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Expression of tuberculosis antigen ESAT-6 in *Nicotiana tabacum* using a potato virus X-based vector

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Summary A good candidate antigen to create a therapeutic vaccine against TB is the ESAT-6 protein. Antigens produced in plants have already been successfully used as experimental vaccines, and small single-stranded RNA plant viruses have emerged as promising tools to rapidly express large amounts of foreign proteins in susceptible host plants. Here, we present the expression of ESAT-6 protein in *Nicotiana tabacum* using a vector based on potato virus X (PVX). The complete ESAT-6 open reading frame is expressed as a fusion protein with the 2A peptide of Foot and Mouth Disease Virus and the amino terminal of the PVX coat protein (CP) (PVXESAT-6). This strategy allows the production of free CP and ESAT-6 as well as fused ESAT-2A-CP to obtain recombinant chimaeric virions expressing ESAT-6 at the surface to be used as particulate antigen in vaccination. ESAT-6 expression was tested in agroinfiltrated tobacco leaves and products of the expected molecular masses corresponding to cleaved CP and ESAT-2A-CP fusion protein were observed, with ESAT-6 yields ranging from 0.5% to 1% of total soluble protein. Our study describes for the first time the expression of the ESAT-6 protein in tobacco plants using a PVX-derived vector. This strategy should serve as a convenient, rapid, low-cost expression system and can also be used for the assessment of ESAT-6 production and function prior to stable plant transformation.

Introduction

Tuberculosis (TB), a chronic illness caused by *Mycobacterium tuberculosis*, is still a major

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world-wide disease, being a considerable public health problem in Latin America, Asia and Africa. In the last years, an increase in the incidence of tuberculosis has been observed and attributed to weak control programs, the AIDS pandemic—which predisposes individuals to develop TB—and to the appearance of *M. tuberculosis* strains resistant to first-line antibiotics.[1] The only approved tuberculosis vaccine is BCG, a prophylactic vaccine made from an attenuated strain of *Mycobacterium bovis* BCG which is effective against severe forms of childhood tuberculosis. Presently, BCG is used worldwide and an estimated 3 billion doses have been used to vaccinate the human population against tuberculosis. However, BCG has a striking variability in its protective activity worldwide[2] and its efficacy against adult pulmonary disease in endemic areas is limited. Hence, new rationally constructed vaccine candidates against tuberculosis are required.

Literature on the production of recombinant protein antigens in plants has increased in the last few years.[3–6] Experimental vaccines using plant-derived antigens have shown many advantages over traditional technologies, including efficacy, increased safety (no pathogen is known to infect both plants and animals), versatility, and economy. Several studies have shown that antigens from diverse pathogens synthesized in plant tissues are able to induce an immune response when administered to laboratory animals by oral, subcutaneous or intramuscular inoculation. In a number of cases, these animals developed a protective response against subsequent pathogen attacks.[7–12]

Two basic strategies can be used to express recombinant proteins in plants: use of plasmids and plant virus vectors for transient expression or the generation of transgenic plants. Transient expression is particularly suited to assess the functionality and stability of gene products before moving onto large-scale production of transgenic plants.[13] In addition, transient expression systems are fast, flexible, and are not influenced by positional effects.[14] *Agrobacterium tumefaciens* can be used in transient expression assays[13] aided by a vacuum infiltration system that allows its delivery into plant tissues[15] and, once optimized, an *Agrobacterium* infiltration protocol can be applied at a larger scale.[13] Transient expression with small single-stranded RNA plant viral vectors have emerged as particularly promising tools, because they can produce large amounts of proteins in susceptible host plants compared with those obtained by stable transformation procedures. We have previously developed a vector based on potato virus X (PVX) to express a major toxoplasma antigen in tobacco leaves.[16] In the present work we present the expression of ESAT-6 protein in tobacco using a PVX-based vector.

Materials and methods

Agrobacterium-mediated transient expression

A. tumefaciens strain GV3101 (RifR GmR) was transformed with constructions pZPVX and pZPVXE-SAT-6 by electroporation. *Agrobacterium* cultures were grown in LB liquid medium overnight at 29 °C on a shaker. A new culture was started the next morning by inoculating fresh medium with the overnight culture (1:10 ratio, v:v). These cultures were grown under the same conditions for an additional 5–7 h. Bacteria were then harvested by centrifugation at 1000*g* and resuspended in dd water. Bacterial densities were adjusted to OD600 = 0.4–0.5 prior to infiltration. Infiltrations were performed by applying pressure against the lower side of a leaf lamina with a syringe containing the bacterial suspension but lacking a needle. Plants were grown under 16-h daylight at 22 °C and observed daily for infection signs. Symptomatic leaves were harvested and stored at −70 °C until use.

Preparation of plant extracts

Crude plant extracts were prepared by grinding the infected leaves (5 g) to a fine powder in liquid nitrogen. The powder was resuspended and homogenized in phosphate buffered saline (1 ml PBS/0.5 g of fresh leaves) containing protease inhibitors. Tissue homogenates were centrifuged at 4 °C, 1000*g*, for 10 min. The supernatant was transferred to a fresh tube and kept at −70 °C until use. Total soluble protein content was estimated by the Bradford assay (Bio-Rad Inc., Segrate, Italy). Homogenized tissues of infected plant were also used to inoculate *N. tabacum* plants to propagate the infectious recombinant PVX particles.

Western blot analysis

Total soluble protein (30 μg) was resuspended using cracking buffer (250 mM Tris–HCl, pH, 6.8, 6% SDS, 5% glycerol, 0.05 mg/mg bromophenol blue), separated by 15% SDS-PAGE and then transferred onto an Immunoblot-ECL membrane (Amersham). The membrane was incubated with anti-ESAT-6 monoclonal antibody (1:1000) or anti-CP polyclonal antiserum (1:1000 followed by anti-mouse or

anti-rabbit IgG alkaline phosphatase conjugate as secondary antibodies (1:4000; NEN Life Science). Prestained protein (Invitrogen) were included in Western blots as molecular weight markers.

Construction of pZPVX and pZPVXESAT-6 vectors

A full-length cDNA copy of PVX strain CP (International Potato Center, Lima, Peru) was subcloned between the Cauliflower Mosaic Virus 35 S promoter and the transcriptional terminator of nopaline synthase (nos) and transferred to a binary vector pZP200 to obtain the pZPVX vector.

For the construction of the pZPVXESAT-6 vector, the 16-amino acid sequence of FMDV 2A peptide was synthesized from four partially overlapping oligonucleotides: 2A-1: 5′-AATTCAATTTTGATCTTTT-GAAGCTTGC-TGGT-3′; 2A-2: 5′-GATGTTGAATCTAA TCCAGGTCCAGCCAACACCACTCAAGCTGTAGGA-TCC-C-3′; 2A-3: 5′-TCGAGGG ATCCTACAGCTTGAGTGGT-GTTG-3′; 2A-4: 5′-GCTGGAC-CTGGATTAGATTCAA CATCACCAGCAAGCTTCAAAAGATCAAAATTG-3′. Oligonucleotides 2A-1 to 4 were annealed together to obtain a PCR fragment that contains a *Bam*HI site corresponding to amino acids 12 and 13 of PVX CP (bold), a three restriction enzyme site-linker (*Sma*I, *Pst*I and *Eco*RI); the sequence encoding 16 amino acids from the FMDV 2A peptide (underlined) and the sequence encoding amino acids 4–13 of the PVX CP (italic), (5′-**GGATCC**CCCGGGCTGCAGGAATTCAAT-TTTGATCTTTT GAAGCTTGCTGGTGATGTTGAATCTA-ATCCAGGT*CCAGCCAACACCACTCAAGCTGTA**GGATCC***-3′). After *Bam*HI digestion, this PCR product was subcloned into pBSNheI, a plasmid containing a NheI fragment of the PVX genome corresponding to positions 4874 to 5792. This fragment has an unique *Bam*HI site at position 5683, between the codons encoding amino acids 12 and 13 of the viral CP, where the PCR product was subcloned to obtain pBSNheI2A plasmid.

An amplicon containing the complete ESAT gene was obtained by PCR with primers Esatup (GAATT-CAACAGAGCAGCAGTGGAAT) and EsatloP (GAATT-CTGCGAACATCCCAGTGAC) and cloned into pGemT plasmid (Promega). The insert was released with EcoRI and ligated to plasmid pBS2A previously digested with *EcoRI*; the resulting construction was named pBS2A-ESAT. Next, the *Bam* HI restriction fragment from pBS2A-ESAT was subcloned into plasmid pNheX to create plasmid pNhe-ESAT. Finally, the *Nhe* I fragment from pNhe-ESAT was subcloned into pZPVX digested with the same enzyme to obtain pZPVXESAT-6.

Results

Construction of a vector carrying the *ESAT-6* antigen

To express the ESAT-6 antigen in plants, we used a vector containing the complete cDNA of PVX strain CP under the control of the 35S promoter of the Cauliflower Mosaic Virus and the transcriptional terminator of nopaline synthase (vector pZPVX, Fig. 1). Since it has been previously demonstrated that PVX chimaeric particles can be assembled from free CP and CP fusion proteins,[17] we used a strategy based on the 2A catalytic peptide of Foot and Mouth Disease Virus.[17] The complete ESAT-6 ORF was cloned into the pZPVX vector in such a way to express the ESAT-6 protein as a fusion with the FMDV 2A peptide and the amino terminal of PVX CP (vector pZPVXESAT, Fig. 1). The 2A sequence promotes a cotranslational cleavage that frees the foreign protein from the CP, although, since cleavage is not 100% efficient, some CP protein fused to the foreign protein is also produced. Thus, this strategy allows the production of free ESAT-6 as well as recombinant chimaeric virions expressing ESAT-6 at the surface as a ESAT-2A-CP fusion protein to be used as particulate antigen in vaccination.

Expression of ESAT-6 in *Nicotiana tabacum* plants

To test ESAT-6 expression, *Nicotiana tabacum* plants were agroinfected with *A. tumefaciens* strain GV3101 harbouring pZPVXESAT, empty pZPVX or no vector.

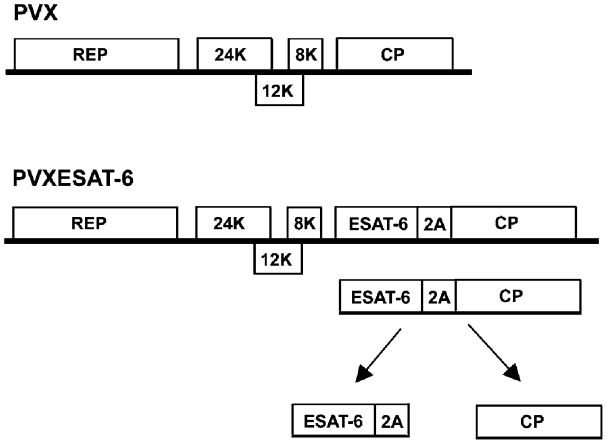

Figure 1 Schematic representation (not to scale) of the wild-type PVX and recombinant PVXESAT-6 viral genomes. Boxes represent coding sequences. REP: viral replicase. 24, 12 and 8 K: viral movement proteins. CP: coat protein. 2A: FMDV 2A peptide. Arrows shows the products produced by the 2A catalytic peptide.

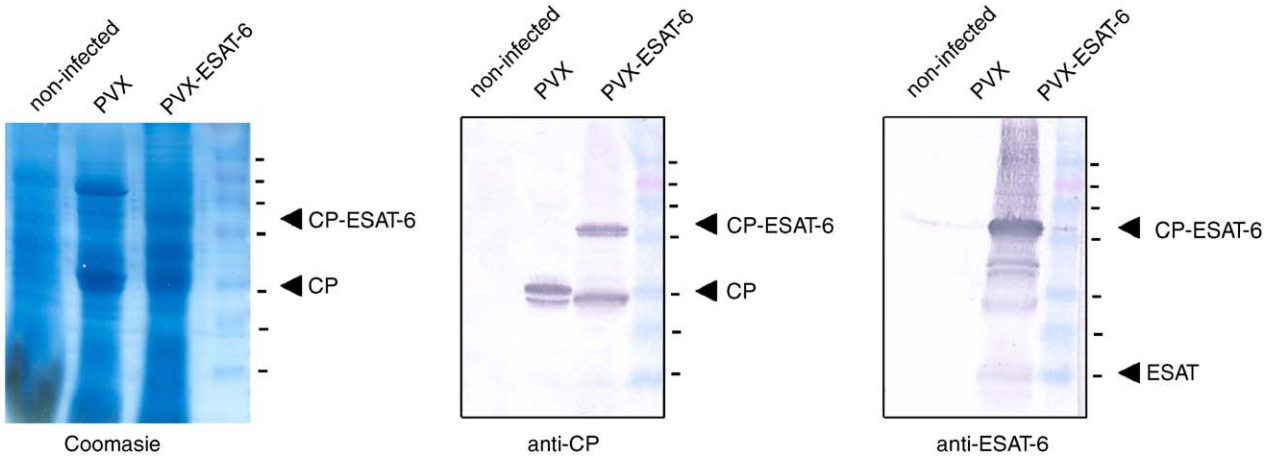

Figure 2 Immunoblot analysis of total leaf protein from *N. tabacum* leaves, probed with either anti-CP antiserum (anti-CP) or anti-ESAT-6 monoclonal antibody (anti-ESAT-6). Plants were agroinfected with *A. tumefaciens* without vector (non-infected), agroinfected with pZPVX (PVX) and agroinfected with pZPVXESAT-6 (PVXESAT-6). Left panel shows a Coomassie Blue Staining of the same protein preparations. The M_r of the native PVX CP is 25 kDa, the M_r of the ESAT-2A-CP fusion protein is 31 kDa and the predicted M_r values of ESAT is 6 kDa. Molecular mass standards (right lane of each panel) are: 83, 62, 47.5, 32.5, 25, 16.5 and 6.5 kDa.

Mock-infected plants showed no symptoms, whereas mottling and mild leaf deformation, typical signs of infection, were observed on inoculated leaves agroinfected with either pZPVXESAT-6 or pZPVX 5-6 days after inoculation. Infection spread systemically to apical leaves 14 days after inoculation.

To determine whether the ESAT-6 peptide accumulated in agroinfected plants, total soluble protein extracts were prepared from apical leaves and subjected to immunobloting. Products of the expected molecular mass, 25 and 31 kDa corresponding to free CP and ESAT-2A-CP fusion protein, respectively, were observed in extracts of leaves infiltrated with pZPVXESAT-6 using anti-PVX-CP antibodies (Fig. 2, middle panel). In PVX infected plants, a band corresponding to CP was observed and no protein was detected in mock-infected plants (Fig. 2, middle panel). When antibodies against ESAT-6 were used, a 31 kDa protein corresponding to ESAT-2A-CP fusion was specifically detected in the pZPVXESAT-6 infiltrated leaves (Fig. 2, right panel). Yields of ESAT-2A-CP in infiltrated leaves ranged from 0.5% to 1% of total soluble protein (Fig. 2, left panel). Successful infection and ESAT-6 expression were obtained after four reinfection cycles, demonstrating the stability of the expression vector.

Discussion

In this study, we describe for the first time the expression of a mycobacterial antigenic protein in tobacco plants, using a PVX-derived vector. Low-molecular-weight antigens such as ESAT-6 and CFP10 have been identified as having great potential for gamma interferon (IFN-g) stimulation.[18–20] ESAT-6 has been considered to be particularly interesting because, although the *esat-6* gene is present in *M. tuberculosis* and virulent *M. bovis*, it is absent from *M. bovis* BCG and major environmental mycobacteria.[21] These characteristics make ESAT-6 a good candidate for a therapeutic vaccine against TB. Recently, another group has reported the production of ESAT-6 in *Arabidopsis thaliana* plants.[22]

The PVX-based vector has proved to be an efficient plant expression system, allowing the production of high levels of ESAT-6 protein in tobacco leaves. Purified or crude plant extracts expressing ESAT-6 will allow the study, in experimental animals, of the cell-mediated immune response against the antigen with the aim of evaluating its potential use as a therapeutic vaccine against tuberculosis. In addition, other tuberculosis antigens could be easily evaluated using the same system and, since PVX has a wide range of hosts, other plants, like potato, could be used to obtain an edible vaccine.

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