Mol Biotechnol
DOI 10.1007/s12033-009-9195-3

RESEARCH

Potato Virus X Coat Protein Fusion to Human Papillomavirus 16 E7 Oncoprotein Enhance Antigen Stability and Accumulation in Tobacco Chloroplast

Mauro Morgenfeld · María Eugenia Segretin · Sonia Wirth · Ezequiel Lentz · Alicia Zelada · Alejandro Mentaberry · Lutz Gissmann · Fernando Bravo-Almonacid

Abstract Cervical cancer linked to infection with human papillomavirus (HPV) is the third cause of cancer-related death in women. As the virus cannot be propagated in culture, vaccines have been based on recombinant antigens with inherited high-cost production. In a search of alternative cheap production system, E7 HPV type 16 protein, an attractive candidate for anticancer vaccine development, was engineered to be expressed in tobacco chloroplast. In addition, E7 coding sequence was fused to potato virus X coat protein (CP) to compare expression level. Results show that E7CP transcript accumulation reached lower levels than non-fused E7. However, antigen expression levels were higher for fusion protein indicating that CP stabilizes E7 peptide in the chloroplast stroma. These results support viability of transplastomic plants for antigen production and the relevance of improving recombinant peptide stability for certain transgenes to enhance protein accumulation in this organelle.

M. Morgenfeld · M. E. Segretin · S. Wirth · E. Lentz · A. Zelada · A. Mentaberry · F. Bravo-Almonacid (✉)
Instituto de Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Vuelta de Obligado 2490, C.C. 1428 Ciudad Autónoma de Buenos Aires, Argentina
e-mail: fbravo@dna.uba.ar

M. Morgenfeld · M. E. Segretin · S. Wirth · A. Zelada · A. Mentaberry
Departamento de Fisiología, Biología Molecular y Celular (FCEN-UBA), Intendente Güiraldes 2620 Pab. II, C.I. 428EHA Ciudad Universitaria, Buenos Aires, Argentina

L. Gissmann
Department of Genome Modifications and Carcinogenesis, German Cancer Research Center, 69120 Heidelberg, Germany

Keywords Human papillomavirus (HPV) · E7 antigen · Fusion protein · Chloroplast transformation · Transplastomic tobacco · Molecular farming

Introduction

Plants offer a potential economic alternative to microbial fermentation and animal cell cultures for the production of recombinant proteins. For pharmaceutical protein, plant systems are inherently safer than native and even recombinant animal or microorganism sources because de absence of human pathogens [1]. In addition, edible plant tissues are well suited for the expression of vaccine antigens and pharmaceuticals for oral delivery [2]. It has also been demonstrated that plant-derived antigens can induce immune responses against important human or animal pathogens [3]. However, one of the main drawbacks of antigen production in plants is the low level of recombinant protein expression when the transgene is integrated into the nuclear genome. An alternative expression system to nuclear transformation is plastid genetic engineering. Transformation of chloroplasts allows higher levels of foreign accumulation than other transformation methods because of the high copy number of the transgene per cell [4–6]. Additional advantages of this system include transgene containment due to maternal inheritance of plastids in most plants, lack of gene silencing, multigene expression in a single transformation step, and expression of foreign proteins lacking methionine as the N-terminal amino acid [7]. This system has been demonstrated to be effective for the expression of vaccine antigens against several human and animal diseases [8, 9]. Mice and rabbits injected with chloroplast-derived vaccines developed specific immune responses and neutralizing antibodies against the pathogens [10, 11].

Published online: 05 July 2009

In a worldwide scenario, human papillomavirus (HPV) infection is the second leading cause of cancer-related morbidity and mortality among women due to its very close association with cervical cancer. It has been estimated that this disease causes almost 260,000 deaths annually, of which about 80% occurred in developing countries where it is the most common cancer affecting women [12]. A prophylactic vaccine against HPV is now available but, due to the long latency period between infection and onset of cancer, the benefits of prophylactic vaccination will not be visible for decades [13]. Thus, a therapeutic vaccine, targeting already infected individuals, is also required. Successful immunotherapy should induce specific cell-mediated immunity that would rapidly clear an established infection and provide protection against future exposure. Treatment of HPV-associated diseases will benefit from therapies that not only boost natural immune-mediated tumor defense mechanisms, but also focus the immune response on the relevant tumor antigens.

HPV type 16 (HPV16) accounts for 50% of all HPV-related cancer cases worldwide [12], therefore a vaccine specifically targeting this type of HPV is particularly desirable. The HPV16 oncoproteins (E5, E6, and E7) are responsible for the onset and maintenance of the transformed state and, therefore, represent appropriate targets for therapeutic vaccines. Several therapeutic HPV-specific E7-based vaccine formulations have been tested in animal models and some have advanced into phases II and III clinical trials [1, 14–16]. Many of these potential HPV therapeutic vaccines eliminate tumors in animal models and some evoke specific cell-mediated immune responses in early phase human trials. However, therapeutic vaccination has been limited by poor presentation of viral antigens that are expressed at low levels and by poor trafficking of effector T-cell populations to non-inflamated mucosal/skin sites. Therefore, the use of adjuvant has been crucial for therapeutic efficacy [17].

Recently, plant-produced HPV16 E7 antigen has been obtained using a transient expression system [18]. Anticancer activity of purified antigen was evaluated in mice for their potential as prophylactic and therapeutic vaccine candidates [19]. Immunization with plant produced E7-induced specific IgG and cytotoxic T-cell responses and protected mice against challenge with E7-expressing tumor cells. Moreover, fusion of the HPV-16 E7 gene with a plant virus coat protein (CP) gene in DNA vaccine has improve antitumor immunity induction despite increasing protein instability and faster degradation via the proteasome [20].

The primary objective of this study was to introduce the HPV16 E7 gene in tobacco chloroplast for the stable expression of this immunotherapeutic antigen. In addition, we engineered HPV16 E7 coding sequence as a fusion to potato virus X (PVX) CP to compare the expression levels of both constructs.

Methods

Cloning

Two transformation vectors were designed to express E7 and E7CP fusion protein in tobacco chloroplasts. For the construction of plasmid pBSW-utrE7, the E7 sequence that codes for the mature E7 (GenBank Accession Number KO2718.1) was digested from pGemE7 with enzymes *Nde* I and *Xba* I and cloned into pBSW-utrEGF plasmid [21] replacing the human epidermal growth factor (hEGF) sequence. A similar strategy was followed for the construction of plasmid pBSWutrE7CP. In this case, *E7* sequence has been upstream fused to PVX CP DNA sequence (GenBank Accession Number X12804.1). Both inserts were cloned under the transcriptional control of the promoter and 5′-untranslated region of tobacco *psbA* gene (5′*psbA*) and downstream the *aadA* sequence that confers spectinomycin resistance, under the transcriptional control of the *rrn* promoter (*Prrn*). For targeting this transcriptional unit by homologous recombination into the intergenic region between *rrn16* and trnI genes in the inverted repeat of the tobacco plastome, the full construction was located between a left flanking region (LFR) that includes 1169 bp of the 3′-region of *rrn16* gene defined by positions 103235 (*Sac* II restriction site) and 104404 (*Avr* II restriction site) of the *Nicotiana tabacum* plastome (GenBank Accession Number NC 001879) and a right flanking region (RFR) that includes the 1015 bp containing the full *trnI* sequence and the 5′-region of *trnA* defined by positions 104404 (*Avr* II restriction site) and 105419 (*Sac* I restriction site) of the *N. tabacum* plastome (see Fig. 1).

Chloroplast Transformation

Chloroplast transformation was carried out as previously described [22], using a PDS 1000/He biolistic particle delivery system (Bio-Rad, USA). Fully expanded leaves of in vitro cultured *N. tabacum* cv. *Petit Havana* plants were bombarded with 50 μg of 0.6 μm gold particles (Bio-Rad) coated with 2 μg of plasmid DNA using 1100 psi rupture discs (Bio-Rad). Transformed shoots were regenerated in selective RMOP (revised medium for organogenesis (shoot regeneration) of *Nicotiana plumbaginifolia*) regeneration medium [23] containing 500 mg/l spectinomycin dihydrochloride. To obtain homoplasmic plants, leaves from PCR-positive shoots were cut into pieces and taken through three additional regeneration cycles in selective medium. Transplastomic plants were seeded in greenhouse and seeds

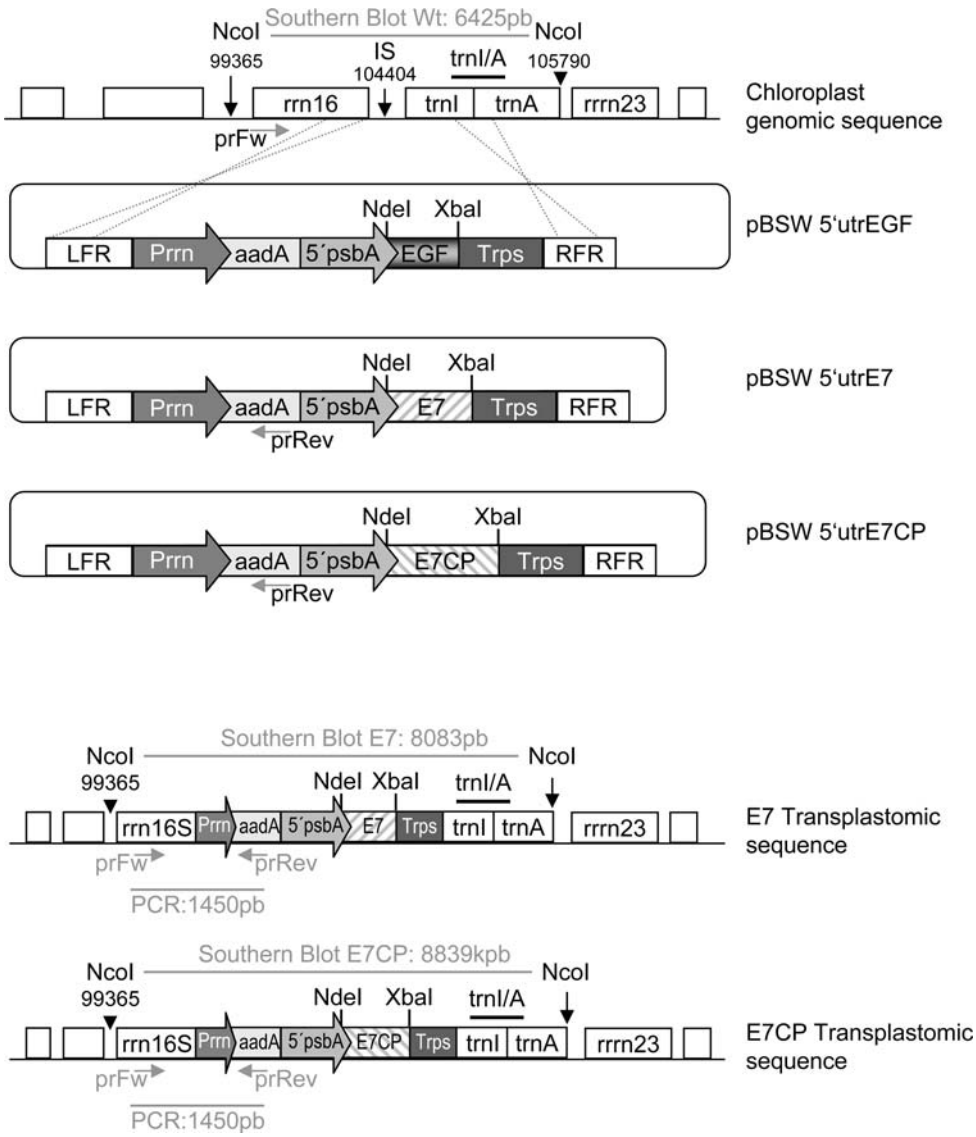

Fig. 1 Chloroplast transformation vectors. pBSW 5′utrE7 and pBSW 5′utrE7CP were obtained by replacing the *hEGF* at *Nde* I and *Xba* I sites of pBSW 5′utrEGF by the E7 and E7CP sequence. This sequence encodes, in a continuous frame, E7 and PVX Capside (CP) sequence. Thus, E7 and E7CP are downstream of the 5′-untranslated sequence and the promoter of the *psbA* gene (5′*psbA*) and upstream of the Trps16 termination sequence. The *aadA* sequence with a ribosome binding inserted upstream of the initial ATG codon is under the control of the *Prrn* promoter. Transgenes constructions are between an LFR (1169 bp) that includes the sequence of the 3′-region of *rrn16* gene and an RFR (1015 bp) containing the full *trnI* and 5′-region of *trnA*. Chloroplast genomic sequence at the insertion site (*IS*) showing homologous recombination region. *rrn23*: sequence encoding the 23S rRNA. *TrnI/A*: probe used in Southern blots assays. The *arrowhead* indicates the primers used for the PCR analysis

were germinated in selective medium to obtain homoplasmic lines.

PCR Analysis

Insertion of different transgenes was verified by polymerase chain reaction (PCR) analysis. DNA samples, obtained from leaf tissues of spectinomycin resistant and wild-type (Wt) plants, were used as template for amplification with primers prFw (5′GTATCTGGGGAATAAGCATCGG3′) and prRev (5′CGATGACGCCAACTACCTCTG3′). prFw hybridize upstream of the LFR in 16S Wt gene and prRev hybridize in the *aadA* sequence. Therefore the 1450-bp fragment was only amplified in transplastomic plants. The PCR reaction was conducted in a total volume of 50 µl, containing 10 ng of leaf total DNA, 10× PCR buffer, 400 µM dNTP mix, 150 ng of each primer, and 1 U *Taq polymerase* (Invitrogen Corp., Carlsbad, USA). The reaction conditions were as follows: initial PCR activation (95°C, 5 min) was followed by 30 amplification cycles (denaturing, 95°C, 30 s; annealing, 55°C, 60 s, and extension 72°C, 90 s) and a 10 min extension step at 72°C.

Southern Blot

Total DNA was extracted from leaves as described by Dellaporta et al. [24]. The DNA (4 µg) was digested with *Nco*I enzyme (New England Biolabs, USA), electrophoresed in 0.8% agarose gels and blotted onto Hybond N^+ Nylon membranes (Amersham Biosciences, USA). Specific DNA sequences were detected by hybridization with ^{32}P-labeled *E7* or *trnI/A* DNA probes. Probes were obtained by random priming with a Prime-a-Gene kit (Promega, USA), pre-hybridization, and hybridization were carried out at 65°C in Church's hybridization solution [25] for 2 and 16 h, respectively. Membranes were washed

twice with gentle shaking for 30 min in 0.2× SSC, 0.1% SDS at 65°C. For re-hybridization, membranes were washed at 95°C with 0.5% SDS to remove probes.

Northern Blot

Total RNA was extracted from fully expanded young leaves using TRiZOL Reagent (Invitrogen Corp., Carlsbad, USA). An aliquot of 4 μg of formaldehyde-denatured RNA was electrophoresed in a 1.5% agarose/formaldehyde gel and blotted onto Hybond N^+ Nylon membranes (Amersham Biosciences). Specific mRNA sequences were detected by hybridization with ^{32}P-labeled E7 DNA probe generated by random priming with a Prime-a-Gene kit (Promega). The blot was pre-hybridized, hybridized, and washed as described for Southern blot.

Western Blot

Total protein from transformed and non-transformed plants were extracted from fully expanded leaves in protein extraction buffer (50 mM Tris–HCl, pH 6.8, 10 mM EDTA–Na_2, 1 mM PMSF, 0.5 μg/ml, 0.01% Triton X-100) that was previously heated to 95°C. Total protein content was quantified by the BCA protein assay (Pierce, Rockford, USA). Analysis of E7 and E7CP fusion recombinant proteins was performed by Western blot. Aliquots of extracts containing 10 μg each of total soluble proteins (TSP) from transplastomic and non-transplastomic plants were separated in a 12% Tris–glycine SDS gel and transferred onto a nitrocellulose membrane. Known amounts of recombinant E7 protein expressed in yeast and purified by a fast protein liquid chromatography method [26] were used as a standard.

The membrane was probed with commercial monoclonal mouse anti-E7 antibody (Zymed Laboratories, San Francisco, USA) (100 ng/ml) followed by three washes with 50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.05% Tween 20, and a second incubation step with alkaline phosphatase-linked goat anti-mouse IgG antibody diluted to 1:3000. After a final wash, phosphatase activity was determined by a chromogenic reaction using 5bromo-4chloro-3indolyl phosphate and nitroblue tetrazolium (Sigma Chemical Co., USA) as substrates.

ELISA Assay

The amount of E7 protein was determined by ELISA. Plant extracts obtained from fully expanded leaves were quantified by the BCA protein assay, and 50 μg of total TSP diluted in PBS buffer (100 μl/well) were added to microtiter plates and incubated over night at 4°C. Coated wells were blocked with PBS containing 5% non-fat dry milk. The presence of E7 protein was detected with a 1:6000 PBS dilution of the monoclonal mouse anti-E7 antibody (Zymed Laboratories, San Francisco, USA), followed by incubation with an HRP-conjugated goat antimouse IgG antibody diluted 1:5000 (Invitrogen Corp., Carlsbad, USA). Between each step microtiter plate was washed thrice with 200 μl PBS-Tween 20, 1%. Enzymatic activity was measured by adding 2,2-azino-di-3-ethylbenz-thiazoline sulfonate substrate, and the absorbance of the samples was read at 450 nm on an ELISA microtiter plate reader (Sensident Scan, Merck, Darmstadt, Germany). Known amounts of recombinant E7 protein diluted in Wt plant extracts were used as a standard.

Results

Chloroplast Transformation

Two transformation vectors were constructed using the pBSW-UTR plasmid for the expression of E7 or E7CP fusion protein in tobacco chloroplasts (see Fig. 1). The transgene was introduced downstream the 5′-untranslated sequence and the promoter of the *psbA* gene. The plasmid contains the *aadA* sequence under the control of *Prrn* promoter for the selection of stable transformants in the presence of spectinomycin.

Since prokaryotic protein synthesis machinery can recognize some plastid transcriptional and translational elements, expression in *Escherichia coli* can be used as a quick method for testing genetic constructions, despite it cannot be used as a precise way to predict protein expression in the chloroplasts. For this purpose, extracts from *E. coli* cells transformed with plasmids pBSW-utrE7 and pBSW utr-E7CP were analyzed by Western blot. Expression of E7 and E7CP fusion proteins was confirmed and used as first broad criterion to verify transformation vectors functionality (data not shown).

Transplastomic tobacco plants were obtained by bombardment of *N. tabacum* cv. *P. Havana* leaves with each of the transformation vectors described. Regenerated plants were initially analyzed by PCR to detect presence of the transgenes (see Fig. 2). Three PCR-positive plants obtained after bombardment with each vector were subjected to two or three additional regeneration rounds in spectinomycin-containing media before grown for further analysis.

Southern Blot

Stable integration of the transgene and homoplasmy of spectynomicin-resistant plant lines were evaluated by Southern blot analysis. Total DNA extracted from leaf was cut with *Nco* I restriction enzyme that recognizes two positions flanking the insertion site outside the left and

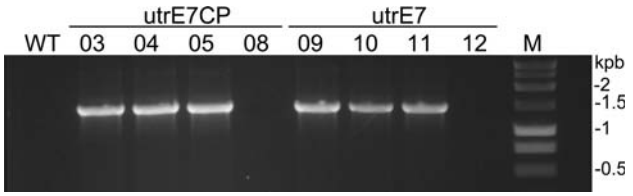

Fig. 2 PCR analysis of spectynomicin-resistant plants. DNA was extracted from leaf tissues of plants regenerated in RMOP medium containing spectynomicin after bombardment with pBSW 5′utrE7CP (03, 04, 05, 08) or pBSW 5′utrE7 (09, 10, 11, 12). *WT* Wt control, *M* DNA marker. Genomic DNA samples (10 ng) were used as templates for amplification with primers prFw and prRev. The 1450-bp amplification product was only obtained on transplastomic lines. Plants 08 and 12 resulted in not transformed spectynomicin-resistant mutants

right recombination regions (see Fig. 1). Therefore, Wt plastomes releases a 6.4-kbp DNA fragment, whereas transformed plastomes release a higher size fragment, depending on the length of the construction inserted (pBSW-utrE7, 8 kbp; pBSW-utrE7CP, 8.7 kbp.). Southern blot analysis revealed with *trnI/A* probe confirm transgene integration for three independent lines transformed with each vector (03, 04, 05, and 09, 10, 11) (see Fig. 3a). Absence of the 6.4 kbp Wt DNA fragment in this plants transformed with pBSW-utrE7 or pBSW-utrE7CP indicates that, within the limit of detection, these lines are essentially homoplasmic.

The same membrane was stripped and re-probed with specific E7 probe. This probe can only hybridize with transformed plastomes. Presence of specific bands only in lines mentioned above confirms previous results (see Fig. 3b).

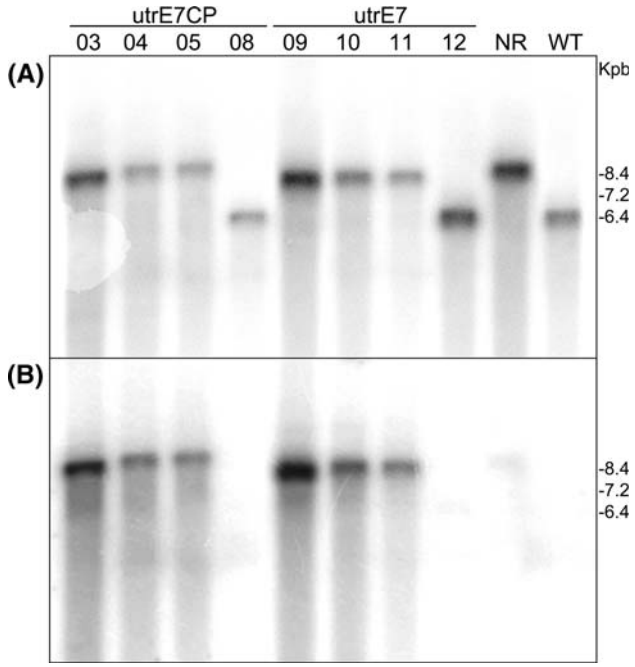

Fig. 3 Southern blot analysis of transplastomic lines. Total cellular DNA was digested with *Nco* I, electrophoresed, and blotted onto membrane. **a** Nylon membrane was hybridized with the *trnI/A* probe. The probes revealed a 6.4-kbp fragment in Wt plastomes and higher size DNA fragments in the transformed plastomes, according to the length of inserted constructs (pBSW-utrE7, 8.1 kbp; pBSW-utrE7CP, 8.8 kbp). **b** Nylon membrane from (**a**) was stripped and re-hybridized with a specific E7 probe that was able to detect the presence of E7 sequence. Each number indicates a different plant line. *WT* DNA from a non-transformed control plant, *NR* DNA from a non-related transplastomic plant. Position of DNA marker (lambda *BstE* II digested DNA, New England Biolabs, USA) is indicated at the right side of both panels

Northern Blot

Transcription of the E7 sequence and the E7CP fusion was confirmed by Northern blot analysis of total leaf RNA extracted from the different transplastomic lines. Specific transcripts were detected by hybridization with the E7 probe. In pBSW-utrE7 transformed lines, three types of transcripts were observed. Monocistronic transcripts corresponding to E7 sequence are produced from *psbA* promoter present in 5′*psbA* sequence, bicistronic transcripts including E7 and *aadA* sequences are produced from *Prrn* promoter and a higher size transcript including sequences of E7, *aadA*, and *rRNA 16S* arising by read-through transcription from the endogenous promoter of the *rrn* operon (see Fig. 4). In the case of pBSW-utrE7CP lines, mono, bi, and tri-cistronic transcripts exhibited higher sizes due to CP fusion. pBSW-utrE7 transcripts levels were higher than pBSW-utrE7CP mRNA transcripts. Differences in band intensities observed between lines pBSW-utrE7 and pBSW utrE7CP were not due to differences in loading.

E7 and E7CP Expression in *N. tabacum*

The presence of the HPV antigen protein was analyzed in three transplastomic lines obtained with each construct by Western blot. Total proteins were extracted from leaves of each line, separated in 15% SDS-PAGE and transferred to nitrocellulose membranes.

Analysis of recombinant protein expression in plants transformed with pBSW-utrE7 revealed a band of the expected size for the E7 protein (~17 kDa), indicating that this system is capable of express E7 as non-fused protein. On the other hand, in plants transformed with pBSW-utrE7CP, a stronger band of the expected size for the E7CP fusion protein (37 kDa) could be observed in Western blot.

The amounts of the recombinant proteins in the transplastomic plants extracts were quantified by ELISA assay and we could detect 0.5% for E7CP and 0.1% for E7 relative to TSP. These results are consistent with Western blot observations.

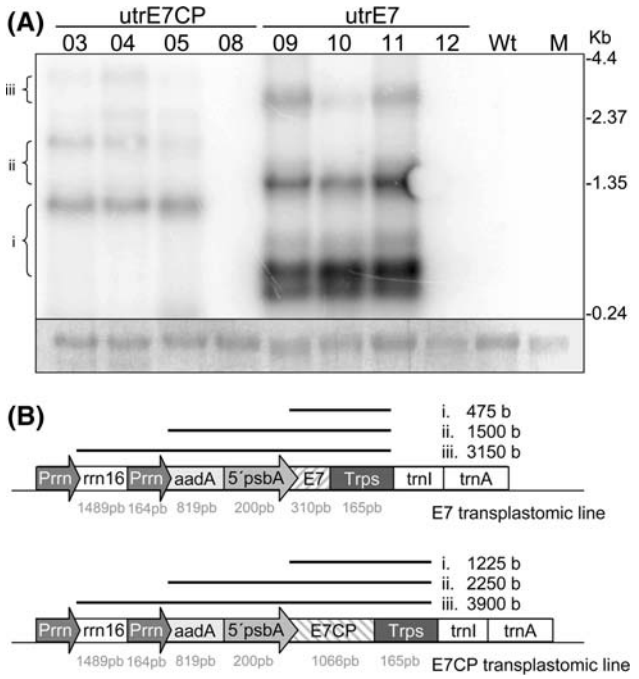

Fig. 4 Northern blot analysis of transplastomic lines. Total RNA was electrophoresed in denaturing conditions, blotted onto a nylon membrane and hybridized with a E7 specific probe. Polycistronic transcripts, synthesized from the *Prrn* promoters incorporated with the constructions and read-trough transcripts synthesized from the *Prrn* promoter of the *rrn* operon, are denoted as (ii) and (iii), respectively. Monocistronic transcripts synthesized from the psbA promoter located into the 5′*psbA* sequence are denoted as (i). **a** Analysis of plants transformed with pBSW 5′utrE7 and pBSW 5′utrE7CP. *M* RNA marker (Invitrogen Corp., Carlsbad, USA). Load reference is at the bottom. **b** The panel shows a transcription map with the expected mRNAs (i)–(iii)

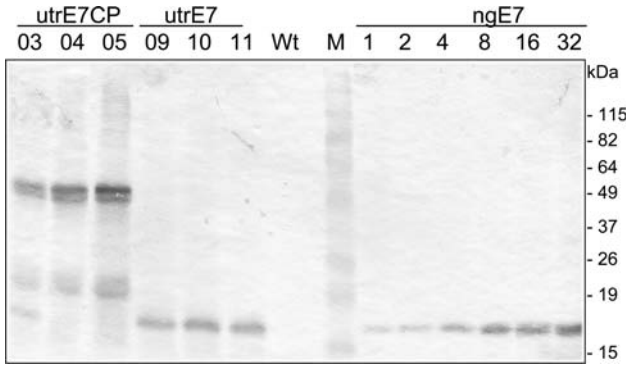

Fig. 5 Expression of recombinant proteins in transplastomic plants. Leaf extracts containing 10 µg of total TSP from the pBSW 5′utrE7CP-transformed plants (03, 04, 05), pBSW 5′utrE7 transformed plants (09, 10, 11), and from non-transformed control plants (WT) were subjected to Western blot analysis using a monoclonal anti-E7 antibody. Different amounts of purified E7 were included as mass reference. Position of pre-stained protein weight marker (M, Invitrogen Corp., Carlsbad, USA) is indicated at the right side of the panel

The E7 protein expression level detected in E7CP lines is around fivefold higher than E7 lines, indicating that this fusion could help to stabilize the E7 peptide and thus to increase the expression level of the recombinant protein in chloroplasts (see Fig. 5).

Conclusion

The primary objective of this study was to introduce the E7 HPV sequence in tobacco chloroplast for the expression of the immunotherapeutic antigen. Results shown here demonstrate the chloroplast system capacity for E7 expression.

The fusion of PVX CP to E7 has been proposed to enable recognition by the immune system [19]. In contrast with DNA E7CP vaccine results, our study shows that CP can stabilize E7 peptide in chloroplast tobacco since higher protein expression levels of E7CP than E7 were obtained. This result is consistent with the stabilization of a hydrophobic core in the C-terminus of the protein [27]. High level of E7 transcript accumulation indicates that transcription level or transcript stability cannot explain expression differences. Since both transcripts share the same sequences surrounding the translation start, 5′-untranslated region and E7 sequence, we conclude that translation speed is not related with higher E7CP accumulation and that the difference could be attributed to higher stability of the fusion protein.

These data suggest that chloroplast-transformed plants are a potential source of E7 antigen for immunotherapeutic vaccines against HPV-related cancer. In addition, our study supports that fusion with stabilization proteins as PVX CP can be a promissory strategy to improve recombinant proteins' expression level in this organelle. However, additional research about how to optimize unstable proteins accumulation in the chloroplast must be done. Other complementary strategies as thylakoid localization, codon optimization, promoter, and untranslated region selection must be considered. Although codon optimization is common for nuclear transformation, this seems not necessary for plastid transformation. Since all codons are relatively frequently used in this organelle, codon optimization yields only a modest increase in protein accumulation levels [4, 28].

E7 and E7CP protein from transplastomic plants will be administered to mice in order to assess and compare antigen effectiveness to generate an immune response against HPV-related cancer. In the future, plant nicotine level could be controlled by different strategies as low nicotine tobacco varieties selection [29] and antigen purification protocol.

Acknowledgment This study was supported by Grant BID 1728/OCAR PID No. 269 from the Agencia Nacional de Promoción

Científica y Tecnológica (ANPCyT). M.M. is an assistant teacher of DFBMyC, FCEN-UBA. E.L., M.E.S., and S.W. are fellows of CONICET. F.B.A., A.Z., and A.M. are Research Scientists of CONICET (Argentina).

References

1. Spok, A., Twyman, R. M., Fischer, R., Ma, J. K., & Sparrow, P. A. (2008). Evolution of a regulatory framework for pharmaceuticals derived from genetically modified plants. *Trends in Biotechnology, 26*, 506–517.
2. Streatfield, S. J., & Howard, J. A. (2003). Plant production systems for vaccines. *Expert Review of Vaccines, 2*, 763–775.
3. Floss, D. M., Falkenburg, D., & Conrad, U. (2007). Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. *Transgenic Research, 16*, 315–332.
4. Maliga, P. (2004). Plastid transformation in higher plants. *Annual Review of Plant Biology, 55*, 289–313.
5. Verma, D., & Daniell, H. (2007). Chloroplast vector systems for biotechnology applications. *Plant Physiology, 145*, 1129–1143.
6. Bock, R. (2007). Plastid biotechnology: Prospects for herbicide and insect resistance, metabolic engineering and molecular farming. *Current Opinion in Biotechnology, 18*, 100–106.
7. Fernandez-San Millan, A., Farran, I., Molina, A., Mingo-Castel, A. M., & Veramendi, J. (2007). Expression of recombinant proteins lacking methionine as N-terminal amino acid in plastids: human serum albumin as a case study. *Journal of Biotechnology, 127*, 593–604.
8. Daniell, H. (2006). Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. *Biotechnology Journal, 1*, 1071–1079.
9. Rybicki, E. P. (2009). Plant-produced vaccines: Promise and reality. *Drug Discovery Today, 14*, 16–24.
10. Molina, A., Veramendi, J., & Hervas-Stubbs, S. (2005). Induction of neutralizing antibodies by a tobacco chloroplast-derived vaccine based on a B cell epitope from canine parvovirus. *Virology, 342*, 266–275.
11. Chebolu, S., & Daniell, H. (2007). Stable expression of Gal/GalNAc lectin of *Entamoeba histolytica* in transgenic chloroplasts and immunogenicity in mice towards vaccine development for amoebiasis. *Plant Biotechnology Journal, 5*, 230–239.
12. Castellsague, X. (2008). Natural history and epidemiology of HPV infection and cervical cancer. *Gynecologic Oncology, 110*, S4–S7.
13. Koutsky, L. A., Ault, K. A., Wheeler, C. M., Brown, D. R., Barr, E., Alvarez, F. B., et al. (2002). A controlled trial of a human papillomavirus type 16 vaccine. *New England Journal of Medicine, 347*, 1645–1651.
14. Hallez, S., Simon, P., Maudoux, F., Doyen, J., Noel, J. C., Beliard, A., et al. (2004). Phase I/II trial of immunogenicity of a human papillomavirus (HPV) type 16 E7 protein-based vaccine in women with oncogenic HPV-positive cervical intraepithelial neoplasia. *Cancer Immunology, Immunotherapy, 53*, 642–650.
15. Roman, L. D., Wilczynski, S., Muderspach, L. I., Burnett, A. F., O'Meara, A., Brinkman, J. A., et al. (2007). A phase II study of Hsp-7 (SGN-00101) in women with high-grade cervical intraepithelial neoplasia. *Gynecologic Oncology, 106*, 558–566.
16. Trimble, C. L., Peng, S., Kos, F., Gravitt, P., Viscidi, R., Sugar, E., et al. (2009). A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. *Clinical Cancer Research, 15*, 361–367.
17. Gerard, C. M., Baudson, N., Kraemer, K., Bruck, C., Garcon, N., Paterson, Y., et al. (2001). Therapeutic potential of protein and adjuvant vaccinations on tumour growth. *Vaccine, 19*, 2583–2589.
18. Franconi, R., Di Bonito, P., Dibello, F., Accardi, L., Muller, A., Cirilli, A., et al. (2002). Plant-derived human papillomavirus 16 E7 oncoprotein induces immune response and specific tumor protection. *Cancer Research, 62*, 3654–3658.
19. Massa, S., Franconi, R., Brandi, R., Muller, A., Mett, V., Yusibov, V., et al. (2007). Anti-cancer activity of plant-produced HPV16 E7 vaccine. *Vaccine, 25*, 3018–3021.
20. Massa, S., Simeone, P., Muller, A., Benvenuto, E., Venuti, A., & Franconi, R. (2008). Antitumor activity of DNA vaccines based on the human papillomavirus-16 E7 protein genetically fused to a plant virus coat protein. *Human Gene Therapy, 19*, 354–364.
21. Wirth, S., Segretin, M. E., Mentaberry, A., & Bravo-Almonacid, F. (2006). Accumulation of hEGF and hEGF-fusion proteins in chloroplast-transformed tobacco plants is higher in the dark than in the light. *Journal of Biotechnology, 125*, 159–172.
22. Daniell, H. (1997). Transformation and foreign gene expression in plants by microprojectile bombardment. *Methods in Molecular Biology, 62*, 463–489.
23. Cséplő, A., & Maliga, P. (1984). Large scale isolation of maternally inherited lincomycin resistance mutations, in diploid *Nicotiana plumbaginifolia* protoplast cultures. *Molecular and General Genetics*, *196*, 407–412.
24. Dellaporta, S., Wood, J., & Hicks, J. (1983). A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter*, *1*, 19–21.
25. Church, G. M., & Gilbert, W. (1984). Genomic sequencing. *Proceedings of the National Academy of Sciences of the United States of America, 81*, 1991–1995.
26. Braspenning, J., Manetti, R., Zumbach, K., Meschede, W., Gissmann, L., & Tommasino, M. (1997). A general purification protocol for E7 proteins from "high- and low-risk" human papillomavirus types expressed in the yeast *Schizosaccharomyces pombe*. *Protein Expression and Purification, 10*, 192–201.
27. Pahel, G., Aulabaugh, A., Short, S. A., Barnes, J. A., Painter, G. R., Ray, P., et al. (1993). Structural and functional characterization of the HPV16 E7 protein expressed in bacteria. *Journal of Biological Chemistry, 268*, 26018–26025.
28. Daniell, H., Ruiz, G., Denes, B., Sandberg, L., & Langridge, W. (2009). Optimization of codon composition and regulatory elements for expression of human insulin like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. *BMC Biotechnology, 9*, 33.
29. Yu, L. X., Gray, B. N., Rutzke, C. J., Walker, L. P., Wilson, D. B., & Hanson, M. R. (2007). Expression of thermostable microbial cellulases in the chloroplasts of nicotine-free tobacco. *Journal of Biotechnology, 131*, 362–369.