04 ng, 0.2 ng and 1.0 ng of the recombinant protein on trek. Simultaneously, the samples of purified recombinant ESAT6-Ag85B(ΔTMD)-6His were used at the same concentrations.

Recombinant protein. E. coli cultivation. *E. coli* strain №120 was cultivated at +37 °C, with shaking at 200 rpm, overnight, in 50 ml LB medium containing 100 μg/ml ampicillin. Then, 5 ml of overnight culture was inoculated in 500 ml Erlenmeyer flask with 100 ml preheated LB medium containing 100 μg/ml ampicillin. Bacterial suspension had been cultivated at +37 °C, with shaking at 200 rpm during 1hour. Then, induction was performed by adding to culture isopropil-b-D-thiogalactoside to a final concentration of 1 mM. After induction, bacterial suspension was cultured under the same condition during 6 hours. *E. coli* biomass was harvested by centrifugation at 5000 g, for 10 min, at +4 °C.

Preparing of biomass lysat for chromatography purification. Biomass of *E. coli* was resuspended in lysis buffer solution (urea — 480 g; Tris-base — 6.06 g; HCl — to pH 8.0; deionized water — to 1 l) in 1:5 ratio. Lysis had been performed overnight, with gently shaking, at +4 C. Obtained lysat was clarified by centrifugation at 8000 g for 30 min, at +4 °C. Supernatant was filtered through membrane filter with 0.45 μm pore size.

Chromatographic purification of recombinant protein. Ni-NTA-agarose (QIAgen, cat. № 30210) and LKB UVICORD II chromatographic photometric system were used to purify recombinant ESAT6-Ag85B(ΔTMD)-6His protein from bacterial lysat. Column, with 2 ml of slurry, was equilibrated whit 10 ml lysis buffer solution. Filtered bacterial lysat was loaded using peristaltic pump (speed: 0.5 ml/min) at room temperature. Washing the column was performed using washing buffer solution (urea — 480 g; NaCl — 117 g; Tris-base — 6.06 g; HCl — to pH 8.0; deionized water — to 1 l) to get base level of optical density at 280 nm wavelength. Recombinant protein was eluted using washing buffer solution containing 250 mM imidazole. Eluate had been preserved at +4 °C.

*IgG fraction from anti-ESAT6-Ag85B(*ΔTMD)-6His *serum. Immunisation of mouse.* To eliminate imidazole from eluate and prepare protein solution for immunization, two step dialysis against 1 l of dialysis buffer solution (urea — 120 g; Tris-base — 6.06 g; HCl — to pH 8.0; deionized water — to 1 l) was performed. Before loading to a dialysis tube, eluate was diluted using dialysis buffer solution to get a final protein concentration below 2 mg/ml. First step of dialysis was performed at +4 °C, with gently mixing using magnetic stirrer, overnight. Second step of dialysis was performed under the same condition, during 4 hours. Dialysate was mixed with Complete Freund's Adjuvant in 1:1 ratio. Obtained mix for immunization was injected to mouse intraperitoneally in a dose 200 μl per mouse. Then injections were repeated twice, with 1 month gap each time.

IgG fraction chromatographic purification. Ten days after third immunization the total bleeding of immune animals was performed. Mouse blood was clarified by centrifugation at 2000 g for 5 min, at room temperature. 1,8 ml of supernatant was added to 16 ml of buffer solution for serum dilution ($Na_2HPO_4 \cdot 12H_2O$– 2.3 g; KH_2PO_4 — 0.2 g; NaCl — 8 g; KCl — 0.2 g; deionized water — to 1 l). Diluted serum was filtered through membrane filter with 0.45 μm pore size.

Protein-G-sepharose (GE, Product code 17-0618-01) and LKB UVICORD II chromatographic photometric system were used to purify IgG fraction from anti-ESAT6-Ag85B(ΔTMD)-6His serum. Column, with 1 ml of slurry, was equilibrated whit 10 ml solution for serum dilution. Filtered diluted serum was loaded using peristaltic pump (speed: 0.5 ml/min) at room temperature. Washing the column was performed using solution for serum dilution to get base level of optical density at 280 nm wavelength. IgG fraction was eluted using 0.1 M glycine-HCl buffer solution, pH 2.5. Eluate was collected in a flask containing 1 ml of 2M Tris-HCl, pH 9.0. Chromatographic purification cycle was repeated using flow-through as a starting material for yield increasing. Immediately after elution, three-stage dialysis was performed against 1 l of solution for serum dilution. First step of dialysis was performed at +4 C, with gently mixing using magnetic stirrer, overnight. Second and third steps of dialysis were performed under the same condition, during 4 hours. Sodium azide was added to dialysate to a final concentration of 0.02%. Purified mouse IgG fraction solution containing sodium azide had been preserved at +4 C.

Western blotting. Mini-PROTEAN Tetra Cell electrophoresis module (BIO-RAD) was used for performing of sodium dodecyl sulfate polyacrilamide gell electrophoresis of proteins (SDS-PAGE). The mix for resolving gel was prepared containing: Tris-base — 46 g; acrylamide — 120 g; sodium dodecyl sulfate — 1 g; ammonium persulfate — 1 g; TEMED — 0.86 ml; HCl — to pH 8.8; deionized water — to 1 l. Immediately after

preparing, mix was loaded between gel glass plates. Then, deionized water was added above gel mix to form a layer of 3–4 mm height. The polymerization time was 1 hour. The mix for stacking gel was prepared containing: Tris-base — 15.73 g; acrylamide — 51 g; sodium dodecyl sulfate — 1 g; ammonium persulfate — 1 g; TEMED — 1 ml; HCl — to pH 6.8; deionized water — to 1 l. Immediately after preparing, mix was loaded between gel glasses above polymerized resolving gel. Then, the 10-teeth comb was placed between gel glass plates. The polymerization time was 20 min. After polymerization, electrophoresis module was assembled and electrophoresis working buffer solution (Tris-base — 3.02 g; glycine — 18.8 g; sodium dodecyl sulfate — 1g; deionized water — to 1 l) was added to upper and lower chambers of electrophoresis module. The analyzed samples and the prestained protein molecular weight marker (Fermentas) were added to gel wells and electrophoresis was performed according manufacturer manual.

After electrophoresis had been completed, the gel was soaked in transfer buffer solution (Tris-base — 5.8 g; glycine — 2.9 g; methanol — 100 ml; deionized water — to 1 l). The gel has been incubated in the transfer buffer for 15 minutes. Simultaneously Immun-Blot PVDF the gel sized piece of membrane (BIO-RAD) and 20 the gel sized pieces of chromatographic paper (Whatman) were soaked in the solution same composition. NOVABLOT Electrophoretic Transfer Kit (LKB) was used to perform semi-dry protein transfer from gel to PVDF membrane. Electrophoretic transfer was performed according manufacturer manual. The point to end the procedure was the full disappearance of prestained protein molecular weight marker from the gel and appearance of marker on membrane surface.

After protein transfer had been completed, the PVDF membrane was soaked in blocking buffer solution containing: $Na_2HPO_4 \cdot 12H_2O$ — 2.3 g; KH_2PO_4 — 0.2 g; NaCl — 8 g; KCl — 0.2 g; skimmed milk — 50 g; Tween-20 — 1 ml; deionized water — to 1 l. The membrane was incubated in blocking buffer solution with gently shaking using orbital shaker (speed: 50 rpm), at +4 C, overnight. Then, the membrane was transferred in mouse anti-ESAT6-Ag85B(ΔTMD)-6His IgG fraction buffer solution containing $Na_2HPO_4 \cdot 12H_2O$ — 2.3 g; KH_2PO_4 — 0.2 g; NaCl — 8 g; KCl — 0.2 g; Tween-20 — 1 ml; mouse IgG — in optimal titer; deionized water — to 1 l. The membrane was incubated in mouse IgG buffer solution with gently shaking using orbital shaker (speed: 50 rpm), at +42 C, for 2 hours. After incubation had been completed, the membrane was washed three times with washing buffer solution containing: $Na_2HPO_4 \cdot 12H_2O$ — 2.3 g; KH_2PO_4 — 0.2 g; NaCl — 8 g; KCl — 0.2 g; Tween-20 — 1 ml; deionized water — to 1 l.

Washed membrane was transferred to the buffer solution of horseradish peroxidase conjugated goat polyclonal antibodies, specific to a mouse IgG (Sigma) containing $Na_2HPO_4 \cdot 12H_2O$ — 2.3 g; KH_2PO_4 — 0.2 g; NaCl — 8 g; KCl — 0.2 g; Tween-20 — 1 ml; horseradish peroxidase conjugated goat polyclonal antibodies — in optimal titer; deionized water — to 1 l. The membrane was incubated in this solution with gently shaking using orbital shaker (speed: 50 rpm), at +37 °C, for 1 hour. After incubation had been completed, the membrane was washed three times with washing buffer solution containing: $Na_2HPO_4 \cdot 12H_2O$ — 2.3 g; KH_2PO_4 — 0.2 g; NaCl — 8 g; KCl — 0.2 g; Tween-20 — 1 ml; deionized water — to 1 l.

Washed membrane was transferred to the ECL substrate solution for horseradish peroxidase detection (BIO-RAD). Chemiluminescence was documented in dark room using x-ray film included developer and fixer solutions.

Results and Discussion

Duckweed transgenic plants (*L. minor)* developed earlier using *Agrobacterium rhizogenes*-mediated method were used in this research [38]. Transgenic duckweed had modified sequence of mycobacterium's genes (*esxA* and *fbpB*) that code recombinant protein ESAT6-Ag85B(ΔTMD)-6His. N-terminal sequence of this protein was represented by polypeptide ESAT6 (98 aminoacids) and C-terminal sequence was represented by polypeptide Ag85B (304 aminoacids). Polypeptide of Ag85B had a deletion of TMD-like sequence (trans-membrane domain) in his N-terminal part and had 6-His tag in C-terminal part. Accumulation of tuberculosis antigens was investigated for both fresh (Fig. 1, *a*) and lyophilized (Fig.1, *b*, *c*) plant material.

Analysis of the protein expression of the fusion protein — analog of secretory antigens ESAT6 and Ag85B from *M. tuberculosis*, — was performed in plant material by Western blotting. Based on the data that recombinant protein accumulation in transgenic plants can reach low values (0.00001% of protein from fresh weight of the plant) we had to

Fig. 1. **Transgenic duckweed plants cultured in vitro (*a*); lyophilized biomass of duckweed accumulated for following storage (*b*, *c*)**

ensure appropriate detection sensitivity of the ESAT6-Ag85B(ΔTMD)-6His protein by Western blotting analysis. Despite of the method parameters (ratio of extraction buffer to plant material, sample volume in gel) the sensitivity should be around 0.1 ng of recombinant protein per track.

Using purified ESAT6-Ag85B(ΔTMD)-6His protein we showed that produced specific antibodies are sensitive enough to detect 0.04 ng of recombinant antigen per track. Such a level of sensitivity was sufficient for analysis of the transgenic plant material. Evaluation of sensitivity of derived antibodies was shown for series of dilution of the purified recombinant protein ESAT6-Ag85B(ΔTMD)-6His (Fig. 2).

If derived antibodies were used for Western blotting analysis of plant extracts the sensitivity of method was lower because of background and nonspecific bands in tracks of crude extracts (Fig. 3, 4). But nonspecific background was not dense in a range of molecular weights that are close to the molecular weight of the recombinant protein ESAT6-Ag85B (ΔTMD)-6His. This made it possible to apply this antibody in Western blotting for detection of recombinant protein ESAT6-Ag85B (ΔTMD)-6His in total protein extracts of transgenic *L. minor* with the expected accumulation level not lower than 0.00001% of recombinant protein in wet weight of the plant sample.

Results of detection of recombinant protein ESAT6-Ag85B (ΔTMD)-6His in fresh biomass of *L. minor* are shown in Fig. 3.

Specific band was found in extract of transgenic duckweed (track 1) corresponding molecular weight 43 kDa of recombinant protein ESAT6-Ag85B(ΔTMD)-6His. However, it was also found specific band that fits smaller molecular weight protein (28–30 kDa), which is probably proteolysis product of 43 kDa protein (track *1*). In extracts from control duckweed plants similar proteins with a molecular weight of 43 kDa and 28–30 were not observed. The approximate amount of antigen in the extract from transgenic duckweed was determined by comparing the intensity of corresponding bands in extract of transgenic plants with bands of control antigen mixed with extract of wild type plants. Control antigen was expressed in *E. coli*, purified chromatographically and used as a control in amounts 0.04, 0.2 and 1 ng per track. Control protein was loaded both in pure form and as a mixture with an extract of wild type *L. minor*. This mixture was prepared by adding purified antigen immediately before grinding of plant biomass. This was done to determine possible losses recombinant protein due to proteolysis during sample preparation. Comparison tracks

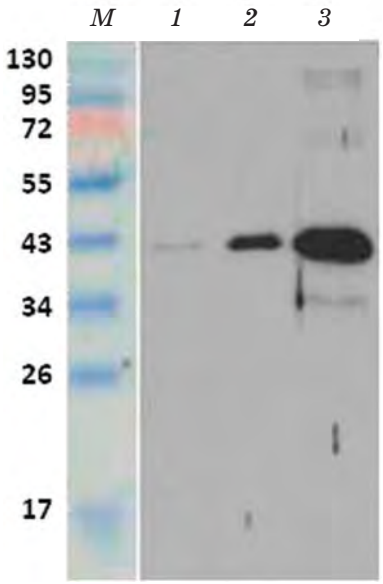

Fig. 2. **Determination of the sensitivity of derived antibodies which are specific to ESAT6-Ag85B(ΔTMD)-6His and was applied as first antibodies in Western blotting method:**
M — molecular weight marker represented as colored bands on the membrane (kDa);
1–3 — purified protein ESAT6-Ag85B(ΔTMD)-6His in amounts 0.04, 0.2 and 1.0 ng per track respectively

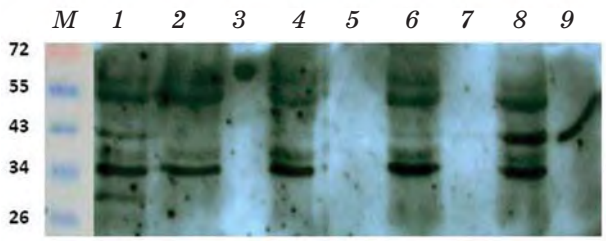

Fig. 3. **Detection of recombinant tuberculosis antigen ESAT6-Ag85B(ΔTMD) via Western blotting analysis in fresh biomass extracts from *L. minor* plants, which are transformed with *esxA-fbpB*$^{\Delta TMD}$ sequence coding fusion protein — analog secretory proteins ESAT6 and Ag85B from *Mycobacterium tuberculosis*:**

1 — extract of transgenic plants; *2* — extract of wild type plants; *3* — track of molecular weight markers (as pre-stained bands on membrane is shown as track M); *4* — extract of wild type plants with addition of purified ESAT6-Ag85B(ΔTMD)-6His protein, 40 pg/track; *5* — purified ESAT6-Ag85B(ΔTMD)-6His protein, 40 pg/track; *6* — extract of wild type plants with addition of purified ESAT6-Ag85B(ΔTMD)-6His protein, 200 pg/track; *7* — purified ESAT6-Ag85B(ΔTMD)-6His protein, 200 pg/track; *8* — extract of wild type plants with addition of purified ESAT6-Ag85B(ΔTMD)-6His protein, 1 ng/track; *9* — purified ESAT6-Ag85B(ΔTMD)-6His protein, 1 ng/track

9 and 8 (Fig. 3), and tracks 7 and 6 (Fig. 3) shows almost identical antigen content in tracks with purified protein and in tracks with mixtures of plant extract with added control protein in the same concentrations. This fact indicates that proteolytic activity of the extract was minimal during the sample preparation and had no significant influence on recombinant protein ESAT6-Ag85B (ΔTMD)-6His in extracts. Taking into account the above we supposed that identified protein degradation (Fig. 3, track *1*) may be due to proteolysis of the recombinant antigen in living cells during synthesis and accumulation. Comparison tracks *1*, *6* and *8* (Fig. 3) allows to determine the approximate amount of product in the extract and plant material — 20-25 ng/ml, which corresponds to 0.4–0.5 μg per 1 g of dry plant weight.

A comparative study of the amount of accumulated antigen was performed for freeze-dried plant material that had been stored after drying in closed plastic tubes at room temperature for 1.5 years. Results of detection of recombinant protein ESAT6-Ag85B (ΔTMD) -6His in dried *L. minor* biomass are presented in Fig. 4.

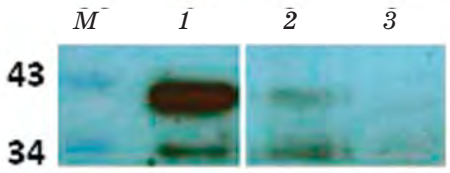

Fig. 4. **Comparative Western blotting analysis of recombinant protein in the extract of freeze-dried and raw biomass of transgenic plants *L. minor*:**

1 — extract of raw biomass of transgenic plants; *2* — extract of freeze-dried biomass of transgenic plants; *3* — extract of wild type duckweed; *M* — marker of molecular weights

The obtained data suggest that the recombinant protein stored for a long time in the lyophilized duckweed plants even without the use of additional techniques — at room temperature and without protective gas environment. However identified protein amount in lyophilized biomass naturally was less than the one in the fresh material.

Approaches to produce recombinant proteins that can be stored for a long time in plants are developing in modern biotechnology. For example, a strategy that is based on the production and accumulation of recombinant proteins in seeds is considered effective and allows improving the stability of the synthesized target proteins in plants [39–41]. However, as was shown by our study, *L. minor* plants long-term storage (for 1.5 year) in lyophilized form has not led to a complete loss of the target recombinant protein, although the concentration of this protein decreased. Thus, a relatively simple and cheap procedure for preparation and storage of biological material does not cause total loss of accumulated protein product for a long period and has the potential for use in the development of edible vaccines.

The method used made it possible to detect recombinant protein ESAT6-Ag85B (ΔTMD)-6His in extracts of transgenic *L. minor* plant. This protein accumulated in transgenic duckweed plants in 0.4–0.5 μg per 1 g of dry plant weight or 0.036 μg per 1 mg of total soluble protein. Storage of freeze-dried plant material for 1.5 years without cooling or freezing did not result in complete degradation of the recombinant protein, although their content was reduced compared to the content in the plants cultured *in vitro*. Duckweed plants, synthesizing recombinant secretory antigens ESAT6 and Ag85B *Mycobacterium tuberculosis* can be offered as a model system to study the possibility of creating of edible plant-derived vaccines.

REFERENCES

1. *Han M., Su T., Zu Y. G., An Z. G.* Research advances on transgenic plant vaccines. *Yi Chuan Xue Bao*. 2006, 33 (4), 285–293. PMID: 16625826.
2. *Schillberg S., Twyman R. M., Fischer R.* Opportunities for recombinant antigen and antibody expression in transgenic plants — technology assessment. *Vaccine*. 2005, 23 (15), 1764–1769. PMID: 15734038.
3. *Tiwari S., Verma P. C., Singh P. K., Tuli R.* Plants as bioreactors for the production of vaccine antigens. *Biotechnol. Adv.* 2009, 27 (4), 449–467. doi: 10.1016/j.biotechadv.2009.03.006.
4. *Fernandez-Fernandez M. R., Mourino M., Rivera J., Rodriguez R., Plana-Duran J., Garcia J. A.* Protection of rabbits against rabbit hemorrhagic disease virus by immunization with the VP60 protein expressed in plants with a potyvirus-based vector. *Virology*. 2001, V. 280, P. 283–291. PMID: 11162842.
5. *Houdebine L. M.* Production of pharmaceutical proteins by transgenic animals. *Comp. Immunol. Microbiol. Infect. Dis*. 2009, V. 32, P. 107–121. PMID: 18243312.
6. *Kumar G. B. S., Ganapathi T. R., Revathi C. J., Srinivas L., Bapat V. A.* Expression of hepatitis B surface antigen in transgenic banana plants. *Planta*. 2005, V. 222, P. 484–493. PMID: 15918027.
7. *Kumar G. B. S., Ganapathi T. R., Srinivas L., Revathi C. J., Bapat V. A.* Secretion of hepatitis B surface antigen in transformed tobacco cell suspension cultures. *Biotechnol. Lett*. 2005, V. 27, P. 927–932. PMID: 16091888.
8. *Kumar G. B. S., Ganapathi T. R., Srinivas L., Revathi C. J., Bapat V. A.* Expression of hepatitis B surface antigen in potato hairy roots. *Plant Sci*. 2006, V. 170, P. 918–925. doi:10.1016/j.plantsci.2005.12.015.
9. *Gao Y., Ma Y., Li M., Cheng T., Li S. W., Zhang J., Xia N. S.* Oral immunization of animals withtransgenic cherry tomatillo expressing HBsAg. *World J. Gastroenterol*. 2003, V. 9, P. 996–1002. PMID: 12717845.
10. *Lauterslager T. G., Florack D. E., Wal T. J., Molthoff J. W., Langeveld J. P., Bosch D., Boersma W. J., Hilgers L. A.* Oral immunization of naive and primed animals with transgenic potato tubers expressing LT-B. *Vaccine*. 2001, V. 19, P. 2749–2755. PMID: 11257419.
11. *Chikwamba R., Cunnick J., Hathaway D., McMurray J., Mason H., Wang K.* A functional antigen in a practical crop: LT-B producing maize protects mice against Escherichia coli heat labile enterotoxin (LT) and cholera toxin (CT). *Transg. Res*. 2002, V. 11, P. 479–493. PMID: 12437079.
12. *Daniell H., Lee S. B., Panchal T., Wiebe P. O.* Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *J. Mol. Biol* . 2001, V. 311, P. 1001–1009. PMID: 11531335.
13. *Mason H. S., Ball J. M., Shi J. J., Jiang X., Estes M. K., Arntzen C. J.* Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl. Acad. Sci. USA*. 1996, V. 93, P. 5335–5340. PMCID: PMC39246.
14. *Tacket C. O., Mason H. S., Losonsky G., Estes M. K., Levine M. M., Arntzen C. J.* Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J. Infect. Dis*. 2000, V. 182, P. 302–305. PMID: 10882612.
15. *Franconi R., Di Bonito P., Dibello F., Accardi L., Muller A., Cirilli A., Simeone P., Donà M. G., Venuti A., Giorgi C.* Plant-derived human papillomavirus 16 E7 oncoprotein induces immune response and specific tumor protection. *Cancer Res*. 2002, V. 62, P. 3654–3658. PMID: 12097270.
16. *Gómez N., Wigdorovitz A., Castañón S., Gil F., Ordás R., Borca M. V., Escribano J. M.* Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus. *Arch. Virol*. 2000, V. 145, P. 1725–1732. PMID: 11003480.
17. *McMurray D. N.* Recent progress in the development and testing of vaccines against human tuberculosis. *Int. J. Parasitol*. 2003, 33 (5–6), 547–554. PMID: 12782054.
18. *Doherty T. M., Andersen P.* Vaccines for Tuberculosis: Novel Concepts and Recent Progress. *Clin. Microbiol. Rev*. 2005, 18 (4), 687–702. PMCID: PMC1265910.
19. *Orme I. M.* Current progress in tuberculosis vaccine development. *Vaccine*. 2005, 23 (17–18), 2105–2108. PMID: 15755579.
20. *Orme I. M., McMurray D. N., Belislea J. T.* Tuberculosis vaccine development: recent progress. *Trends Microbiol*. 2001, 9 (3), 115–118. PMID: 11239788.
21. *Wang Bao-Lin, Ying Xu, Chao-Qun Wu.* Cloning, expression, and refolding of a secretory protein ESAT-6 of Mycobacterium tuberculosis. *Prot. Exp. Purif*. 2005, 39 (2), 184–188. PMID: 15642469.
22. *Brodin P., Rosenkrands I., Andersen P., Cole S. T., Brosch R.* ESAT-6 proteins: protective antigens and virulence factors? *Trends Microbiol*. 2004, V. 12, P. 500–508. PMID: 15488391.
23. *Young D., Dye C.* The development and impact of tuberculosis vaccines. *Cell*.

2006, V. 124, P. 683–687. doi:10.1016/j.cell.2006.02.013.

24. *Lozes E., Huygen K. 1., Content J.* Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. *Vaccine.* 1997, 15 (8), 830–833. PMID: 9234526.
25. *Weinrich Olsen A., van Pinxteren L. A., Meng Okkels L., Birk Rasmussen P., Andersen P.* Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. *Infect. Immun.* 2001, V. 69, P. 2773–2778. PMID: 11292688.
26. *Palendira U., Spratt J. M., Britton W. J., Triccas J. A.* Expanding the antigenic repertoire of BCG improves protective efficacy against aerosol *Mycobacterium tuberculosis* infection. *Vaccine.* 2005, V. 23, P. 1680–1685. PMID: 15705472.
27. *Zhu D., Jiang S., Luo X.* Therapeutic effects of Ag85B and MPT64 DNA vaccines in a murine model of *Mycobacterium tuberculosis* infection. *Vaccine.* 2005, 23 (37), 4619–4624. PMID: 16053938.
28. *Zhang H., Peng P., Miao S., Zhao Y., Mao F., Wang L., Bai Y., Xu Z., Wei S., Shi C.* Recombinant Mycobacterium smegmatis expressing an ESAT6-CFP10 fusion protein induces anti-mycobacterial immune responses and protects against Mycobacterium tuberculosis challenge in mice. *Scand. J. Immunol.* 2010, 72 (4), 349–357. PMID: 20883320.
29. *Dorokhov Y. L., Sheveleva A. A., Frolova O. Y.* Superexpression of tuberculosis antigens in plant leaves. *Tuberculosis.* 2007, 87 (3), 218–224. PMID: 17182283.
30. *Rigano M. M., Dreitz S., Kipnis A. P.* Oral immunogenicity of a plant-made, subunit, tuberculosis vaccine. *Vaccine.* 2006, 24 (5), 691–695. PMID: 16169639.
31. *Permyakova N. V., Zagorskaya A. A., Belavin P. A., Uvarova E. A., Nosareva O. V., Nesterov A. E., Novikovskaya A. A., Zav'yalov E. L., Moshkin M. P., Deineko E. V.* Transgenic Carrot Expressing Fusion Protein Comprising M. tuberculosis Antigens Induces Immune Response in Mice. *Biomed. Res. Int.* 2015, V. 2015, Article ID 417565. doi: 10.1155/2015/417565.
32. *Mishra S., Yadav D. K., Tuli R.* Ubiquitin fusion enhances choleratoxin B subunit expression in transgenic plants and the plant-expressed protein binds GM1 receptors more efficiently. *J. Biotechnol.* 2006, V. 127, P. 95–108. doi: 10.1016/j.jbiotec.2006.06.002.
33. *Sharma M. K., Singh N. K., Jani D., Sisodia R., Thungapathra M., Gautam J. K., Meena L. S., Singh Y., Ghosh A., Tyagi A. K., Sharma A. K.* Expression of toxin co-regulated pilus subunit A (TCPA) of Vibrio cholerae and its immunogenic epitopes fused to choler atoxin B subunit in transgenic tomato (Solanum lycopersicum). *Plant. Cell. Rep.* 2008, V. 27, P. 307–318. PMID: 17962948.
34. *Gasdaska J. R., Spencer D., Dickey L.* Advantages of Therapeutic Protein Production in the Aquatic Plant Lemna. *BioProces. J.* 2003, 2 (2), 49–56.
35. *Yılmaz E., Akyurt I., Güna G.* Use of Duckweed, Lemna mino, as a Protein Feedstuff in Practical Diets for Common Carp, Cyprinus carpio, Fry. *Turk. J. Fish. Aquatic Sci.* 2004, V. 4, P. 105–109.
36. *De Leede L. G., Humphries J. E., Bechet A. C., Van Hoogdalem E. J., Verrijk R., Spencer D. G.* Novel controlled-release Lemna-derived IFN-alpha2b (Locteron): pharmacokinetics, pharmacodynamics, and tolerability in a phase I clinical trial. *J. Interfer. Cytokine Res.* 2008, 28 (2), 113–122. PMID: 18279106.
37. *Murashige T., Skoog F.* A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plantarum.* 1962, V. 15, P. 473–497.
38. *Matvieieva N. A., Kishchenko O. M., Shakhovsky A. M., Kuchuk M. V.* Transfer of ESAT6 and Ag85B antigens to *Lemna minor* L. plants using *Agrobacterium rhizogenes. Biotechnology.* 2011, 4 (2), 46–53.
39. *Cunha N. B., Murad A. M., Ramos G. L., Maranhão A. Q., Brígido M. M., Araújo A. C. G., Lacorte C., Aragão F. J. L., Covas D. T., Fontes A. M., Souza G. H. M. F., Vianna G. R., Rech E. L.* Accumulation of functional recombinant human coagulation factor IX in transgenic soybean seeds. *Transg. Res.* 2011, V. 20, P. 841–855. doi: 10.1007/s11248-010-9461-y.
40. *Kim W. S., Krishnan H. B.* Expression of an 11 kDa methionine-rich delta-zein in transgenic soybean results in the formation of two types of novel protein bodies in transitional cells situated between the vascular tissue and storage parenchyma cells. *Plant Biotechnol. J.* 2004, V. 2, P. 199–210. PMID: 17147611.
41. *Yamada Y., Nishizawa K., Yokoo M., Zhao H., Onishi K., Teraishi M., Utsumi S., Ishimoto M., Yoshikawa M.* Anti-hypertensive activity of genetically modified soybean seeds accumulating novokinin. *Peptides.* 2008, V. 29, P. 331–337. PMID: 18226422.

НАКОПИЧЕННЯ РЕКОМБІНАНТНОГО ЗЛИТОГО ПРОТЕЇНУ–АНАЛОГА СЕКРЕТОРНИХ ПРОТЕЇНІВ Ag85B ТА ESAT6 *Micobacterium tuberculosis* — У ТРАНСГЕННИХ РОСЛИНАХ *Lemna minor* L.

А. А. Петерсон
М. Ю. Василенко
Н. А. Матвєєва
М. В. Кучук

Інститут клітинної біології та генетичної інженерії НАН України, Київ

E-mail: joyna56@gmail.com

Метою роботи було визначення наявності рекомбінантного протеїну ESAT6-Ag85B(ΔTMD)-6His із *Mycobacterium tuberculosis* та вивчення рівнів його накопичення у рослинах ряски (*Lemna minor* L.). ESAT6 та Ag85B є секреторними протеїнами *M. tuberculosis* і запропоновані для створення нових вакцин проти туберкульозу. Отримані раніше шляхом опосередкованої *Agrobacterium rhizogenes* трансформації рослини ряски містили злиту послідовність генів: *esxA-fbpB*$^{\Delta TMD}$. Для визначення рівнів накопичення туберкульозних антигенів у рослинах було одержано поліклональні мишачі антитіла шляхом імунізації лабораторних тварин рекомбінантним протеїном ESAT6-Ag85B(ΔTMD)-6His, експресованим в *E. coli*. Методом вестерн-блот виявлено рекомбінантний туберкульозний антиген ESAT6-Ag85B(ΔTMD)-6His в екстрактах із трансгенних рослин *L. minor*. Визначено рівень накопичення антигену, який становив 0,4–0,5 мкг протеїну на 1 г сирої маси рослин. Також вивчено можливість зберігання та ступінь деградації туберкульозного антигену в ліофілізованому рослинному матеріалі, що зберігався впродовж 1,5 року без охолодження чи заморожування. Отримані рослини ряски *L. minor*, які синтезують рекомбінантний антиген, аналог секреторних протеїнів ESAT6 та Ag85B *M. tuberculosis*, можуть бути об'єктом досліджень під час створення їстівних рослинних вакцин.

Ключові слова: *Lemna minor*, ESAT6, Ag85B(ΔTMD), генетична трансформація, *Agrobacterium rhizogenes*.

НАКОПЛЕНИЕ РЕКОМБИНАНТНОГО СЛИТОГО ПРОТЕИНА — АНАЛОГА СЕКРЕТОРНЫХ ПРОТЕИНОВ Ag85B И ESAT6 *Micobacterium tuberculosis* — В ТРАНСГЕННЫХ РАСТЕНИЯХ *Lemna minor* L.

А. А. Петерсон
М. Ю. Василенко
Н. А. Матвеева
Н. В. Кучук

Институт клеточной биологии и генетической инженерии НАН Украины, Киев

E-mail: joyna56@gmail.com

Целью работы было определение наличия рекомбинантного протеина ESAT6-Ag85B (ΔTMD)-6His из *Mycobacterium tuberculosis* и изучение уровней его накопления в растениях ряски (*Lemna minor* L.). ESAT6 и Ag85B являются секреторными протеинами *M. tuberculosis* и предлагаются для создания новых вакцин против туберкулеза. Полученные ранее путем опосредованной *Agrobacterium rhizogenes* трансформации растения ряски содержали слитую последовательность генов: *esxA-fbpB*$^{\Delta TMD}$. Для определения уровней накопления туберкулезных антигенов в растениях были получены поликлональные мышиные антитела путем иммунизации лабораторных животных рекомбинантным протеином ESAT6-Ag85B(ΔTMD)-6His, экспрессированном в *E. coli*. Методом вестерн-блот выявлен рекомбинантный туберкулезный антиген ESAT6-Ag85B(ΔTMD)-6His в экстрактах из трансгенных растений *L. minor*. Определен уровень накопления антигена, соответствующий 0,4–0,5 мкг протеина на 1 г сырой массы растений. Также изучена возможность сохранения и степень деградации туберкулезного антигена в лиофилизированном растительном материале, который хранился в течение 1,5 года без охлаждения или замораживания. Полученные растения ряски, синтезирующие рекомбинантный антиген, аналог секреторных протеинов ESAT6 и Ag85B *M. tuberculosis*, могут быть объектом исследований при создании съедобных растительных вакцин.

Ключевые слова: *Lemna minor*, ESAT6, Ag85B(ΔTMD), генетическая трансформация, *Agrobacterium rhizogenes*.