Tuberculosis (2007) **87**, 218–224

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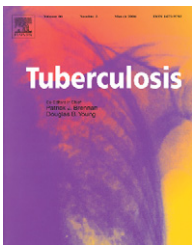

Superexpression of tuberculosis antigens in plant leaves

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Received 3 June 2006; received in revised form 17 August 2006; accepted 5 October 2006

KEYWORDS
ESAT6:Ag85B;
Plant virus;
Gene silencing

Summary
Recent developments in genetic engineering allow the employment of plants as factories for 1/foreign protein production. Thus, tuberculosis (TB) ESAT6 antigen was expressed in different plant systems, but the level of vaccine protein accumulation was extremely low. We describe the technology for superexpression of TB vaccine proteins (Ag85B, ESAT6, and ESAT6:Ag85B fusion) in plant leaves which involves: (i) construction of tobacco mosaic virus-based vectors with the coat protein genes substituted by those for TB antigens; (ii) *Agrobacterium*-mediated delivery to plant leaf tissues of binary vectors containing the cDNA copy of the vector virus genome; and (iii) replication of virus vectors in plant cells under conditions suppressing the virus-induced gene silencing. This technology enables efficient production of the TB vaccine proteins in plants; in particular, the level of Ag85B antigen accumulation was not less than 800 mg/kg of fresh leaves. Expression of TB antigens in plant cells as His_6-tagged proteins promoted their isolation and purification by Ni-NTA affinity chromatography. Deletion of transmembrane domains from Ag85B caused a dramatic increase in its intracellular stability. We propose that the strategy of TB antigens superproduction in a plant might be used as a basis for the creation of prophylactic and therapeutic vaccine against TB.

Introduction

Tuberculosis (TB), caused primarily by the facultative intracellular bacterium *Mycobacterium tuberculosis*, is the leading cause of death from a single infectious agent despite the availability of effective short-course chemotherapy. Currently, the only available TB vaccines is the Bacille Calmette-Guérin (BCG), attenuated vaccine derived from *Mycobacterium bovis*. Although BCG has been widely used for decades, its efficacy has been shown to be highly variable.[1] While BCG is routinely given at birth to infants and generally protective against miliary and meningial TB in

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doi:10.1016/j.tube.2006.10.001

children, it is unable to protect against lung infections including adult pulmonary TB. To improve TB vaccines, several projects were undertaken in the 1980s in an attempt to find new immunogens that could constitute subunit or recombinant vaccines to replace the BCG.[2] It is noteworthy that genome DNA microarray techniques have identified 129 *M. tuberculosis* specific open reading frames that are absent in the genome of the BCG vaccine strains.[3] Apparently, the proteins present in *M. tuberculosis* and absent in BCG represent the putative vaccine antigens. Consequently, short-term culture filtrate proteins were purified and their antigenicity assessed in proliferation and interferon (IFN-γ) production assays with lymphocytes isolated from mice infected with *M. tuberculosis*.[4] The "antigen 85 complex" (Ag85 A, B and C) as a family of proteins secreted in large amounts into the growth medium of *M. tuberculosis* cultures have been used in a vaccine.[5] The ESAT6 antigen is another prominent target found in culture filtrates with a partner protein, CFP10.[6] The vaccines based on ESAT6 and Ag85B antigens have now entered clinical trials.[7,8]

In the last few years, substantial evidence accumulated that plant-derived vaccines are feasible.[9] The main advantages provided by plants as factories for foreign proteins production are (i) low cost of cultivation: plants need only soil, water and sun, and no expensive media or sterility are needed; (ii) plants expressing vaccine proteins (vaccinogens) can be used to produce edible vaccines that could be administered through mucosal surfaces, i.e. orally or intranasally; (iii) the technology is entirely safe, since only selected target proteins are produced as vaccinogens. It is particularly important that transgenic plants usually do not provide advantages for vaccine protein production, since the level of foreign proteins production by transgenic plants obtained by stable nuclear transformation is commonly very low (about 0.1% of the total soluble protein). Therefore, instead of stable transgenic plants, the system based on transient replication of plant virus vectors producing the TB antigens was used in the present work. To this end the coat protein (CP) gene was substituted by that of Ag85B, ESAT6 or Ag85B:ESAT6 fusion in the genomes of tobacco mosaic virus (TMV) infecting cruciferous plants (crTMV) or TMV U1. It should be noted that the productivity of the TMV CP genes is very high. Thus, the CP represents about 70% of the total soluble proteins of infected leaf. *Agrobacterium tumefaciens* with binary vectors containing a cDNA copy of the RNA virus vector were delivered to *Nicotiana benthamiana* leaf cells by injection (agroinjection). Transcription of viral cDNA resulted in a virus RNA genome synthesis, replication and foreign protein production instead of the CP. It is widely known that antiviral plant defense reaction, the virus-induced gene silencing (VIGS), is developed in virus-infected plants to serve as a sequence-specific viral genome inactivation system. However, most of plant viruses induce a counter-defense reaction by expressing so called protein suppressors of VIGS. In order to overcome VIGS in our expression system, an efficient protein-suppressor of VIGS (P19) encoded by tomato bushy stunt virus[10] was co-expressed with the virus vector. This technology provided a way for an efficient production of TB vaccine proteins in plant cells. We also report that removal of a putative Ag85B transmembrane (TM) domain from TB vaccine proteins resulted in a great enhancement of their intracellular stability. Expression of His-tagged TB antigens promoted their isolation and purification by Ni-NTA affinity chromatography. On the whole, this technology provided an efficient system for TB vaccine proteins production.

Materials and methods

Cloning of TB antigen-expressing genes into pGEM3 vector

The gene ESAT6 and Ag85B were amplified from *M. tuberculosis* genome H37Rv (type strain; ATCC 27294) DNA by PCR. The two pairs of primers were ESAT6-BamH1p(1): CGGGATCCATGACAGAGCAGCAGTGG, ESAT6-EcoRIm(1): GAGAATTCCTATGCGAACATCCCAGTG; Ag85-EcoR1p(1): ACGAATTCATGATCGGCACGGCAGCGGCT, Ag85-SalIm(1): ACGCGTCGACCTAGCCGGCGCCTAACGAACT for amplification ESAT6 and Ag85B genes, respectively. PCR amplifications were done with 35 cycles at 94 °C for 1 min, 66 °C for 1 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 2 min, using a DNA Thermal Cycler. The reaction was performed in 100 μl of buffer containing 50 mM $MgSO_4$, 250 mM KCl, 50 mM $(NH_4)_2SO_4$, 200 μM dNTPs, 100 mM Tris–HCl (pH 8.85), and 2.5 U Taq-polymerase. The PCR products were inserted into the pGEM3 vector and transferred into *Escherichia coli* strain XL-1. To create translationally fused chimeric gene ESAT6:Ag85B, two DNA fragments after PCR with pairs of primers [ESAT6-BamH1p(1) and ESAT6-EcoRIm(2): GAGAATTCTGCGAACATCCCAGTGACG] and [Ag85-EcoRIp(2): ACGAATTCGCTGCTACAGACGTGAGCCGAAAGATTC and Ag85-SalIm(1): ACGCGTCGACCTAGCCGGCGCCTAACGAACT] were digested with EcoRI and cloned into pGEM3 vector. TM domain of Ag85B was removed by PCR using primer GGGGACGCCGAGCGGGCGCGTT.

Creation of *E. coli* producer of ESAT6:Ag85B-$(His)_6$

ESAT6:Ag85B chimera fused gene after PCR with primers [ESAT6-BamHIp(2): CGGGATCCCTGACAGAGCAGCAGTGG and Ag85-SalIm (2): ACGCGTCGACCTAGCCGGCGCCTAACGAACTCTGG] was cloned into pQE30 vector and transferred into *E. coli* XL1. *E. coli* SG transformed with pQE30::ESAT6:Ag85B was plated on LB solid medium containing ampicillin (100 μg/ml), and grown overnight at 37 °C. An overnight culture of the resulting strain was used to inoculate LB with ampicillin medium and grown at 37 °C. When the A_{600} reached 0.7, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, the cells were incubated for another 4 h. After fermentation, the cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C. Ni–NTA column was used to purify ESAT6:Ag85B-$(His)_6$. The supernatant of solubilized inclusion body from 6 M guanidine–HCl or 8 M urea was applied, respectively, to Ni^{2+}-charged HiTrap columns pre-equilibrated with 6 M guanidine–HCl in 20 mM sodium phosphate buffer or 8 M urea in 20 mM sodium phosphate buffer, pH 7.4. After sample loading, the column was washed with 6 M guanidine–HCl or 8 M urea, pH 7.4. ESAT6:Ag85B-$(His)_6$ was eluted using a linear gradient with imidazole (10–500 mM) in 6 M guanidine–HCl or 8 M urea, pH 7.4, separately. Our analysis of amino acid sequence

revealed the TM domain in Ag85B protein (L_{15}–T_{37} in Fig. 1a). It was not unlikely that the TM inhibited the protein production. In line with this assumption, the TM deletion increased drammatically the level of accumulation in plants of ESAT6:Ag85B-$(His)_6$-(TM−) protein produced by pQE30::ESAT6:Ag85B (TM−) vector. This Ni–NTA column purified 43 kDa protein was used for immunization of mice and generation of antibodies.

Plasmids used for agroinjection

For construction of the 35S-based TB antigen-expressing binary vectors and their TM(−) and His-tagged variants NcoI-SalI fragments encoding ESAT6 and Ag85B or ESAT6:Ag85 fusion construct were cloned into pCAMBIA1300 after linearization with NcoI-SalI. Binary Actin2-based crTMV:GFP vectors[11] were used as the basic constructs for obtaining TB antigen-expressing binary vectors. Two fragments: (i) NcoI-SalI TB antigen-expressing and (ii) XhoI-ApaI fragment corresponding 3′NTR of crTMV genome, were inserted into pCambia-crTMV:GFP vector linearized by EcoRI-ApaI. For construction of TMV UI-based ESAT6 and Ag85B-expressing binary vectors, we used the *Arabidopsis thaliana* Actin2 promoter-based TMV U1-GFP vector where GFP was fused with N-terminal part of the CP gene.[12] The whole cassette was inserted into the binary vector pBin19 between KpnI and SalI sites. Next, three DNA fragments: (i) HindIII-BamHI cDNA containing C-terminal part of TMV UI MP and N-terminal part of CP; (ii) BamHI-XbaI cDNA containing ESAT6 or Ag85B, and (iii) XbaI-NotI cDNA containing TMV UI 3′NTR, were ligated with plasmid linearized with HindIII-NotI to get the respective vectors. To construct crTMV-CP:Ag85B vector, the EcoRI/HindIII fragment containing the C-terminal part of crTMV MP gene, N-terminal (26 aa) part of the CP gene, Ag85B gene and the3'NTR was inserted into crTMV-CP-GFP.

Agroinjection procedure

Agrobacterium tumefaciens strain GV3101 was transformed with individual constructs, grown in LB-medium supplemented with rifampicin 50 mg/l, carbencilin 50 mg/l and 100 μM acetosyringone at 28 °C. *Agrobacterium* cells from an overnight culture (5 ml) were collected by centrifugation (10 min, 4500*g*) and resuspended in 10 mM MES (pH 5.5) buffer supplemented with 10 mM $MgSO_4$ and100 μM acetosyringone. The bacterial suspension was adjusted to a final OD_{600} of 0.8. Prior to infiltration, equal volumes of *Agrobacteria* containing vectors expressing TB vaccine proteins were mixed with those expressing the P19[10] gene of tombusvirus (a well known suppressor of VIGS). Agroinjection was conducted on near fully expanded leaves that were still attached to the intact plant. A bacterial suspension was injected using a 5 ml syringe. After injection, plants were further grown under greenhouse conditions at 22 °C and 16 h light.

Western blot analysis

Three days after agroinjection the proteins from sites of agroinjection were isolated and tested with ESAT6-Ag85B-specific antibodies. Fully expanded leaves (1 g) were

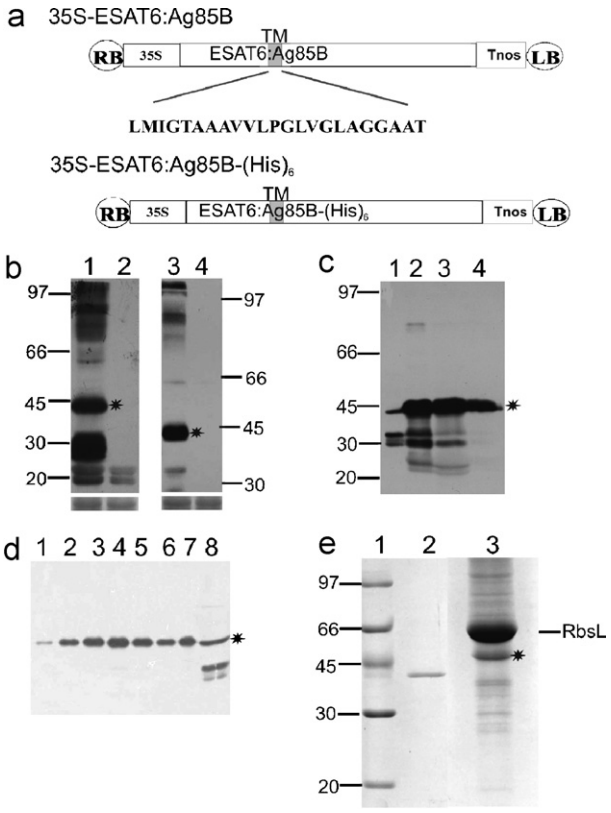

Figure 1 Expression of the 35S promoter-based TB vaccine protein genes by non-viral vectors in agroinjected *N. benthamiana* leaves. (a) Schematic representation (not to scale) of the 35S based T-DNA clone of ESAT6:Ag85B and ESAT6:Ag85B-$(His)_6$ vectors. Grey box indicates TM domain with the amino acid sequence shown. All constructs were based on the T-DNA of the pBin19 binary vector. LB and RB indicate the left and right borders of T-DNA, respectively. Tnos, terminator of nopaline synthase (NOS). (b) Western blot analysis of protein samples from leaves agroinjected with 35S-ESAT6:Ag85B (lane 1) and 35S-ESAT6:Ag85B-$(His)_6$ (lane 3) vectors. Antiserum to bacterially expressed ESAT6:Ag85B was used. The samples from non-injected leaves were used as the negative control (lanes 2 and 4). The equal loading of leaf proteins was confirmed by appearance of the large subunit of RUBISCO (RbsL). The positions of molecular mass of protein standards are shown. Protein bands corresponding to ESAT6:Ag85B are marked by asterisk. (c) Western analysis of proteins in subcellular fractions from leaf agroinjected with the 35S-ESAT6:Ag85B-$(His)_6$. The subcellular fractions represent S30 (lane 1), P30 (lane 2), P1 (lane 3), CW (lane 4). (d) and (e) Purification of recombinant ESAT6:Ag85B protein. His-tagged ESAT6:Ag85B was purified by affinity chromatography using Ni–NTA agarose. The clarified cell extract and various chromatography fractions were analyzed by 15% SDS–PAGE. Proteins were probed with antiserum to ESAT6:Ag85B after western blotting (d) and visualized by Coomassie blue staining (e). (d) Lanes 1–7, column fractions eluted with 8 M urea pH 5.9 (lanes 1,2) and pH 4.5 (lanes 3-7); lane 8, cell extract before column chromatography. (e) lane 1, molecular weight markers; lane 2, 43 kDa *E.coli*-expressed ESAT6:Ag85B-$(His)_6$-(TM−) protein (0.5 μg); lane 3 column fractions eluted with 8 M urea pH 4.5. 45 kDa ESAT6:Ag85B protein is marked by asterisk.

homogenized in 15 ml of buffer A (250 mM Tris–HCl, pH 8, 10 mM KCl, 1 mM EDTA, 0.1 mM $MgCl_2$, 0.1% BSA, 10% (wt/vol) sucrose, 1 mM DTT, 1 mM PMSF). The homogenate was filtered through Miracloth (Calbiochem) to obtain the cell wall (CW) and supernatant (S1) fraction. The S1 was further fractioned to give the 30,000*g* pellet (P30) and a supernatant (S30) fractions. CW was washed by buffer A, three times to remove cytoplasmic contaminants. The CW fraction was homogenized in 10 volumes of PBS (7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl; pH 7.5) plus 1 mM PMSF and 0.1% Triton X-100 and centrifuged again. This procedure was repeated three times followed by three washes in PBS buffer plus 1 mM PMSF. The proteins of different fractions were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride membranes (Amersham, Arlington Heights, Ill). The membranes were probed with affinity-purified rabbit antibodies to ESAT6-Ag85B. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) were used as the secondary antibody and the reaction was visualized by chemiluminescence (ECL system, Amersham Pharmacia). To estimate the level of TB antigen accumulation in leaf we compared autograph band density of the protein under examination and those produced by known amounts of bacterially expressed ESAT6-Ag85B.

Purification of TB vaccine proteins from plant leaves

Purification of TB vaccine proteins was carried out by affinity chromatography on a Ni–NTA agarose column with certain modifications. The column was washed with water and equilibrated with buffer A (8 M guanidine, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 8). Leaf material (1 g) was homogenized with buffer A (15 ml). Guanidine-solubilized proteins were centrifuged at 12,000*g* for 20 min to remove the debris and insoluble material and the supernatant was gently stirred with 1.6 ml Ni–NTA agarose resin for 1 h at room temperature. The mixture was loaded onto a column previously equilibrated with buffer A. Briefly, the column was washed with buffer B (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 6.8). Finally, the TB vaccine proteins were eluted with buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 6.3), D (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 5.9) and buffer E (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 4.5). The sample fractions were analyzed by SDS–PAGE.

Results

Expression of the 35S promoter-based TB vaccine protein genes by non-viral vectors

In the first series of experiments, *N. benthamiana* leaves were agroinjected with the 35S promoter-based binary vectors, expressing directly the ESAT6-Ag85B or ESAT6:Ag85B-$(His)_6$ genes (Fig. 1a). Three days later, the total proteins were analyzed by Western blotting. Several bands reacting with antiserum to ESAT6-Ag85B could be revealed in Fig. 1b (lane 1): (i) the separate 45-kDa band corresponded to the expected size of ESAT6-Ag85B (10-kDa+35-kDa) fuse; (ii) the slow bands (around 90-kDa) presumably corresponded to ESAT6-Ag85B dimers and higher aggregates and (iii) less than 30-kDa bands apparently represented the putative products of degradation or premature termination of the 45-kDa fusion. Surprisingly, the presence of the His_6 tag at the C terminus of ESAT6:Ag85B-$(His)_6$ somewhat reduced its production (Fig. 1b, lane 3). Subcellular fractionating of leaf extracts (Fig. 1c) showed that the major part of ESAT6:Ag85B-$(His)_6$ was accumulated in membrane-enriched fractions P30 (lane 2), P1 (lane 3) and the CW (lane 4). To estimate the level of TB antigen accumulation, the density of appropriate band autograph was compared with that produced by known amount of bacterially expressed ESAT6-Ag85B. The levels of the TB ESAT6:Ag85B and ESAT6:Ag85B-$(His)_6$ antigens accumulation were not more than 100 mg per kg of fresh leaf weight (FLW). A single band was revealed in ESAT6:Ag85B protein preparation isolated by Ni-NTA affinity chromatography (Fig. 1d). However, in addition to ESAT6:Ag85B, a major band corresponding to the large subunit of plant RUBISCO (RbsL) complex was revealed by Coomassie staining (Fig. 1e, lane 3). It should be noted that RbsL is normally present at a concentration of 240 mg/ml in the stroma of chloroplasts and constitutes 30–50% of the soluble leaf proteins of C_3 plants.[13]

Figure 1a shows that a characteristic TM domain revealed in the N-proximal region of Ag85B represented the 23-aa segment enriched with hydrophobic amino acids preceded by a more positive flanking sequence. To examine the influence of the TM domain on TB vaccine protein stability in plant cells we removed it from Ag85B and constructed two types of 35S-based vectors expressing His_6-tagged TB antigens: (i) TM-containing ESAT6:Ag85B(TM+), Ag85B(TM+), and (ii) TM-lacking, ESAT6:Ag85B(TM−) and Ag85B(TM−). Figure 2 shows that production of ESAT6:Ag85B (A) and Ag85B (B) was drammatically increased after deletion of the TM domain from Ag85B.

Expression of TB antigens in *N. benthamiana* leaves agroinjected with TMV-based vectors

The first two virus vectors expressing Ag85B and ESAT6 were constructed on the base of TMV U1 genome (the type member of a tobamovitus group). To retain full activity of the CP subgenomic promoter, we fused the TB antigens with the N-terminal 25 codons of the CP (Fig. 3a). Ag85B antigen accumulation in agroinjected leaves could be readily detected (Fig. 3b, lane 2) at the level of 200 mg/kg FLW, whereas the level of ESAT6 expression was rather low (not more that 2 mg/kg of FLW) (Fig. 3b, lane 8). Remarkably, leaf necrosis developed 3–4 days after agroinjection with the ESAT6-expressing vector virus, indicating that ESAT6 may cause a toxic effect in leaves.

The next two virus vectors expressing Ag85B and ESAT6 (Fig. 3a) were constructed on the base of the crTMV, which has been isolated and characterized by our group.[14] One of the distinctive features of crTMV genome is that the movement protein and CP genes of crTMV are overlapped by 75 nucleotides. The crTMV-ESAT6:Ag85B vector virus contained the CP subgenomic promoter lacking the N-terminal CP codons and the crTMV MP-CP overlapping

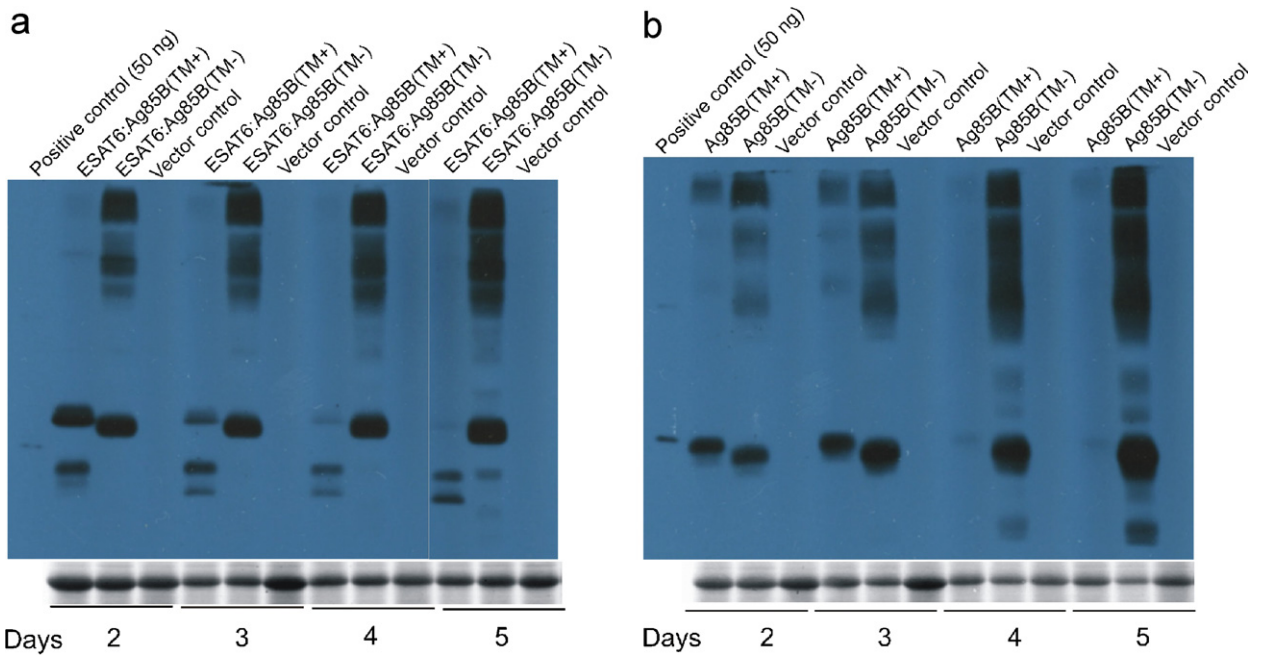

Figure 2 Deletion of Ag85B TM domain increases TB antigen accumulation in agroinjected plant leaves. (a) and (b) Influence of Ag85B TM domain on the levels of accumulation ESAT6:Ag85B-$(His)_6$ (a) and Ag85B-$(His)_6$ (b). 2–5 days after agroinjection with the 35S- ESAT6:Ag85B(TM+) or 35S-ESAT6:Ag85B(TM−) (a) and with the 35S-Ag85B(TM+) or 35S-Ag85B(TM−) (b) constructs the proteins were isolated from *N. benthamiana* leaves and probed with antiserum to ESAT6:Ag85B in Western blot analyses. The proteins from leaves injected with empty vector were used as negative control (vector control). Equal loading of leaf proteins was confirmed by appearance of large subunit of RUBISCO (RbsL). Purified 43 kDa *E-coli* ESAT6:Ag85B-$(His)_6$-(TM−) protein (50 ng) was used as a positive control.

region. By contrast, the crTMV-CP:Ag85B vector retained natural MP-CP genes overlapping arrangement: the N-terminal 25 codons of crTMV CP gene were fused with Ag85B gene. Both vectors directed high level of TB antigens syntheses (Fig. 3b, lanes 3 and 6); the level of the CP:Ag85B production was not less than 800 mg/kg of FLW.

PAGE analyses and Coomassie staining of the proteins in subcellular fractions from leaves agroinjected with crTMV-ESAT6:Ag85B-(TM−)-His or crTMV-Ag85B (TM−)-His was also done. The results showed that: (i) in P30 and S30 fractions the TB antigens were highly contaminated with normal cellular proteins, including RbsL as a major component (Fig. 4a), whereas (ii) TB antigens free of substantial contamination were revealed in CW fraction by Coomassie staining. The TB antigens could be significantly purified by Ni-NTA affinity chromatography, however a considerable amount of RbsL was still revealed by Coomassie staining (Fig. 4b). Remarkably, the cross-reactions between TB antigens and plant RbsL were entirely absent.

Discussion

In recent years, there has been a growing interest in the use of transgenic plants to produce foreign proteins, including human proteins and enzymes, different forms of antibodies and vaccines.[15] However, an important factor restricting use of stably transformed transgenic plants for foreign protein production is the low level of transgene expression. Thus, *Arabidopsis thaliana* plants were made transgenic for a fusion construct composed of *E. coli* heat-labile enterotoxin B subunit and ESAT6 genes.[16] The levels of the fusion protein production were very low and varied in the range from 11 to 24.5 μg/g of FLW. Recently ESAT6 expression was tested in tobacco leaves agroinfected with a PVX-based vector producing virions with a chimeric capsid composed of two types of CP: the fusion ESAT-2A-CP and native PVX CP.[17] The level of fusion CP production was as low as 0.5–1% of total soluble leaf protein. In our experiments, co-expression of even non-viral (non-replicatable) 35S promoter-based ESAT6:Ag85B construct with the P19 gene (VIGS suppressor) resulted in a notable production of TB antigen. Products of the expected molecular mass corresponding to ESAT6 (10 kDa)+Ag85B (35 kDa) fusion monomer and its aggregates were revealed by Western blotting in extracts of agroinjected leaves. The total TB antigen contents estimated by autograph densitometry in leaves agroinjected with non-viral vectors was about 100 μg/g of FLW.

It is important that the levels of TB antigen accumulation are much higher when the plant virus vectors are used. Our approximate estimations showed that expression of Ag85B by crTMV- and TMV U1-based vectors ranged up to about 800 mg/kg of FLW. However, the expression level of a particular protein in agroinjection experiments can hardly be predicted. Thus, production of ESAT6 by vector virus was no more than 2 mg/kg of FLW. As mentioned above, it is likely that ESAT6 is toxic for plant cells.

Purification of proteins by Ni-NTA affinity chromatography is impeded by the presence of Ni-binding His-containing proteins in leaf extracts.[18] Our results showed that the

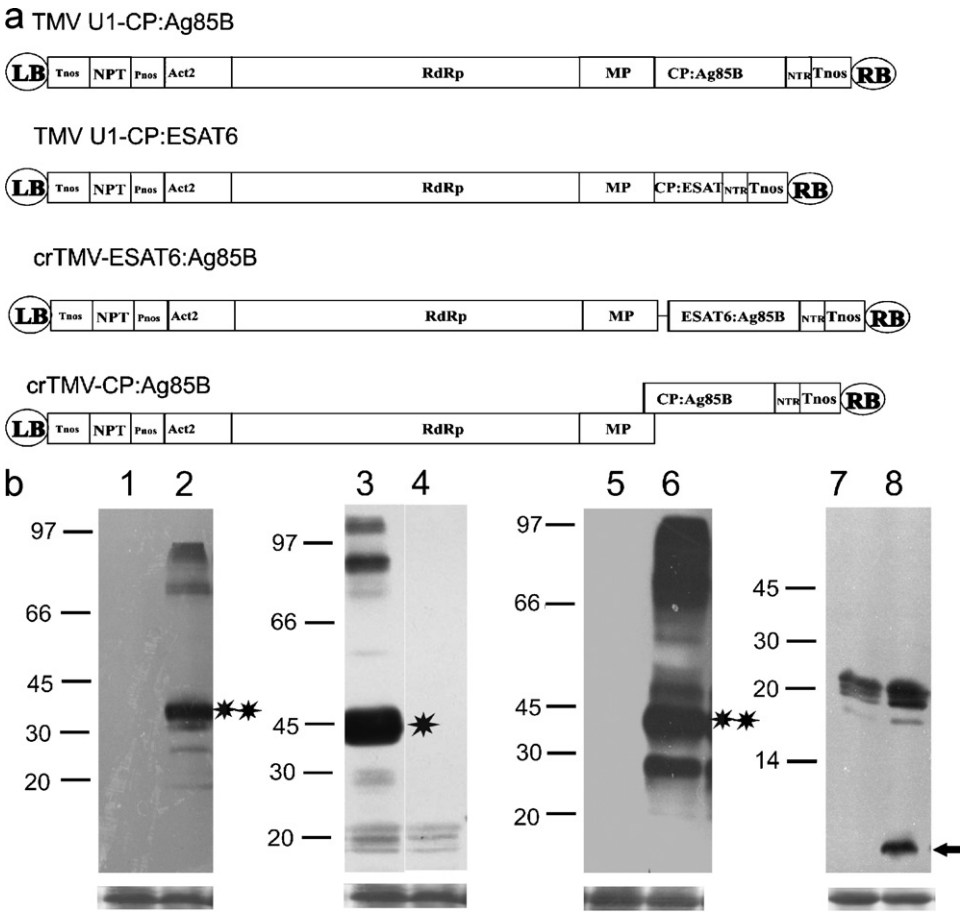

Figure 3 TB vaccine protein production in *N.benthamiana* leaves agroinjected with TMV-based vector. (a) Schematic representation of the binary TMV U1- based (TMV U1-CP:Ag85B and TMV U1-CP:ESAT6) and crTMV-based (crTMV-ESAT6:Ag85B and crTMV-CP:Ag85B) vectors where Act2, *Arabidopsis thaliana* Act2 promoter; MP, movement protein gene, RdRp, RNA dependent RNA polymerase gene. (b) Western blot analysis of leaves agroinjected with TMV U1-CP:Ag85B (lane 2), TMV U1-CP:ESAT6 (lane 8), crTMV-ESAT6:Ag85B (lane 3) and crTMV-CP:Ag85B (lane 6). Protein samples from non-agroinjected leaves (lanes 1, 4, 5, 7) were used as a negative control. The protein bands corresponding ESAT6:Ag85B and Ag85B are marked, respectively, by one or two asterisks. Arrow shows the position of CP:ESAT6.

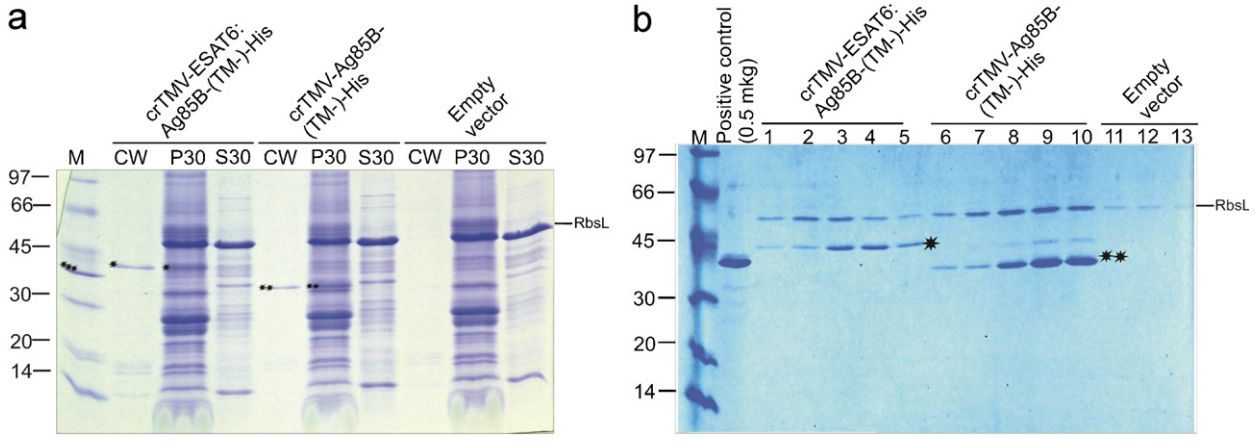

Figure 4 Purification of TB vaccine proteins from leaves agroinjected with crTMV-based vector. (a) Subcellular fractioning and Coomassie blue staining of proteins from leaves agroinjected with crTMV-ESAT6:Ag85B(TM−)-His and crTMV-Ag85B(TM−)-His. Lane M, marker proteins containing 43 kDa *E-coli* ESAT6:Ag85B protein (0.5 μg, designated by three asterisks). The protein bands corresponding to ESAT6:Ag85B and Ag85B are marked, respectively, by one or two asterisks. (b) Affinity chromatography of TB vaccine proteins and Coomassie staining of 15% SDS–PAGE with different 8 M urea, pH 4.5 eluates. The position of RbsL is marked.

efficiency of His-tagged TB antigens purification improved with an increase in of its contents in extracts (cf. Fig. 1e, lane 3 and Fig. 4b, lanes 1–10) and it is likely that the CW fraction (Fig. 4a) will serve as the best source for TB antigens purification.

Collectively, our results indicate that transient expression of vector viruses with concomitant suppression of VIGS represents a technology for fast and inexpensive production of significant amounts of TB antigens using small quantities of *N. benthamiana* plants.

Acknowledgements

This work was partly supported by Russian Foundation for Basic Research (Grants 04-04-48490, 05-04-48674, 05-04-08002, 06-08-01473) and Russian Federal Agency of Science and Innovations (Contract 02.435.11.3012).

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