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RESEARCH

Translational Fusion and Redirection to Thylakoid Lumen as Strategies to Enhance Accumulation of Human Papillomavirus E7 Antigen in Tobacco Chloroplasts

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Abstract Human papillomavirus (HPV) is the causal agent of cervical cancer, one of the most common causes of death in women worldwide, and its E7 antigen is the major candidate for a therapeutic vaccine. The large scale production of E7 by molecular farming that would lead to the development of a safe and inexpensive vaccine is impaired by its low accumulation level in the plant cell. To enhance antigen production in the plastids, two alternative strategies were carried out: the expression of E7 as a translational fusion to β-glucuronidase enzyme and redirection of E7 into the thylakoid lumen. The use of the β-glucuronidase as a partner protein turned out to be a successful strategy, antigen expression levels were enhanced between 30 and 40 times relative to unfused E7. Moreover, best accumulation, albeit at a high metabolic cost that compromised biomass production, was obtained redirecting E7 into the thylakoid lumen by the incorporation of the N-terminal transit peptide, Str. Following this approach lumenal E7 production exceeded the stromal by two orders of magnitude. Our results highlight the relevance of exploring different strategies to improve recombinant protein stability for certain transgenes in order to exploit potential advantages of recombinant protein accumulation in chloroplasts.

Keywords Human papillomavirus · E7 antigen · Transplastomic tobacco · Fusion protein · Thylakoid translocation · Molecular farming

Introduction

Compared to other technologies for recombinant protein production, plant molecular farming is an attractive alternative because of its relative lower cost [1]. Plants produce large quantities of biomass without the need for expensive culture media and allow easy scaling up of production [2]. Chloroplast transformation has many advantages for the production of recombinant proteins when compared to other expression systems in plants [3, 4]. Transgene integration is accomplished by homologous recombination between plastid-targeting sequences in the transformation vector and the targeted region in the plastid genome. As a consequence, the positional effects that can hinder transgene expression in nuclear transformation are not observed [5]. In addition, photosynthetic cells contain hundreds of polyploid chloroplasts; therefore, the integrated transgene can be present up to thousands of copies per cell. As a result and since gene silencing has not been reported in plastids, high expression levels of heterologous proteins can potentially be achieved in transplastomic plants [6, 7]. However, it has been reported that heterologous protein accumulation in chloroplasts could be hindered by post-transcriptional mechanisms. These mechanisms, although not yet fully understood, could be related with the quality and quantity control of plastid proteins by proteases and chaperones [8].

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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, about 80 % of which takes place in developing countries where cervical cancer is the most common cancer affecting women [9]. These data support the need for the development of an efficient vaccination strategy but, since the virus cannot be propagated in vitro, vaccines have been based on recombinant antigens with the associated high production cost. A prophylactic vaccine against HPV is now available with demonstrated efficacy [10]. However, there are still many infected women who need to be treated [11]. Thus, a therapeutic vaccine, targeting already infected individuals, is also required. Several therapeutic HPV-specific E7-based vaccine formulations have been tested in animal models [12] and some have advanced into clinical trials [13, 14]. HPV16 E7 is 98 amino acids long with a M_r 11,000 based on amino acid composition and an apparent electrophoretic M_r of 16,000–19,000 [15]. The high stability of the E7 structure responsible for the anomalous electrophoretic behavior is capable to resist thermal denaturation even in the presence of SDS [16].

Recently, the production of the HPV16 E7 antigen using transplastomic expression systems was reported [17, 18]. However, accumulation levels of E7 in chloroplast were low because of protein instability which has been related to "natively unfolded" or "intrinsically disordered" proteins [19]. In a previous work, E7 coding sequence was fused to potato virus X coat protein (CP) to improve antigen stability and immunogenicity. E7CP accumulation reached higher levels than non-fused E7 indicating that CP stabilizes E7 peptide in the chloroplast stroma [17]. There are other examples, such as human somatotropin [20], 5-enolpyruvylshikimate-3-phosphate synthase [21], cholera toxin B subunit [22], and cyanovirin-N [23] where high levels of expression were only obtained when the candidate products were synthesized as fusion proteins. As no increments in mRNA accumulation were observed in comparison with non-fused proteins, it was suggested that the fusion could be acting by protecting the recombinant protein against degradation.

The primary objective of this study was to explore alternative strategies to increase HPV16 E7 antigen accumulation in tobacco chloroplasts. For this purpose, the original E7 was fused to *Escherichia coli* β-glucuronidase and expressed in the chloroplast stroma as translational N-terminal (E7GUS) and C-terminal (GUSE7) fusion proteins.

A better understanding of the post-transcriptional mechanisms involved in chloroplast genetic regulation would allow the design of strategies to increase heterologous protein accumulation. Previous works suggest that in some cases redox potential could greatly influence recombinant protein stability [24]. Cysteine residues constitute the main target for redox regulation in proteins due to the reactivity of the thiol group [25]. The E7 cysteines could play a role in redox regulation of E7 structure and function, as observed for many other proteins [26], including the papillomavirus L1 major capsid protein [27]. The thylakoid lumen is an inner compartment in the chloroplast with different characteristics from the stroma, including different redox potential. In this work, we explored the possibility to accumulate in the thylakoid lumen recombinant unstable proteins such as E7. For this approach, E7 protein was targeted to this compartment by addition of the transit peptide from the 23 kDa protein of the oxygen-evolving system of photosystem II (Str), previously used to translocate recombinant proteins coded in the nuclear genome into the thylakoid lumen of *Arabidopsis thaliana* plants [28]. Folded proteins containing the Str transit peptide are translocated across the thylakoidal membrane by the ΔpH/TAT pathway [29]. In the present study, we expressed chimeras consisting of E7 and E7CP fused to Str. These recombinant proteins encoded in the *Nicotiana tabacum* plastome were translated and then targeted to the thylakoid lumen, presenting enhanced expression levels.

This work contributes to optimizing expression of E7 in transplastomic plants either as a fusion protein with β-glucuronidase or by accumulation in the thylakoid lumen. Both strategies will require further optimization before these recombinant proteins can be considered for therapeutic treatment (i.e., removing all tags or fused proteins to prevent unexpected immunological reactions). Overall the results presented in this paper reinforce the potential of the plastid system within the field of molecular farming.

Materials and Methods

Cloning

Four transformation vectors were designed from pBSW-utr E7 plasmid [17] which was previously digested with *Nde*I and *Xba*I restriction enzymes to remove the *hEGF* gene. All inserts were cloned between both restrictions sites and located under the transcriptional control of the promoter and 5′-untranslated region of the 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 construct was located

between a left flanking region (LFR) that includes 1,169 bp of the 3′-region of *rrn16* gene defined by positions 103,235 and 104,404 of the *N. tabacum* plastome (GenBank accession number NC 001879) and a right flanking region (RFR) that includes the 1,015 bp containing the full *trnI* sequence and the 5′-region of *trnA* defined by positions 104,404 and 105,419 of the *N. tabacum* plastome (Fig. 1).

DNA fragments coding for E7 and GUS fusion proteins were obtained by PCR from pGemE7 (GenBank accession number KO2718.1) and pBI121 [30]. E7 sequence for the E7GUS construct was amplified using primers 5′E7*Nde*I (GACATATGCACGGAGATAC) and 3′E7*Hin*dIII (GGA TTAAGCTTTCTGAGAAC) and then subcloned in the pZErO-2 vector (Invitrogen, Carlsbad, CA, USA). The GUS sequence was amplified using primers 5′GUS*Hin*dIII (CAAGCTTTACGTCCTGTAG) and 3′GUS*Xba*I (GGAG AGTTCTAGATTCATTG) and then subcloned in a pGEM vector (Promega, Madison, WI, USA). pGemGUS was digested with *Hin*dIII and *Xba*I in order to obtain a GUS-containing fragment that was ligated to pZErO-E7 previously treated with the same restriction enzymes. Finally, the E7GUS sequence was cut with *Nde*I and *Xba*I and ligated with the aforementioned digested plasmid to obtain the transformation vector pBSW-utr E7GUS. Analogous procedure was used to obtain pBSW-utr GUSE7 using primers 5′GUS*Nde*I and 3′GUS*Hin*dIII for GUS sequence and 5′E7*Hin*dIII and 3′E7*Xba*I for E7 sequence.

Str signal sequence (used to construct pBSW-utr StrE7 and pBSW-utr StrE7CP) was obtained by PCR from *N. tabacum* DNA using primers 5′StrSpeAse (TAACTAG

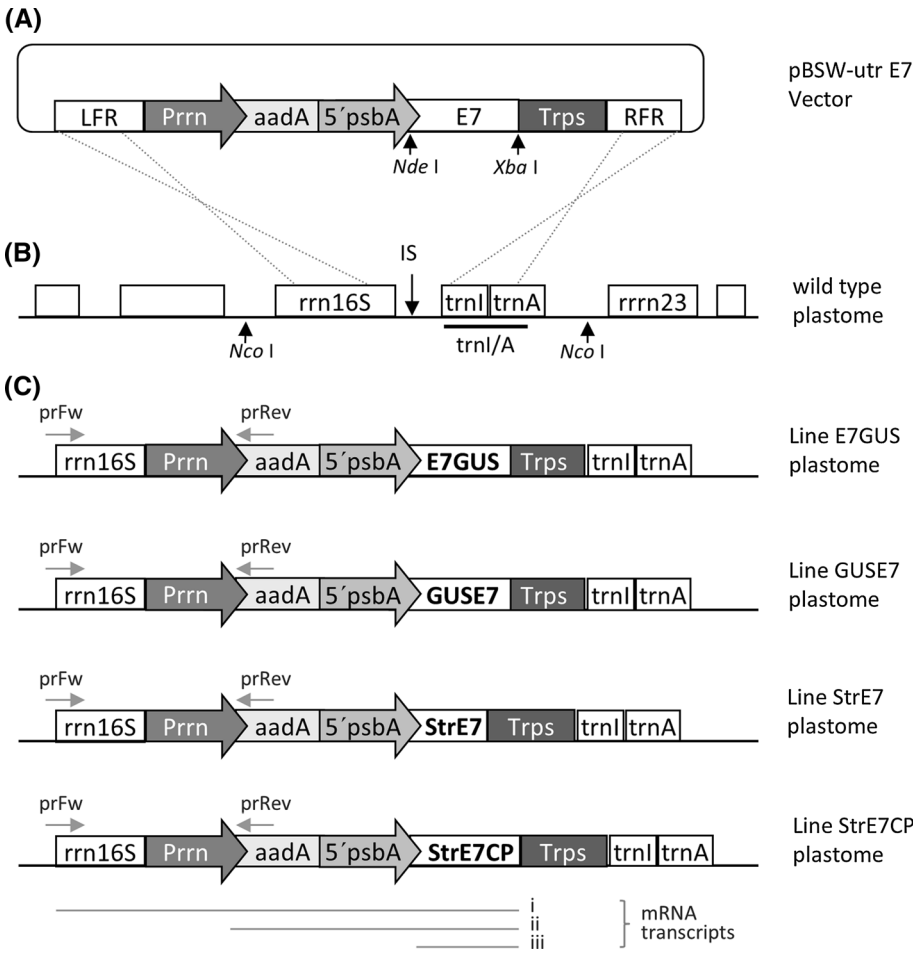

Fig. 1 Chloroplast transformation vectors. **a** Transformation vectors were obtained by replacing the E7 at *Nde*I and *Xba*I sites of pBSW-utr E7 by the E7GUS, GUSE7, StrE7, or StrE7CP fusion sequences that were inserted downstream the 5′-untranslated sequence and promoter of the *psbA* gene (5′psbA) 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 *rrn* promoter (Prrn). Transgenes constructs are between the left flanking region (LFR 1,169 bp) that includes the sequence of the 3′-region of *rrn16* gene and the right flanking region (RFR 1,015 bp) containing the full *trnI* and 5′ region of *trnA*. **b** Chloroplast genomic sequence of wild type plastome at the insertion site (IS) showing homologous recombination region. **c** Chloroplast genomic maps for the transplastomic lines showing the expected primary mRNA transcripts that contain the E7 sequence (i)–(iii). *rrn23*: gene encoding the 23S rRNA. *trnI/A*: probe used in Southern blots. prFw and prRev indicate the primers used for the PCR analysis

TATTAATGGCTTCAACAC) and 3′LumNdeXba (TCTA GACATATGAGCATCTGCAGGG). After amplification, the PCR product was subcloned in a pZErO-2 vector. The Str fragment was digested with *Ase*I and *Xba*I and subcloned into pBSW-utr E7, which was previously digested with the *Nde*I and *Xba*I. The same enzymes were used to cut plasmid pBSW-utr Str (NdeXba) and to obtain inserts from pBSW-utr E7 and pBSW-utr E7CP. After ligation, the final plasmids pBSW-utr StrE7 and pBSW-utr StrE7CP were obtained. All the intermediary constructs prepared in the pZErO-2 plasmid were sequenced.

Chloroplast Transformation

Chloroplast transformation was carried out as previously described [31], 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 1,100 psi rupture disks (Bio-Rad). Transformed shoots were regenerated in selective RMOP (revised medium for organogenesis (shoot regeneration) of *Nicotiana plumbaginifolia*) regeneration medium [31] containing 500 mg/l spectinomycin dihydro-chloride.

To obtain homoplasmic plants, leaves from PCR-positive shoots were cut into pieces and taken through two additional regeneration cycles in selective medium. Alternatively transplastomic plants were seeded in greenhouse and seeds were germinated in selective medium to obtain homoplasmic lines. After rooting, plants were transferred to soil and grown under greenhouse conditions. In the greenhouse, natural light was supplemented 16 h/day by sodium lamps providing 100–300 μmol/s/m^2, temperature was set at 26 °C during day and 19 °C in the night.

Southern Blot

Total DNA was extracted from leaves as described by Dellaporta et al. [32]. The DNA (4 μg) was digested overnight with the *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 α-^{32}P-labeled *trnI/A* DNA probe. Probe was 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 [33] 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. The blot was exposed to a storage phosphor screen, which was analyzed in a Storm 840 PhosphorImager system (Amersham).

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 α-^{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 was extracted from fully expanded leaves in protein extraction buffer (0.2 g/ml) (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).

Aliquots containing total soluble proteins (TSP), from transplastomic and non-transplastomic plants, were separated in a 12 % Tris–glycine SDS gel and Coomassie blue stained or transferred onto a nitrocellulose membrane. 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 1:3,000. After a final wash, phosphatase activity was determined by a chromogenic reaction using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Sigma Chemical Co., USA) as substrates. Membranes were scanned and the protein band intensities were quantified using the ImageJ software (NIH, http://rsbweb.nih.gov/ij).

Microscopy

Samples for the ultrastructural characterization of leaf material were fixed overnight at 4 °C in 0.1 M phosphate buffer (pH 7.2) containing 2.5 % glutaraldehyde. Then, the tissues were incubated for 2 h at 4 °C in 1 % (w/v) OsO_4 and dehydrated by sequential immersion in ethanol–acetone solutions of higher content. Finally, the samples were included in Spurr resin and cross sections were obtained by ultramicrotome. The semi-thin sections for light microscopy were stained with 1 % (w/v) toluidine blue. Light microscopy was also used to observe living tissue corresponding to the cell monolayer in the lower epidermis. Ultrathin sections for electron microscopy were stained

with dye Reynolds and contrasted with 2 % (w/v) uranyl acetate. The electron microscopy studies were performed with a transmission electron microscope Zeiss EM 10 C (Zeiss, Oberkochen, Germany).

Results

Generation of Transgenic Plants by Chloroplast Transformation

In vitro tobacco plants (*N. tabacum* L. cv. Petit Havana) were transformed by particle bombardment using the different tobacco chloroplast transformation vectors (Fig. 1). Several plastid-transformed (transplastomic) lines were obtained and three independent lines for each construct were characterized in more detail by PCR (data not shown). These selected transplastomic lines were regenerated three times in selective media and transferred to soil under greenhouse conditions for further analysis. Seeds from these plants were germinated in spectinomycin-containing media to confirm the inheritance of the selection marker and the absence of wild-type plastomes (data not shown).

Analysis of Transgene Integration into the Plastid Genome

Stable integration of the transgene into the plastome of spectinomycin-resistant lines was evaluated by Southern blot. Total DNA extracted from leaves was cut with the *Nco*I restriction enzyme that recognizes two positions flanking the insertion site outside the left and right recombination regions (Fig. 1b). Therefore, wild-type plastomes release a 6.4-kbp DNA fragment, whereas transformed plastomes release a higher size fragment, depending on the construct inserted. Southern blot analysis revealed with *trnI/A* probe confirmed transgene integration for all independent lines evaluated (E7GUS: 41–43, GUSE7: 51–52, StrE7: 21–23, StrE7CP: 31–33) (Fig. 2).

A remaining presence of the 6.4-kbp wild-type DNA fragment was observed in all the transplastomic lines. Given that these transplastomic plants were regenerated three times and then subjected to germination in selective media, the likelihood of heteroplasmy would be low. The wild-type-like hybridization signal may be due to the presence of promiscuous plastid DNA in one of the other two genomes of the plant cell [34]. It is well established that during evolution large fragments of chloroplast DNA integrated in the nuclear and mitochondrial genomes [35–37].

Line 32 showed a band of higher size than expected. According to previous works, plastome rearrangements can occur by flip–flop recombination events because of the presence of duplicated expression sequences in the transformed plastome [38]. This recombination could possibly explain the unexpected pattern observed for this transplastomic line.

Analysis of Transgenes Transcription

We confirmed transcription of E7-containing constructs by northern blot analysis of total leaf RNA extracted from the different transplastomic lines. Three types of transcripts were observed after hybridization with the E7 probe: monocistronic transcripts corresponding to transgene sequence transcribed from the *psbA* promoter (present in the 5′*psbA* sequence), bicistronic transcripts transcribed from the *rrn* promoter (P*rrn*) included in the cassette, and a higher size transcript arising by read-through transcription from the endogenous promoter of the *rrn* operon (Fig. 3). The electrophoretic mobility for each transcript supported the differences in the sequences included in the transformation plasmids. The expected transcripts sizes were observed for all the transplastomic lines analyzed (E7GUS: i 4.9 kb, ii 3.3 kb, iii 2.2 kb; GUSE7: i 5.1 kb, ii 3.4 kb, iii

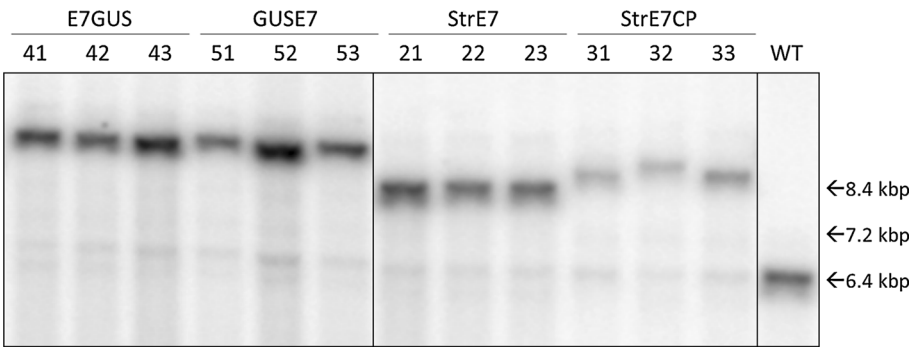

Fig. 2 Plastomic integration of transgenes. Southern blots analysis of transplastomic lines. DNA digested with *Nco*I and hybridized with the *trnI/A* probe. The probes revealed a 6.4-kbp fragment in Wt plastomes and a higher size DNA fragments in the transformed plastomes, according to the length of inserted constructs

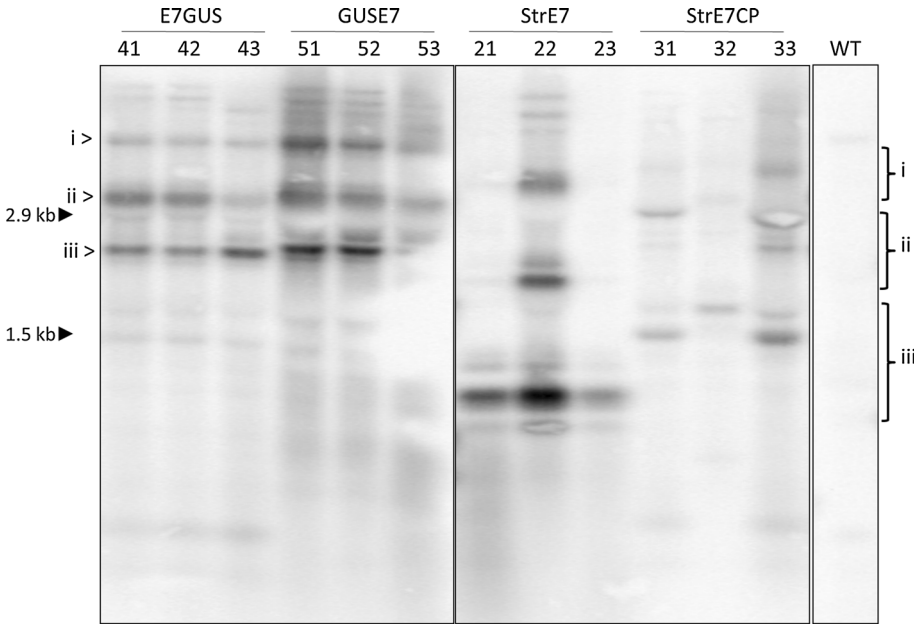

Fig. 3 Transgenes transcription. Northern blots of transplastomic lines. Total RNA hybridized with an E7-specific probe. Polycistronic transcripts, synthesized from the Prrn promoter incorporated with the constructs and read-trough transcripts synthesized from the endogenous promoter of the *rrn* operon, are denoted as (*ii*) and (*i*), respectively. Monocistronic transcripts synthesized from the *psbA* promoter located into the 5′psbA sequence are denoted as (*iii*). Positions of 23S rRNA (2.9 kb) and 16S rRNA (1.5 kb) are denoted by *arrows*

2.4 kb; StrE7: i 3.4 kb, ii 1.7 kb, iii 0.7 kb and StrE7CP: i 4.1 kb, ii 2.5 kb, iii 1.5 kb) except for line 32 (StrE7CP) which showed transcripts of higher size, presumably because the aforementioned reasons.

Analysis of Recombinant Proteins Accumulation

Expression of recombinant proteins in the leaves was confirmed by western blot using a monoclonal antibody against E7. E7GUS and GUSE7 plants revealed a band of the expected size (~80 kDa) and a second band probably generated by partial proteolysis (Fig. 4a), indicating that this system is able to accumulate E7 as fused protein. The differences observed in the migration of the two fusion proteins can be attributed to intrinsic anomalous folding of E7, which is resistant to denaturing conditions. Moreover, the six transplastomic lines showed β-glucuronidase activity in foliar tissue (as evidenced by histochemical assays) confirming GUS functionality in both N- and C-terminal fusion proteins (data not shown).

StrE7CP transplastomic plants presented a band of the expected size for the E7CP protein and an additional band of higher size was also observed in the line 32, which could be attributed to the anomalous characteristics previously described (Fig. 4b). StrE7 lines showed higher expression levels and a complex pattern probably associated to the capacity of E7 to form aggregates that resist denaturation in SDS-containing sample buffer. E7 and StrE7 as well as E7CP and StrE7CP showed a similar size when expressed in plants suggesting that the Str signal peptide was processed after translocation into the thylakoid lumen (Fig. 4c). The analysis of *E. coli* carrying these same constructs clearly allowed distinguishing between E7CP protein that either lacks or includes the Str peptide, given that the signal peptide is not processed in the bacteria transformed with the same vector. Minor differences observed in electrophoretic mobility between E7 expressed in plants and bacteria could be explained by dissimilarities in posttranslational processing of proteins in both organisms.

Due to the intrinsic properties of E7, it was very difficult to quantify antigen expression levels by ELISA. Therefore, a comparative analysis of expression levels in the different transplastomic plants was done by western blot (Fig. 5a) and densitometric analysis of the blot (Fig. 5b). For this purpose, dilutions of proteins extracts corresponding to different amounts of leaf material (equivalent to 30–3,000 μg of LM) were used, including the previously characterized E7 and E7CP plants. In that work, unfused E7 accumulation in transplastomic lines was estimated at 0.1 % of total soluble protein [17]. Both GUS fusion proteins showed expression levels between 30 and 40 times higher than the unfused E7 included as standard. Similar results could be observed for StrE7CP. Interestingly, the highest increase of E7 accumulation level was obtained with StrE7 construction: nearly 80 times higher.

Evaluation of Transplastomic Lines Phenotypes

Mature transplastomic lines were phenotypically indistinguishable from wild-type control plants (Fig. 6a) with the exception of the StrE7 plants that presented retarded growth, lighter green color. In these lines, ultrastructure analysis of leaf cells showed abnormal organization of

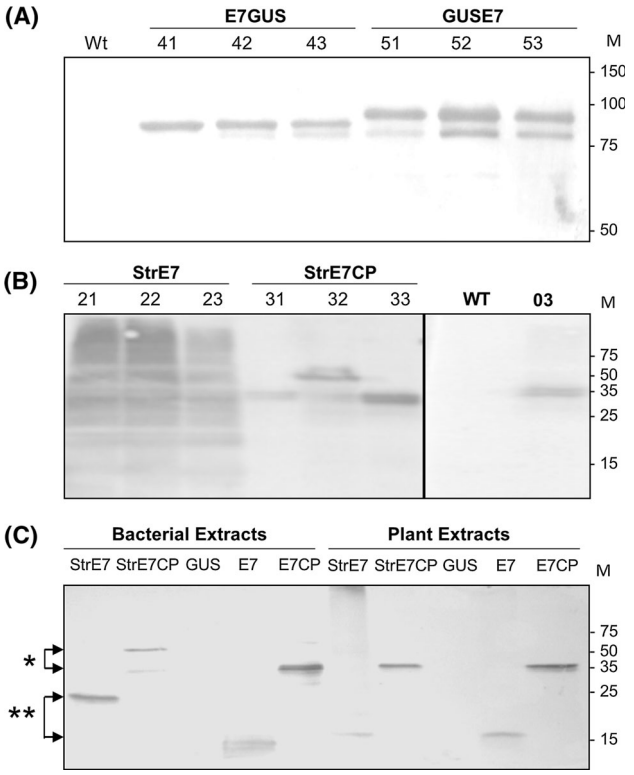

Fig. 4 Expression of recombinant fusion proteins in transplastomic plants. **a** Western blot of leaf extracts containing 10 μg of TSP from the E7GUS (*lines 41–43*) and GUSE7 (*lines 51–53*) and from non-transformed control plants (Wt). **b** Western blot of leaf extracts containing 10 μg of total soluble protein from StrE7 (*lines 21–23*), StrE7CP (*lines 31–33*), from non-transformed plants (WT) as negative control and from E7CP (*line 03*) as positive control. **c** Western blot of bacterial and plant extracts to evaluate processing of Str signal peptide. In order to see a unique band, proteins extracted from *E. coli* (equivalent to 10 μg TSP) and from leaf material (equivalent to 30 μg for StrE7, 75 μg for StrE7CP, and 3,000 μg for E7 and GUS as control) were analyzed. A monoclonal anti-E7 antibody was used in all the assays. Position of pre-stained protein weight marker (M, Invitrogen Corp., Carlsbad, USA) is indicated at the right side of the panel

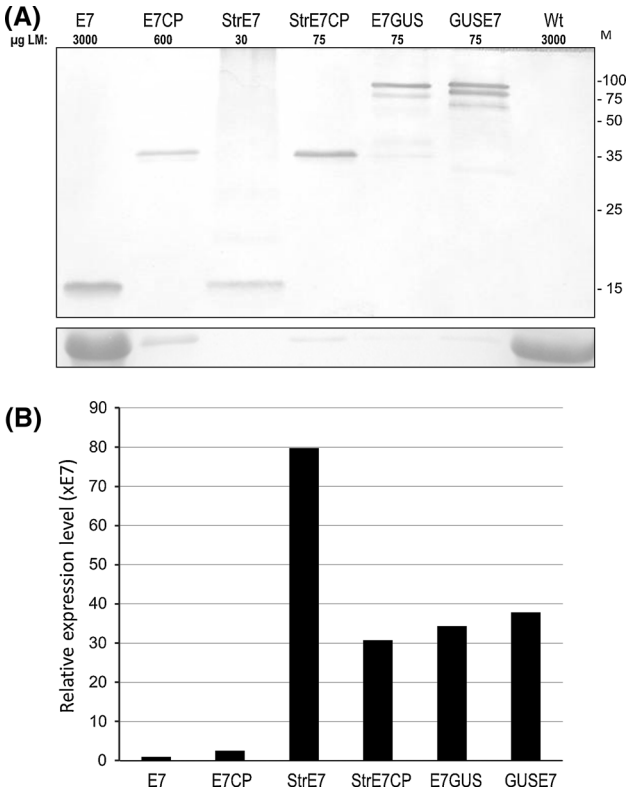

Fig. 5 Relative expression level of recombinant proteins. **a** Different amounts of leaf material (LM) extracts (30–3,000 μg) from six transplastomic lines including E7 and E7CP as references and positive controls were analyzed by western blot using a monoclonal anti-E7 antibody. *Lower panel* shows the band corresponding to the large subunit of RuBisCO in the Ponceau S-stained nitrocellulose membrane to illustrate the relative loading of the samples. **b** Relative expression level calculated after densitometric analysis of the blot shown in **a** normalized to the tissue mass loaded on the gel. *Graph* shows results relative to the E7 line (=1)

plastoglobuli, grana, and lamella of the chloroplasts (Fig. 6b). Despite the StrE7 plants were fertile, the number and viability of seeds from StrE7 lines were notably lower than wild type and the others lines.

Discussion

In spite of the high potential of plants as efficient bioreactors to produce vaccines against HPV [39], the accumulation level of recombinant proteins cannot be predicted and each case represents a unique challenge [3, 4]. This is especially relevant when protein instability constitutes the primary limiting factor [40] as occurs with the HPV E7 antigen that shows properties shared by "natively unfolded" or "intrinsically disordered" proteins [41].

Based on publications in the field and our own experience with expression of E7 in the plastid stroma [17], we decided to explore two alternative strategies to improve antigen accumulation in chloroplasts: translational fusion of E7 to a stable protein (β-glucuronidase) and redirection of the recombinant antigen to the thylakoid lumen.

The translational fusion to another protein that presents important stability has been previously used for nuclear transgenic plants rendering positive results [42–44], as well as for transplastomic plants [20, 45–47]. In this work, we chose the β-glucuronidase, a remarkably stable protein in the chloroplast stroma [48] which proved useful as a carrier to achieve high expression levels in transplastomic tobacco plants [49]. Both N- and C-terminal GUS fusions also proved to be useful to increase E7 accumulation. Although degradation products were also observed, GUS was capable of protecting E7 against proteolysis and improving its stabilization. Expression levels were between 30 and 40 times higher than the unfused E7, which represents 0.1 %

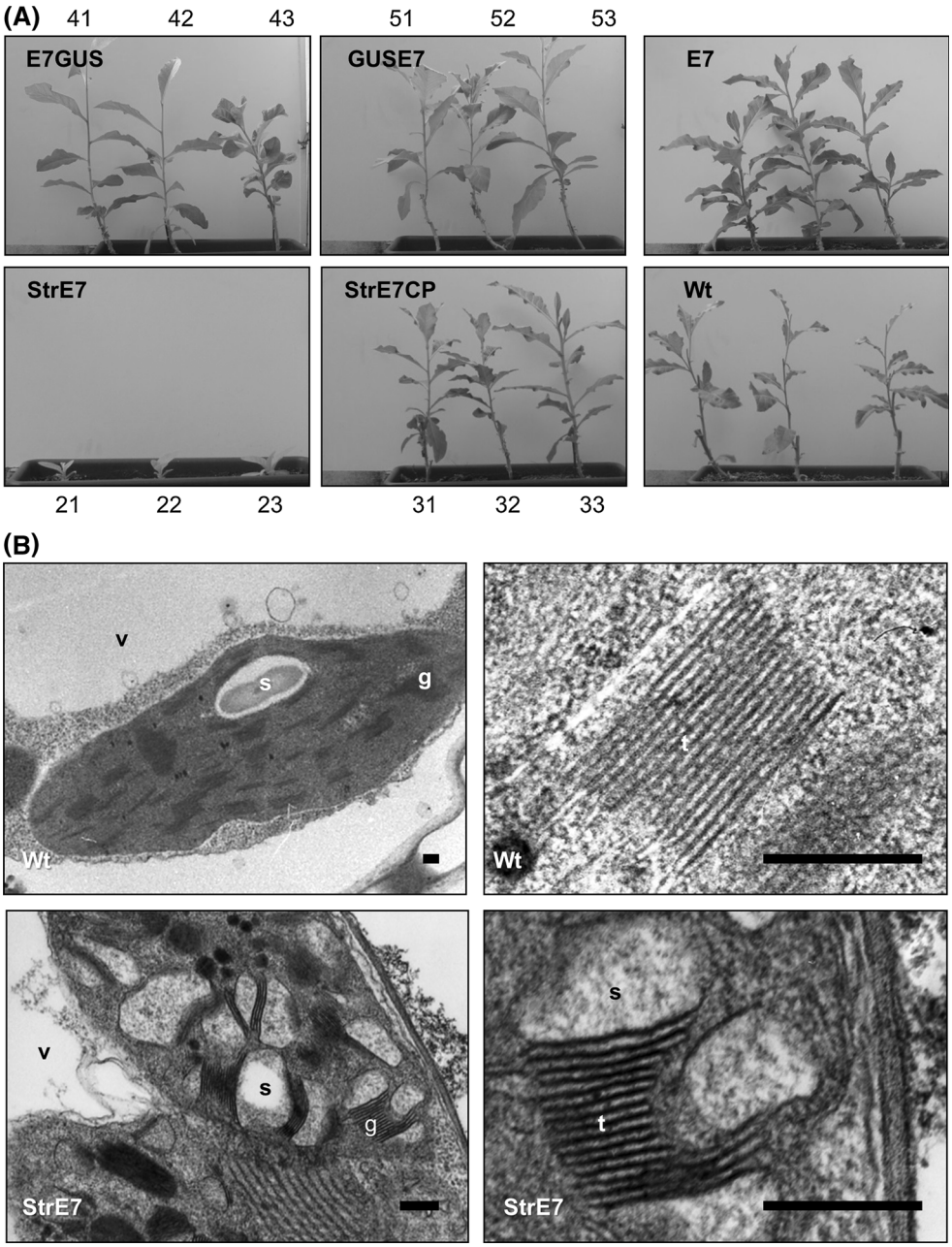

Fig. 6 Phenotypic comparison of transplastomic lines. **a** Phenotype and development. Transplastomic plants grown under greenhouse conditions are phenotypically indistinguishable from wild-type tobacco with the exception of StrE7 *lines* (*21–23*). Pictures were taken 3 months after sowing the seeds. **b** Chloroplast morphology. Representative electron micrographs of chloroplast ultrastructure from wild type (WT) and StrE7 (*line 21*) grown under normal conditions. g, grana; s, starch granule; t, thylakoid; v, vacuole. *Scale bars* in each panel = 200 nm

of total soluble proteins in the leaf cells of transplastomic plants [17]. In spite of this increase in expression of fusion proteins, plants had normal development and phenotype, indicating that tobacco chloroplast can cope with the accumulation of higher amounts of E7.

Interestingly, the accumulation levels of plastid E7GUS and GUSE7 are notably lower than the 41 % of unfused GUS enzyme previously reported [48]. This fact suggests partial stabilization of E7 by GUS, limiting accumulation level below the maximum potential of the system. Complementary studies with other carrier proteins may optimize the fusion strategy.

An alternative approach to improve plastid E7 accumulation was evaluated redirecting E7 from chloroplast stroma into the thylakoid lumen. The bipartite N-terminal peptide from the 23-kDa subunit of the oxygen-evolving complex (Str) is coded in the nuclear genome and is able to target proteins from cytoplasm to the lumen of thylakoids [50]. Str contains a transit peptide that is cleaved after translocation into the stroma, followed by a signal peptide (SP) which is removed during translocation process into the thylakoid by a specific peptidase located in the lumen. The lumen import mechanism targeted by Str occurs via twin-arginine translocation (TAT) pathway that allows transport

of folded proteins. Our results show that both constructs, StrE7 and StrE7CP, were processed in the transplastomic plants but not in bacteria where signal peptidase is absent. Processing of Str releasing mature peptides E7 and E7CP suggest successful translocation from stroma to the thylakoid lumen.

According to the primary hypothesis of this work, antigen accumulation levels were enhanced in two orders of magnitude in StrE7 plants relative to E7 lines and one order of magnitude in StrE7CP plants compared to E7CP lines. These results confirm the potential of lumen localization to enhance accumulation of certain recombinant proteins. They also suggest that E7 aggregation occurs after import into the thylakoids, where it is not as susceptible to degradation as in the stroma. However, the reason for the E7 enhanced accumulation in the thylakoid lumen remains elusive. Its biochemical context and more oxidative environment could favor stable folding of E7. Another possibility is linked to the lower complexity and limited diversity of the protease machinery in this sub-organellar compartment. Nevertheless, alternative explanations, such as the direct action of a putative redox-dependent enzymatic mechanism, cannot be discarded on the basis of the present evidence.

Despite results obtained in the stroma, the presence of CP fused to E7 reduces antigen accumulation in the thylakoid lumen. This could be a consequence of lower translocation efficiency due to not yet characterized properties of the CP sequence. Alternatively, the PVX coat protein could present instability in the biochemical context of the thylakoid.

Translocation of StrE7 was linked to an important decrease in growth rate, a pale-green leaf phenotype, and poor seeding. Morphological alterations of chloroplast structure were observed by electronic microscopy. These unintended pleiotropic effects on plant growth and physiology were shown in some cases for transplastomic plants [51]. However, StrE7CP plants presented a normal phenotype suggesting that Str fusion and Tat translocation is not intrinsically related to phenotypic alteration. Atypical characteristics of StrE7 plants could be caused by cytotoxic effects of E7 accumulation in the lumen. Another explanation for the phenotypic alteration could be related to saturation of the translocation Tat machinery by StrE7 transport. Interestingly, growth arrest and leaf color fading have also been observed in transplastomic tobacco plants that expressed a recombinant protein directed to thylakoid lumen by a heterologous prokaryotic signal peptide [47, 52]. These problems must be solved before considering this approach as an effective way for producing functional therapeutic E7 as unfused antigen. In many cases, this can be attenuated by a properly agronomic management as grafting [53] and/or the use of fertilizers [54]. Also molecular approaches could be explored as complementary strategies including alternative translocation signals and inducible promoters.

To conclude, both alternative strategies explored in this work improved the expression of E7 in transplastomic plants despite some drawbacks described. We believe that the results presented in this work contribute to expand and optimize the expression of recombinant proteins in transplastomic plants. At the same time, the molecular mechanisms regulating foreign protein folding, targeting, and accumulation in these organelles remain highly hypothetical and the specific knowledge is still incomplete. More than one model could be postulated to explain our observations. To further elucidate this issue, each of the possible mechanisms discussed here, must be addressed in future research.

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