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Highly Efficient Expression of *Escherichia coli* Heat-Labile Enterotoxin B Subunit in Plants Using Potato Virus X-Based Vector

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Abstract—A synthetic gene of the B-subunit of *Escherichia coli* heat-labile toxin, optimized for expression in plants, was designed and synthesized. The recombinant viral vector was constructed on the basis of potato virus X containing the LTB gene instead of the removed triple block of transport genes and the coat protein gene, which provides for LTB expression in plants. The vector is introduced into the plant cells during cell infiltration by agrobacteria incorporating a binary vector, the T-DNA region of which contains a cDNA copy of the recombinant viral genome. Under conditions of posttranscriptional gene silencing inhibition, the LTB yield in *Nicotiana benthamiana* plants is 1-2% of total soluble protein; in this case, LTB synthesized in plants forms pentameric complexes analogous to those found in the native toxin. The designed viral system of LTB transient expression can be used to obtain in plants a vaccine against enteropathogenic *Escherichia coli*.

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Key words: plant as "biofactories", LTB, vaccine, viral vector

Diarrhea caused by enteropathogenic *Escherichia coli* is one of the most widespread diseases of humans and farm animals. Heat-labile enterotoxin (LT) is one of the pathogenicity factors, resembling cholera toxin in its immunological and physicochemical properties [1, 2]. LT belongs to the AB_5 class of toxins and includes one 27-kD A-subunit, exhibiting proper toxic ADP-ribosyl transferase activity, and a pentamer formed due to noncovalent binding of five 11.6-kD B-subunits (LTB). The interaction of LT with intestinal cells is the result of specific binding of the pentamer formed by B-subunits to the cellular receptor—GM1 ganglioside.

LTB is an efficient immunogen providing for formation of protective immunity against *E. coli* diarrhea [3]. Immunization by LTB can be oral with generation of a high titer of specific serum and mucosal antibodies [4, 5]. Moreover, in the case of oral or intranasal immunization, LTB can be used as an adjuvant providing for formation of the efficient mucosal immune response to the simultaneously introduced antigen [6]. All these properties make LTB an interesting object for creation of plant systems for its production with the promise of orally administered "edible" vaccine.

Production in plants of various proteins for medicine, agriculture, and industry is a promising trend in biotechnology. The advantage of plant "biofactories" over animal or microbial cells is the easy growing, determining the low final cost of the product, and the absence of pathogens in common for humans and plants. Transgenic

Abbreviations: GFP) green fluorescent protein; LT) heat-labile toxin of *Escherichia coli*; LTB) B-subunit of *Escherichia coli* heat-labile toxin; PME) pectin methyl esterase; PTGS) post-transcriptional gene silencing; PVX) potato virus X; RDRP) RNA-dependent RNA polymerase; 35S) 35S RNA of cauliflower mosaic virus; *sLTB*) synthetic gene of LTB; Sgp) subgenomic RNA promoter.

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potato plants have been designed [7, 8], and it was shown that the obtained LTB was able to induce both systemic and mucosal immune response. However, the LTB expression levels in transgenic plants did not exceed 0.01 and 0.19% of total soluble protein in transgenic tobacco [9] and potato [7] plants, respectively, which is an obstacle in the economically competitive production of vaccine preparations [10]. Higher levels of LTB expression were achieved in seeds of transgenic maize plants [11-13], but expression levels varied greatly both between different transgenic lines and between plants of different generations of the same line. Thus, in transgenic maize plants expressing LTB under gamma-zein promoter control the LTB production levels in seeds in most cases was only one hundredth part of a percent, while in some cobs they reached 1-3% of total soluble protein [13].

An alternative to transformation of the plant nuclear genome is genetic transformation of chloroplasts, which makes it possible to achieve significantly higher expression levels due to the gene dose effect [14]. Thus, LTB expression levels in tobacco transplastome plants were 2-2.5% [15]. However, it should be noted that genetic transformation of chloroplasts is complicated and time consuming, and so far it has been achieved for only a few plant species.

An alternative to transgenic plants is the use of viral expression systems allowing rapid expression in plants of significant amounts of target proteins, in some cases up to 30-40% of total soluble protein [16]. Vectors based on plant virus genomes are successfully used for production in plants of proteins for medical purpose, including vaccine proteins for rabies [17] and plague [18] pathogens, antibodies [19], and many others (see review 20]). The goal of this work was to design a system for LTB production in plants using a recombinant vector based on the potato virus X genome.

MATERIALS AND METHODS

Media, reagents, and synthetic oligonucleotides. Bacteria were grown in LB broth or in plates with LB agar at 37°C (*E. coli*) or at 30°C (*Agrobacterium tumefaciens*). If necessary, the following antibiotics were added to the media: ampicillin (100 μg/ml), kanamycin (50 μg/ml), rifampicin (50 μg/ml), or gentamicin (24 μg/ml). To clone the sequence encoding the mature part of LTB in expression vector pQE30, the oligonucleotides LTB-F (GCGGATCCGCTCCCCAGTCTATTACAGAACTA) and LTB-R (AACCTGCAGGCTAGTTTTCCATACTGATTGCC) were used.

To obtain the synthetic gene of LTB the following synthetic oligonucleotides were used: LTBP-1 (CGGGATCCAAAAAAATGGCTAA); LTBP-2 (GGTTAAGTGTTATGTTCTTTTTACTGCTCTTCTTTCTTCTCTTTGTGCTTATGGAGCACC); LTBP-3 (ACAATCTATTACTGAACTTTGTTCTGAATATAGGAATACTCAAATTTATACTATTAATGA); LTBP-4 (TAAGATTCTTTCTTATACTGAATCTATGGCTGGAAAGAGGGAAATGGTTATTATTACTTT); LTBP-5 (TAAGTCTGGAGCTACTTTTCAAGTTGAAGTTCCAGGATCTCAACATATTGATTCTCAAAAGA); LTBP-6 (AAGCTATTGAAAGGATGAAGGATACTCTTAGGATTACTTATCTTACTGAAACTAAGATT); LTBP-7 (GATAAGCTTTGTGTTTGGAATAATAAGACTCCAAATTCTATTGCTGCTATTTCTATGGA); LTBP-8 (AAATTCTGAAAAGGATGAACTTTAAATTTCTAGATTGAGCTCCG); LTBP-9 (CGGAGCTCAATCTAGAAATTTAAAG); LTBP-10 (TTCATCCTTTTCAGAATTTTCCA); LTBP-11 (TAGAAATAGCAGCAATAGAATTTGGAGTCTTATTATTCCAAACACAAAGCTTATCAATC); LTBP-12 (TTAGTTTCAGTAAGATAAGTAATCCTAAGAGTATCCTTCATCCTTTCAATAGCTTTCTT); LTBP-13 (TTGAGAATCAATATGTTGAGATCCTGGAACTTCAACTTGAAAAGTAGCTCCAGACTTAAAAG); LTBP-14 (TAATAATAACCATTTCCCTCTTTCCAGCCATAGATTCAGTATAAGAAAGAATCTTATCAT); LTBP-15 (TAATAGTATAAATTTGAGTATTCCTATATTCAGAACAAAGTTCAGTAATAGATTGTGGTG); LTBP-16 (CTCCATAAGCACAAAGAGAAGAAAGAAGAGCAGTAAAAAGAACATAACACTTAACCTTAG); LTBP-17 (CCATTTTTTTGGATCCCG). Before assembly of the synthetic gene, the oligonucleotide 5′ ends were phosphorylated using T4 polynucleotide kinase.

Producing vectors used in plant agroinfiltration experiments. Plasmid pBIN_HcPro is a binary vector in which the *HcPro* gene is cloned under 35S promoter control. In binary vector pBIN_PME, the full-sized pectin methyl esterase (*PME*) gene of *Nicotiana tabacum* was cloned under control of the 35S promoter in direct orientation, and in vector pBIN_asPME in reverse orientation [21]. Gene *p19* of the tomato bushy stunt virus was cloned in vector pBIN_P19 under 35S promoter control.

Viral vector pA7248LTBP was constructed in two steps. In the first step, the *sLTB* synthetic gene was excised from plasmid pUC-LTBP using restriction endonucleases BamHI and Ecl136II and cloned in auxiliary plasmid pA7231 (IC2 in [22]) instead of *GFP* genes at BamHI and BsrGI sites. In the second step, the AvrII-SacI of the resulting plasmid pA7231LTBP was transferred into vector pVXdt-GFP [22]. The scheme of the resulting vector pA7248LTBP is shown further.

Agroinfiltration of plants. Agrobacteria containing recombinant binary vectors were grown for 12 h on a shaker at 30°C. Cells (1.5 ml) were pelleted by centrifugation (4000*g*, 5 min), the pellet was resuspended in 1.5 ml buffer containing 10 mM MES (pH 5.5) and 10 mM $MgCl_2$, and the optical density was adjusted to $A_{600} = 0.2$. The *N. benthamiana* leaves were injected with agrobacterial suspension using a syringe without needle.

Protein isolation from plant tissue. Leaf material (10 mg) was ground to a homogeneous suspension in

extraction buffer (0.4 M sucrose, 50 mM Tris buffer, pH 8.0, 5 mM $MgCl_2$, 10% glycerol, 5 mM β-mercaptoethanol). The resulting mixture was centrifuged at 14,000*g* for 15 min, and the supernatant containing proteins was taken for protein determination according to Bradford.

Raising of antibodies to LTB. To obtain hyperimmune serum, mice were intraperitoneally injected with 100 μg purified LTB with incomplete Freund's adjuvant. The mice were immunized four times with 14-day intervals; sera were taken on the fifth day after the last injection.

Western-blot analysis of LTB production. Protein electrophoresis was carried out in 10% polyacrylamide gel in the presence of SDS. Proteins were transferred from the gel onto Hybond-P membrane (Amersham, Great Britain) by electroblotting. To exclude unspecific binding of antibody to the membrane, the latter was treated in 5% solution of dry milk in TBS-T buffer. Then the membrane was hybridized with polyclonal mouse antibodies to LTB, and finally with the peroxidase-conjugated second-layer antibodies. Specific protein complexes were detected using a Western Blot ECL Plus kit (Amersham). The revealed band intensities were quantified by analysis of a digital photo using the TINA-PCBas program.

RESULTS AND DISCUSSION

Design and synthesis of an artificial *LTB* gene optimized for expression in plants. Since the nucleotide sequence of the natural *LTB* gene from enteropathogenic *E. coli* is not optimal for expression in plants owing to codon composition and the existence of potential internal sites of polyadenylation and splicing, we designed a synthetic *LTB* gene. The nucleotide sequence of the synthetic *sLTB* gene was constructed on the basis of amino acid sequence of LTB protein using synonymic codons most frequent in the highly-expressed potato and tobacco genes. For this purpose, computer analysis of context characteristics of mRNA of these species was carried out using bioinformatic methods [23-25], which revealed the optimal synonymic codons and structure of translation initiation and termination signals. Along with codon composition, the existence in the *sLTB* sequence of potential sites of mRNA processing, which can be recognized in plant cells, was taken into consideration. Thus, the use of synonymic codons made it possible to remove from the sequence a potential polyadenylation site AATAAA (Fig. 1). To stabilize LTB within the plant cell, nucleotides encoding the signal of localization in the

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sLTB     AAA AAA ATG GCT AAG GTT AAG TGT TAT GTT CTT TTT ACT GCT CTT CTT TCT TCT
nLTB             ATG AAT AAA GTA AAA TGT TAT GTT TTA TTT ACG GCG TTA CTA TCC TCT
Amino acids        M   A   K   V   K   C   Y   V   L   F   T   A   L   L   S   S

CTT TGT GCT TAT GGA GCA CCA CAA TCT ATT ACT GAA CTT TGT TCT GAA TAT AGG AAT ACT
CTA TGT GCA TAC GGA GCT CCC CAG TCT ATT ACA GAA CTA TGT TCG GAA TAT CGC AAC ACA
  L   C   A   Y   G   A   P   Q   S   I   T   E   L   C   S   E   Y   R   N   T

CAA ATT TAT ACT ATT AAT GAT AAG ATT CTT TCT TAT ACT GAA TCT ATG GCT GGA AAG AGG
CAA ATA TAT ACG ATA AAT GAC AAG ATA CTA TCA TAT ACG GAA TCG ATG GCA GGT AAA AGA
  Q   I   Y   T   I   N   D   K   I   L   S   Y   T   E   S   M   A   G   K   R

GAA ATG GTT ATT ATT ACT TTT AAG TCT GGA GCT ACT TTT CAA GTT GAA GTT CCA GGA TCT
GAA ATG GTT ATC ATT ACA TTT AAG AGC GGC GCA ACA TTT CAG GTC GAA GTC CCG GGC AGT
  E   M   V   I   I   T   F   K   S   G   A   T   F   Q   V   E   V   P   G   S

CAA CAT ATT GAT TCT CAA AAG AAA GCT ATT GAA AGG ATG AAG GAT ACT CTT AGG ATT ACT
CAA CAT ATA GAC TCC CAA AAA AAA GCC ATT GAA AGG ATG AAG GAC ACA TTA AGA ATC ACA
  Q   H   I   D   S   Q   K   K   A   I   E   R   M   K   D   T   L   R   I   T

TAT CTT ACT GAA ACT AAG ATT GAT AAG CTT TGT GTT TGG AAT AAT AAG ACT CCA AAT TCT
TAT CTG ACC GAG ACC AAA ATT GAT AAA TTA TGT GTA TGG AAT AAT AAA ACC CCC AAT TCA
  Y   L   T   E   T   K   I   D   K   L   C   V   W   N   N   K   T   P   N   S

ATT GCT GCT ATT TCT ATG GAA AAT TCT GAA AAG GAT GAA CTT TAA
ATT GCG GCA ATC AGT ATG GAA AAC TAG
  I   A   A   I   S   M   E   N   S   E   K   D   E   L   -
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Fig. 1. Sequences of the plant-optimized synthetic *sLTB* gene and original bacterial *nLTB*. Different nucleotides in sequences are shown in gray; a potential site of mRNA polyadenylation in the *nLTB* sequence is underlined. Amino acid sequences of the N-terminal signal peptide (amino acids 1-21) and those of the signal of localization in the endoplasmic reticulum (SEKDEL), introduced into *sLTB*, are italicized.

endoplasmic reticulum, SEKDEL, were introduced into the 3′ terminus of the *sLTB* sequence (Fig. 1).

The sequence of N-terminal signal peptide, providing for export of the mature part of the protein into the periplasm space in bacteria [26] and guiding it into the endoplasmic reticulum in yeasts [27], was preserved in *sLTB*.

To obtain the synthetic *LTB* gene, equimolar amounts of synthetic oligonucleotides were mixed (see "Materials and Methods"), the mixture was denatured by heating to 94°C, then the oligonucleotides were hybridized by slow cooling of the mixture to 25°C. To obtain the full-sized gene, the annealed oligonucleotides were ligated by T4 DNA ligase and used as template for amplification by PCR with terminal primers LTBP1 and LTBP9. The PCR product was cloned in pUC19 vector at BamHI and SacI sites, whose recognition sites were introduced into sequences of the terminal primers. The correctness of the synthesis was confirmed by sequencing *sLTB* in the recombinant plasmid pUC-LTBP.

Preparation of LTB-producing *E. coli* strain. Preparations of LTB proteins and corresponding antibodies are necessary for the following analysis of producing plants by Western blotting. To obtain the producing strain, the *LTB* gene sequence corresponding to the mature part of the protein (minus the 21-amino acid N-terminal signal peptide) was obtained from genomic DNA of enteropathogenic *E. coli* by PCR with LTB-F and LTB-R primers. The sequences were cloned in expression vector pQE30 with formation of hybrid genes encoding LTB fused with six histidine residues at the N-terminus. The recombinant protein was isolated and purified by standard methods using metal-affinity chromatography. The isolated LTB protein was used for immunization of mice and following production of antibody preparations. The anti-LTB antibodies could detect 0.2 ng LTB protein by Western-blotting.

Construction of the recombinant viral vector based on potato virus X genome providing for sLTB expression in *Nicotiana benthamiana*. Earlier we constructed a number of recombinant PVX-based viral vectors providing for sLTB expression, but the product yield was far below the expected level (about 0.01% of total soluble protein). These vectors contained the *sLTB* gene under control of subgenomic RNA promoter inserted in the PVX genome between the triple gene block and the coat protein gene. However, it is known that: (i) the closer to the 5′ end of the PVX genome the target gene is located the higher is its expression level, and (ii) the incorporation of a second gene into subgenomic RNA decreases the efficiency of expression of the first gene. Keeping this in mind, we have designed a vector in which the *sLTB* gene under control of subgenomic RNA promoter was inserted immediately after the PVX replicase gene. In this case, the triple gene block and the coat protein gene were removed from this recombinant vector.

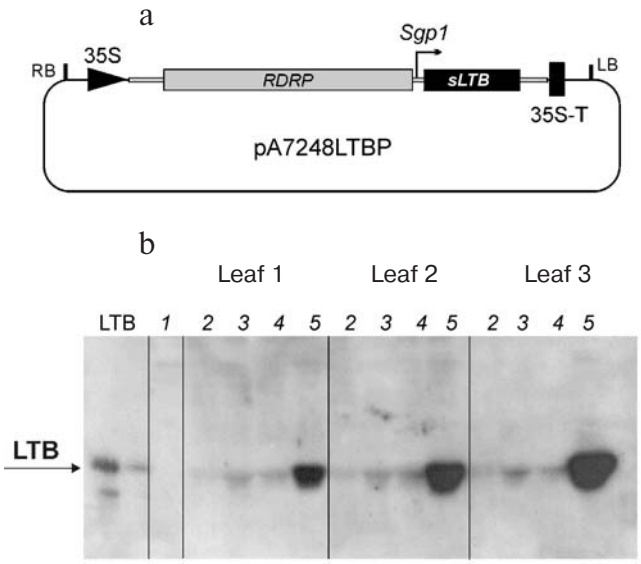

Fig. 2. LTB production in *N. benthamiana* cells. a) Structure of the pA7248LTBP vector. Sequences from the cDNA copy of the PVX genome are shown in gray, and the subgenomic RNA promoter 1 is shown by the bent arrow. *sLTB* is synthetic gene encoding LTB with the attached peptide SEKDEL at the C end; 35S is promoter; 35S-T is terminator of 35S RNA of cauliflower mosaic virus; RB and LB are positions of boundaries of the binary vector T-DNA region. b) Western-blot analysis of LTB production in agroinfiltrated plants. LTB is mature protein expressed in *E. coli*, its calculated molecular mass being 13.1 kD (25 and 10 ng). Ten micrograms of protein from producing plants infiltrated by agrobacteria were applied on each lane. The agrobacteria contained the following vectors: *1*) PVXdt-GFP (negative control); *2*) pA7248LTBP; *3*) pA7248LTBP + pBIN_HcPro; *4*) pA7248LTBP + pBIN_asPME; *5*) pA7248LTBP + pBIN_PME + pBIN_P19.

Viral vector PVXdt-GFP, based on the genome of PVX strain UK3 [22], was used as the basis for vector design. This vector includes 5′-nontranslated region of the PVX genome, polymerase gene, the first promoter of subgenomic RNA, the target gene, the last 60 nucleotides of the coat protein gene, and the 3′-nontranslated region of the PVX genome. The whole construct was placed between 35S promoter and 35S terminator and cloned in the binary vector pBIN19 within the T-DNA region. The delivery into plant cells using agroinfiltration of leaves is accompanied by infection of most leaf cells in the infected region, T-DNA transfer into the nucleus, and transcription of the viral RNA copy from 35S promoter. The following stage includes viral vector replication and product synthesis at a high level [22]. The scheme of recombinant vector pA7248LTBP is shown in Fig. 2.

Production of LTB in *N. benthamiana*. For experiments on sLTB expression in *N. benthamiana* plants, vector pA7248LTBP was introduced into the strain *A. tumefaciens* GV3101, and the recombinant agrobacteria were used for infiltration of *N. benthamiana* leaves. The non-recombinant vector pVXdt-GFP was used as negative control.

One of the main factors restricting viral infection and, in particular, expression of target proteins in viral expression systems is the development of posttranscriptional gene silencing (PTGS) activated by double-stranded replicative forms of viral RNA [28]. In this connection, the influence of PTGS suppressors on the efficiency of LTB expression was studied. Two known PTGS suppressor proteins were tested: HcPro of potato virus Y and P19 of tomato bushy stunt virus. In addition, we used the tobacco pectin methyl esterase (*PME*) gene, a factor of the cell antivirus protection. It was shown previously [29] that inhibition of expression of this gene in tobacco plants sharply stimulates reproduction of viral vector. To estimate the effect of PTGS suppressors on the level of sLTB production, the *N. benthamiana* leaves were simultaneously infiltrated by agrobacterial strain GV3101/pA7248LTBP, as well as by the GV3101-containing plasmids pBIN_HcPro, or pBIN_asPME, or GV3101/pBIN_PME together with GV3101/pBIN_P19.

The plant tissue preparations from agroinfiltration zones were taken for LTB immunodetection seven days after beginning of infection. In plants agroinfiltrated with GV3101/PVXdt-GFP, accumulation of GFP was estimated visually by fluorescence intensity under ultraviolet light, and the maximum of expression was observed on the seventh day. The yield of target product sLTB was determined by Western blot analysis (Fig. 3). The LTB protein preparation obtained in *E. coli* (mature protein without signal sequence) was used as control. It was found that the sLTB expression level was about 5 ng per 10 μg total soluble protein (0.05%) in the case of infiltration by GV3101/pA7248LTBP strain without PTGS suppressors. The use of known PTGS suppressors HcPro and asPME several times increased the sLTB yield. However, the combination of P19 with PME provided 20-50-fold increase in sLTB synthesis compared to the level obtained in the absence of PTGS suppressors. In this case, the yield of sLTB was 1.5-2.0% of total soluble protein. This result exceeded by more than one order of magnitude the maximal LTB yield obtained in transgenic plants [30, 31].

Fig. 3. Formation of pentameric forms by LTB synthesized in *N. benthamiana* cells. *1*) The sLTB preparation was isolated from producing plants. Buffer for isolation contained 2% SDS, and before application the protein preparation was heated for 5 min at 95°C; *2*) same as on lane *1*, but buffer for isolation contained 0.2% SDS, and before application the protein preparation was heated for 10 min at 37°C.

In the only publication on LTB production in plants using viral vectors, a vector based on the tobacco mosaic virus genome was applied, and the product yield was about 0.75% of total soluble protein [32]. The bacterial *LTB* gene was cloned under control of the additional subgenomic RNA promoter between genes of transport and coat proteins. However, the vector used by those authors was intruded into the plant cells by mechanical inoculation by the mRNA transcript, obtained *in vitro*, with following systemic infection of the plant by recombinant virus. On one hand, this is labor-consuming and makes difficult process scaling, while on the other hand this suggests generation of infectious virus, which restricts the use of this expression system in accordance with biosafety requirements.

Posttranslational processing and pentamer formation by sLTP expressed in plants. The amino acid sequence of sLTB contains the N-terminal signal peptide providing for the localization of LTB in the periplasm space of *E. coli*. Later this peptide is split off and is absent from the mature protein. Upon expression in yeasts, the signal peptide guides the protein to the endoplasmic reticulum and is also split off as soon as the mature LTB form appears [27]. In previous works on LTB expression in transgenic plants, the full-sized *LTB* gene was used along with its fragment corresponding to the mature part, and the correct processing of the leader peptide was observed in tobacco leaves [9]. Western-blot analysis carried out in our laboratory on sLTB isolated from plant producers has shown that the leader peptide is efficiently split off with formation of mature protein: the molecular mass of LTB obtained in plants corresponds to the mature part with account for the SEKDEL signal, namely to 12.4 kD, whereas the size of LTB with the leader peptide would be 14.7 kD.

An important characteristic of LTB expressed in plants is its ability to form pentamers, which are an immunogenic form of the protein [33]. It is known that LTB efficiently forms pentamers in *E. coli* cells, but pen-

tamers might dissociate with formation of monomers under heating in the presence of SDS. Note that for the above-described Western-blot analysis of expression level, protein preparations were obtained by extraction from the plant tissue using the buffer for application on SDS-PAGE, which contains SDS and mercaptoethanol, and before application the preparations were heated for 5 min at 95°C. Therefore, as expected, the bulk of LTB was in the form of monomers.

To check the supposition that LTB expressed in plant cells *in vivo* forms pentamers, we modified the procedure for obtaining and analyzing the protein preparations. For this purpose, the protein was extracted in buffer containing SDS and mercaptoethanol at different concentrations, and before application the preparations were heated at different temperatures. The result of one experiment is shown in Fig. 3. In this case the protein was extracted using buffer containing 0.2% SDS, and before electrophoresis the preparation was heated for 10 min at 37°C. Results of Western-blot analysis show that a significant portion of the LTB is present in the form of pentamers. Thus, LTB synthesized using phytoviral vectors in *N. benthamiana* is able to form pentamers, the immunogenic form of the protein.

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REFERENCES

1. Clements, J. D., and Finkelstein, R. A. (1979) *Infect. Immun.*, **24**, 760-769.
2. Clements, J. D., Yancey, R. J., and Finkelstein, R. A. (1980) *Infect. Immun.*, **29**, 91-97.
3. Clements, J. D., and Finkelstein, R. A. (1978) *Infect. Immun.*, **21**, 1036-1039.
4. Spangler, B. D. (1992) *Microbiol. Rev.*, **56**, 622-647.
5. Dickinson, B. L., and Clements, J. D. (1995) *Infect. Immun.*, **63**, 1617-1623.
6. Dickinson, B. L., and Clements, J. D. (1996) in *Mucosal Vaccines* (Kiyono, H., Ogra, P. L., and McGhee, eds.) Academic Press, N. Y., pp. 73-87.
7. Mason, H. S., Haq, T. A., Clements, J. D., and Arntzen, C. J. (1998) *Vaccine*, **16**, 1336-1343.
8. Tacket, C. O., Mason, H. S., Lasonsky, G., Clements, J. D., Levine, M. M., and Arntzen, C. J. (1998) *Nature Med.*, **4**, 607-609.
9. Haq, T. A., Mason, H. S., Clements, J. D., and Arntzen, C. J. (1995) *Science*, **268**, 714-716.
10. Stoger, E., Sack, M., Fischer, R., and Christou, P. (2002) *Curr. Opin. Biotechnol.*, **13**, 161-166.
11. Streatfield, S. J., Major, J. M., Barker, D. K., Brooks, C., Woodard, S. L., Horn, M., Nikolov, Z. L., Hood, E. E., Jilka, J. M., and Howard, J. A. (2002) *In vitro Cell. Dev. Biol. Plant*, **38**, 11-17.
12. Streatfield, S. J., Lane, J. R., Brooks, C. A., Barker, D. K., Poage, M. L., Mayor, J. M., Lamphear, B. J., Drees, C. F., Jilka, J. M., Hood, E. E., and Howard, J. A. (2003) *Vaccine*, **21**, 812-815.
13. Chikwamba, R., McMurray, J., Shou, H., Frame, B., Peggs, S. E., Scott, P., Mason, H., and Wang, K. (2002) *Molec. Breeding*, **10**, 253-265.
14. Bock, R. (2001) *J. Mol. Biol.*, **312**, 425-438.
15. Kang, T. J., Loc, N. H., Jang, M. O., Jang, Y. S., Kim, Y. S., Seo, J. E., and Yang, M. S. (2003) *Transgenic Res.*, **12**, 683-691.
16. Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V., and Gleba, Y. (2005) *Nat. Biotechnol.*, **23**, 718-723.
17. Yusibov, V., Hooper, D., Spitsin, S., Fleysh, N., Kean, R., Mikheeva, T., Deka, D., Karasev, A., Cox, S., Randall, J., and Koprowski, H. (2002) *Vaccine*, **20**, 3155-3164.
18. Santi, L., Giritch, A., Roy, C. J., Marillonnet, S., Klimyuk, V., Gleba, Y., Webb, R., Arntzen, C. J., and Mason, H. S. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 861-866.
19. Giritch, A., Marillonnet, S., Engler, C., van Eldik, G., Botterman, J., Klimyuk, V., and Gleba, Y. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 14701-14706.
20. Canizares, M. C., Nicholson, L., and Lomonossoff, G. P. (2005) *Immunol. Cell. Biol.*, **83**, 263-270.
21. Dorokhov, Y. L., Skurat, E. V., Frolova, O. Y., Gasanova, T. V., Ivanov, P. A., Ravin, N. V., Skryabin, K. G., Makinen, K. M., Klimyuk, V. I., Gleba, Y. Y., and Atabekov, J. G. (2006) *FEBS Lett.*, **580**, 3329-3334.
22. Komarova, T. V., Skulachev, M. V., Zvereva, A. S., Shwarts, A. M., Dorokhov, Yu. L., and Atabekov, J. G. (2006) *Biochemistry* (Moscow), **71**, 846-850.
23. Kochetov, A. V., Ischenko, I. V., Vorobiev, D. G., Kel, A. E., Babenko, V. N., Kisselev, L. L., and Kolchanov, N. A. (1998) *FEBS Lett.*, **440**, 351-355.
24. Kochetov, A. V., Ponomarenko, M. P., Frolov, A. S., Kisselev, L. L., and Kolchanov, N. A. (1999) *Bioinformatics*, **15**, 704-712.
25. Likhoshvai, V. A., and Matushkin, Y. G. (2002) *FEBS Lett.*, **516**, 87-92.
26. Hirst, T. R. (1995) in *Handbook of Natural Toxins*, Vol. 8, *Bacterial Toxins and Virulence Factors in Disease* (Moss, J., Ihlewski, B., Vaughan, M., and Tu, A. T., eds.) Marcel Dekker, N. Y., pp. 123-184.
27. Schonberger, O., Hirst, T. R., and Pines, O. (1991) *Mol. Microbiol.*, **5**, 2663-2671.
28. MacDiarmid, R. (2005) *Annu. Rev. Phytopathol.*, **43**, 523-544.
29. Dorokhov, Y. L., Frolova, O. Y., Skurat, E. V., Ivanov, P. A., Gasanova, T. V., Sheveleva, A. A., Ravin, N. V., Makinen, K. M., Klimyuk, V. I., Skryabin, K. G., Gleba, Y. Y., and Atabekov, J. G. (2006) *FEBS Lett.*, **580**, 3872-3878.
30. Lauterslager, T. G., Florack, D. E., van der Wal, T. J., Molthoff, J. W., Langeveld, J. P., Bosch, D., Boersma, W. J., and Hilgers, L. A. (2001) *Vaccine*, **19**, 2749-2755.
31. Chikwamba, R. K., Cunnick, J., Hathaway, D., McMurray, J., Mason, H., and Wang, K. (2002) *Transgenic Res.*, **11**, 479-493.
32. Wagner, B., Hufnagl, K., Radauer, C., Wagner, S., Baier, K., Scheiner, O., Wiedermann, U., and Breiteneder, H. (2004) *J. Immunol. Meth.*, **287**, 203-215.
33. Nashar, T. O., Webb, H. M., Eaglestone, S., Williams, N. A., and Hirst, T. R. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 226-230.