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ORIGINAL PAPER

Translocation from the chloroplast stroma into the thylakoid lumen allows expression of recombinant epidermal growth factor in transplastomic tobacco plants

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Abstract Chloroplast transformation has many potential advantages for the production of recombinant proteins in plants. However, it has been reported that chloroplast expression of many proteins, such as human epidermal growth factor (hEGF), results hindered by post-transcriptional mechanisms. hEGF degradation has been related to the redox potential of the stroma and protein misfolding. To solve this problem, we proposed the redirection of hEGF into the thylakoid lumen where the environment could improve disulfide bonds formation stabilizing the functional conformation of the protein. We generated transplastomic tobacco plants targeting hEGF protein to the thylakoid lumen by adding a transit peptide (Str). Following this approach, we could detect thylakoid lumen-targeted hEGF by western blotting while stromal accumulation of hEGF remained undetectable. Southern blot analysis confirmed the integration of the transgene through homologous recombination into the plastome. Northern blot analysis showed similar levels of egf transcripts in the EGF and StrEGF lines. These results suggest that higher stability of the hEGF peptide in the thylakoid lumen is the primary cause of the increased accumulation of the recombinant protein observed in StrEGF lines. They also highlight the necessity of exploring different suborganellar destinations to improve the accumulation levels of a specific recombinant protein in plastids.

Keywords Human epidermal growth factor, EGF · Transplastomic tobacco · Thylakoid · Translocation · Molecular farming

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Introduction

Plants as bioreactors display a huge potential to produce biopharmaceuticals (Giddings et al. 2000; Habibi et al. 2019; van Eerde et al. 2019). Compared to traditional cells bioreactors, molecular pharming offers lower costs, simple scaling up and improved biosafety because of the absence of human or animal pathogens (Obembe et al. 2011; Sparrow and Twyman 2009). However, the accumulation level of recombinant proteins cannot be predicted beforehand and each case represents a unique challenge (De Marchis et al.

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2012; Giorgi et al. 2010). This is especially relevant for transplastomic plants. Chloroplast transformation has many additional advantages to produce therapeutic proteins when compared to other plant-based expression systems (Bock 2014; Maliga and Bock 2011; Scotti et al. 2012).

Integration into a specific site of the plastid genome by homologous recombination avoids positional effects and together with the high ploidy level of the plastid genome and the absence of gene silencing reults in high expression levels of recombinant proteins (De Cosa et al. 2001; Lentz et al. 2010; Oey et al. 2009).

There are several examples of recombinant proteins for therapeutic use that have been produced in transplastomic plants (Burnett and Burnett 2020), including human growth hormone (hGH) (Staub et al. 2000), serum albumin (Fernandez-San Millan et al. 2003), proinsulin (Ruhlman et al. 2007), interferon-a2b (Arlen et al. 2007), exendin-4 (Kwon et al. 2013) and human glutamic acid decarboxylase (Wang et al. 2008).

However, sometimes instability or posttranscriptional mechanisms can prevent the accumulation of heterologous proteins. A clear example of this limitation has been described in our first attempt to accumulate in the plastid stroma the mature form of human epidermal growth factor (hEGF) (Wirth et al. 2006), a 6.2 kDa non-glycosylated polypeptide that has therapeutic value for the treatment of tissue lesions such as epidermis wounds (Brown et al. 1989). We previously showed that, in spite of the presence of the specific mRNAs in all the transplastomic plants analyzed, recombinant hEGF was only detected when stabilized as a fusion to an N-terminal fragment of *Escherichia coli* β-glucuronidase (GUS). Moreover, the accumulation of hEGF in the chloroplasts was higher in the dark than in the light. These results suggested that hEGF accumulation is dependent on correct folding in an environment with proper redox potential (Wirth et al. 2006).

To achieve high levels of expression, it is necessary to explore different compartments. In the case of expression in plastids, proteins have been expressed in the stroma and in the lumen of thylakoids. These compartments have their own repertoire of proteases and chaperones and differences in redox potential that are known to affect the stability and accumulation of the recombinant proteins (De Marchis et al. 2012). In the present work, we explored the redirection of recombinant hEGF into the thylakoid lumen to enhance protein accumulation.

This strategy has been successfully exploited to produce unstable proteins such as phaseolin from bean (De Marchis et al. 2016) or E7 from human papillomavirus (Morgenfeld et al. 2014) and to improve the expression of alkaline phosphatase (Bally et al. 2009). We decided to evaluate the feasibility of accumulating hEGF in the lumen of the thylakoids. To do this, the hEGF protein was targeted to this compartment by the addition of the transit peptide sequence from the 23 kDa protein of the photosystem II oxygen-evolving system (Str), which was previously used to translocate recombinant proteins into the thylakoid lumen (Morgenfeld et al. 2014). Proteins containing the Str transit peptide are translocated in a folded conformation across the thylakoidal membrane by the ΔpH/TAT pathway (Albiniak et al. 2012).

In the present study, we expressed the chimera StrEGF consisting of hEGF fused on its N-terminal region to the Str transit peptide. StrEGF plants resulted in higher accumulation levels of hEGF compared to sromal localization. Unfortuately, the recombinant protein remained in the insoluble fraction hindering further progress.

Our work contributes to optimizing hEGF expression in transplastomic plants by accumulating this recombinant protein in the thylakoid lumen. This strategy will require further optimization before this recombinant protein can be considered for therapeutic treatment. Overall, the results presented in this work reinforce the potential of the plastid system within the field of molecular farming.

Materials and methods

Chloroplast transformation vector construction

Transformation vector pBSW-utr StrEGF was designed from pBSW-utr StrE7 plasmid (Morgenfeld et al. 2014) which was previously digested with *Nde*I and *Xba*I restriction enzymes to remove the *E7* sequence leaving the vector with the *Str* signal. The hEGF sequence was cut from pBSW-utr EGF (Wirth et al. 2006) with the same restriction enzymes and subcloned into the aforementioned digested plasmid to obtain the final transformation vector pBSW-utr

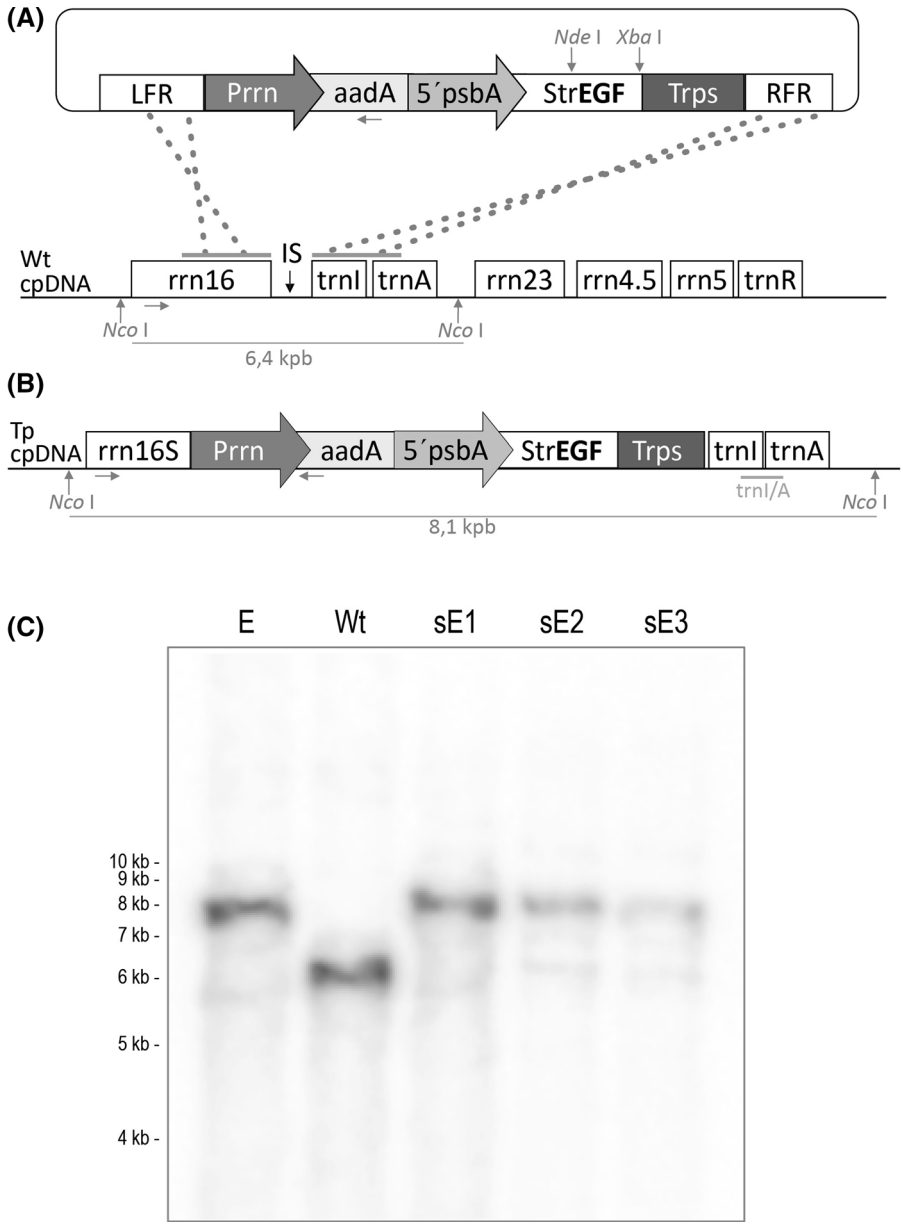

Fig. 1 Chloroplast transformation vector and analysis of transgene integration. **a** Vector pBSW-utr StrEGF contains the Str bipartite signal from *oec23* sequence fused to *hEGF* (StrEGF) under the 5′ untranslated sequence and the promoter of the *psbA* gene (5′psbA). The *aadA* sequence is under the control of the *rrn* promoter (*Prrn*). The left flanking region (LFR) contain the 3′ region of *rrn16*, and the right flanking region (RFR) the full *trnI* and the 5′ region of *trnA*. Vertical arrows indicate *Nde*I and *Xba*I sites utilized for cloning. **b** Chloroplast genome physical maps at the insertion site (IS) of wild type plants (Wt cpDNA) and predicted structure of the transplastomic genome plant (Tp cpDNA). *Nco*I restriction sites and the *trnI/A* probe used in the Southern blot are indicated. Horizontal arrows indicate positions of primers utilized for PCR. Thick lines indicate both regions where recombination is targeted at *rrn16* and *TrnIA* sequences. **c** Southern blot using *trnI/A* probe ^{32}P-labeled to confirm integration and homoplasmy. DNA was digested with *Nco*I. Position of 1 kb dna Ladder plus (Invitrogen) is indicated at the left. E, transplastomic line transformed with unfused Hegf; Wt, wild-type; sE1-3, transplastomic lines transformed with pBSW-utr StrEGF

StrEGF (Fig. 1). This vector carries the *StrhEGF* sequence under the 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*). Flanking regions were included to allow homologous recombination with *N. tabacum* plastome. The left flanking region (LFR) included the 3′ region of *rrn16* and the right flanking region (RFR) contained the full *trnI* and the 5′ region of *trnA* from *N. tabacum*.

Chloroplast transformation and molecular characterization of transgenics plants

Chloroplast transformation was carried out as previously described (Svab et al. 1990), using a PDS 1000/He biolistic device (Bio-Rad, USA). Briefly, leaves of in vitro cultured *N. tabacum* cv. Petit Havana plants were bombarded with 50 mg 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 regeneration medium containing 500 mg/l spectinomycin dihydrochloride. To obtain homoplastic plants, leaves from PCR-positive shoots were taken through two additional regeneration rounds in selective medium. Then, plants were transferred to soil and grown in a greenhouse.

For PCR analysis, DNA obtained from leaf material was used as template for amplification with primers Cl Fw (5′-GTATCTGGGGAATAAGCATCGG-3′) and Cl Rev (5′-CGATGACGCCAACTACCTCTG-3′). Cl Fw hybridizes upstream of the LFR to *16S* wt gene and Cl Rev hybridizes to the *aadA* sequence. Therefore, a 1450 bp fragment was only amplified from transplastomic plants.

All samples for molecular characterization were extracted from the third fully expanded leaves starting from the top of the plant, 5 h after the lighting cycle begun. The age of the plants sampled was 60–90 days post-germination.

Southern blot (Dellaporta et al. 1984)

Total DNA was extracted from leaves as described by Dellaporta et al. (1984). The DNA (2 µg) was digested overnight with *NcoI* enzyme (New England Biolabs, USA), electrophoresed and blotted onto a Hybond Nylon membrane (Amersham Biosciences, USA). Specific sequences were detected by hybridization with α-32P-labeled trnI/A DNA probe. The probe was generated by random priming with a Prime-a-Gene kit (Promega, USA). The blot was pre-hybridized, hybridized, and washed as previously described (Morgenfeld et al. 2014). The blot was exposed to a storage phosphor screen, which was analyzed in a Storm 840 PhosphorImager system (Amersham).

Northern blot

TRiZOL Reagent (Invitrogen Corp., Carlsbad, USA) was used to extract total RNA from leaves. 4 µg of denatured RNA was electrophoresed in a 1.5% agarose/formaldehyde gel and subsequently blotted onto Hybond *N*-Nylon membranes (Amersham Biosciences).

Specific mRNA sequences were detected by hybridization with α-32P-labeled 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.

Protein extraction and analysis

Total protein extracts were obtained from fully expanded leaves by processing 25 mg of leaf tissue in 125 µl of Laemmli sample buffer. For SDS-PAGE, 20 µl of each extract was electrophoresed in 15% gels and transferred onto nitrocellulose membrane for later antibody detection.

The blot was probed with a mouse monoclonal antibody raised against mature EGF of human origin (Santa Cruz Biotechnology, Inc.). As a secondary antibody, we use goat IgG anti-mouse IgG linked to alkaline phosphatase (Cell Signalling Tec.) and diluted to 1:3000. Phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical Co., USA) as substrates. For the solubility analysis, 0.2 g of leaf tissue were ground in liquid nitrogen, mixed with 1 mL of 50 mM Tris–HCl pH 8, 30 mM NaCl, centrifuged for 15 min at 10,000×*g*, 4 °C, to obtain an insoluble fraction (I) and a soluble one (S).

For E coli total protein extracts 1 ml culture was pelleted into microcentrifuge tubes and the supernatant was discarded. The pellet was resuspended in 200 µl of Laemmli buffer.

Results

Generation of transplastomic *N. tabacum* plants expressing StrEGF

The chloroplast transformation vector pBSW-utr-StrEGF (Fig. 1a) was delivered by particle bombardment to *Nicotiana tabacum* foliar explants. The vector includes homology arms that allowed the insertion by recombination of StrEGF to the intergenic space between *rrn16S* and *trnI loci* (Fig. 1b). From 10 bombardments, we recovered 12 Independent spectinomycin-resistant shoots. 9 of them were analyzed by western blot and hEGF expression was confirmed in only 6 lines. Three out of these were further characterized by PCR using primers that anneal to the 16S plastidic gene and to the *aadA* gene. Therefore, a 1450 bp fragment was amplified only in transplastomic plants (data not shown). These plants were subjected to two additional regeneration rounds on selective media. Finally, they were transferred to soil and grown under greenhouse conditions. These plants were employed for subsequent analysis.

Analysis of transgene integration in transformed plants

Transgene integration into the plastome and homoplasmic state were assessed by Southern blot. Total DNA, extracted from leaves from three different spectinomycin-resistant lines previously characterized by PCR, was employed for restriction enzyme digestion with *NcoI*. This enzyme, which is absent in the transgenic cassette, recognize sequence both upstream and downstream of the integration site. After digestion wild-type and transplastomic plastomes are expected to generate a 6.4 kbp and 8.1 kbp DNA fragment, respectively. A trnI/A probe was employed for Southern blot analysis, confirming the transgenic state for all three independent StrEGF lines (Fig. 1c).

A residual band of 6.4 kbp, corresponding to the wild-type plastome could still be observed in all transplastomic lines. Considering that the transplastomic plants were subjected to three consecutive rounds of regeneration under antibiotic selection, followed by germination in spectinomycin-containing media (Fig. 2b), it would be highly unlikely that these plants exhibited a heteroplasmic state. The observed additional hybridization signal could be explained by additional copies of plastid DNA in the nuclear or mitochondrial genome in the plant cells (Ruf et al. 2000). It has been proven that large copies of plastid DNA integrated into the other genomes during evolution (Ayliffe et al. 1998; Nakazono and Hirai 1993; Stern and Lonsdale 1982).

Phenotypic comparison between transplastomic StrEGF plants,a transplastomic EGF line and wild-type counterparts revealed stunted growth in the three StrEGF lines (Fig. 2a). Despite this observation, all transplastomic lines exhibited normal plant architecture. The fact that all seeds were able to germinate in spectinomycin containing media (Fig. 2b) confirmed the inheritance of the selection marker and absence of untransformed plastomes suggesting plants homoplasmy.

Analysis of transcript generation

Total leaf RNA extraction followed by northern blot assay was employed to evaluate the presence of expected RNAs containing the StrEFG sequence for all transplastomic lines. An EGF probe was employed to detect the three main transcription products: monocistronic transcripts transcribed from the *psbA* promoter (0.6 kbp 1), bicistronic transcripts generated from the rrn promoter (*Prrn*) (1.6 kbp 2) and a polycistronic transcript generated by read-through from the endogenous *rrn* operon promoter (3.3 kbp 3) (Fig. 3a).

Total leaf RNA extracted from a stromal localized EGF transplastomic line was employed as a molecular weight reference. As expected, all the different types of StrEGF containing transcripts showed retarded electrophoretic mobility when compared to the EGF sample, due to the additional Str sequence. In order to further confirm the identity of the transcripts, the hEGF probe was removed and the membrane hybridized with an *aadA* probe. For this particular probe, only polycistronic and bicistronic transcript detection was expected. As observed, all the transcripts exhibited the expected molecular weight sizes (Fig. 3b). The third northern blot done on the same membrane, was revealed with a probe corresponding to the TrnI/A sequence. In this blot it is possible to verify the equal loading of all samples including the wild type, the specificity of the hybridization in the previous assays and the complexity of the different transcripts considering precursors, intermediates and

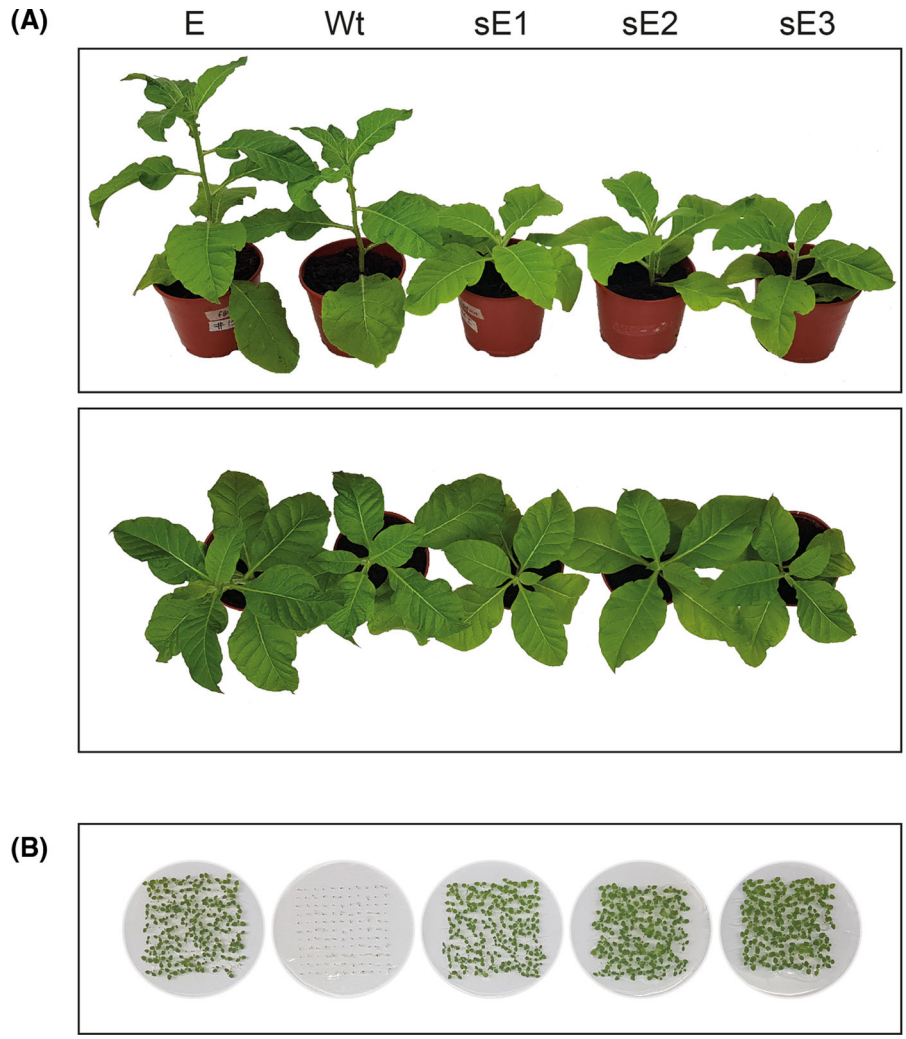

Fig. 2 Phenotypic characterization of transplastomic plants. **a** *Nicotiana tabacum* (L. cv. Petit Havana) lines were grown under greenhouse conditions and phenotypes were registered 16 weeks post sowing (side and top views). **b** 100 seeds of each line were germinated in MS medium containing spectinomycin (0.5 g/l). The pictures were taken 7 days after sowing the seeds. E, transplastomic line for unfused EGF; Wt, wild-type; sE1-3, transplastomic strEGF lines

matured molecules. This complexity is enhanced not only by alternative sites of transcription initiation and termination but also by RNA specific process as maturation and splicing (Stern et al. 2010).

Analysis of recombinant StrEGF expression

The expression of StrEGF in transplastomic leaves was assessed by western blot employing a monoclonal antibody raised against EGF (Fig. 4). Total protein extracts obtained from leaves of transplastomic StrEGF lines (sE) exhibited specific bands that were absent in both EGF (E) and wild-type (Wt) samples included as controls. The molecular weight observed for these specific bands ($\sim$ 12 kDa) is compatible with hEGF being in a dimer conformation. Additional bands with higher molecular weight could probably represent multimeric aggregated states of EGF that resisted denaturing SDS-PAGE conditions (Fig. 4). Protein extracted from *E. coli* previously transformed with pBSW-utrStrEGF vector (sE) showed a band of $\sim$ 14 kDa consistent with an unprocessed StrEGF monomer still containing the Str signal peptide ($\sim$ 8 kDa) fused to the EGF peptide ($\sim$ 6 kDa).

This observation could be easily explained by the lack of a bacteria-specific thylakoid-specific protease.

According to western blot analysis the vast majority of recombinant StrEGF was found in insoluble extracts (I) and could not be detected in soluble extracts (S) (Fig. 4).

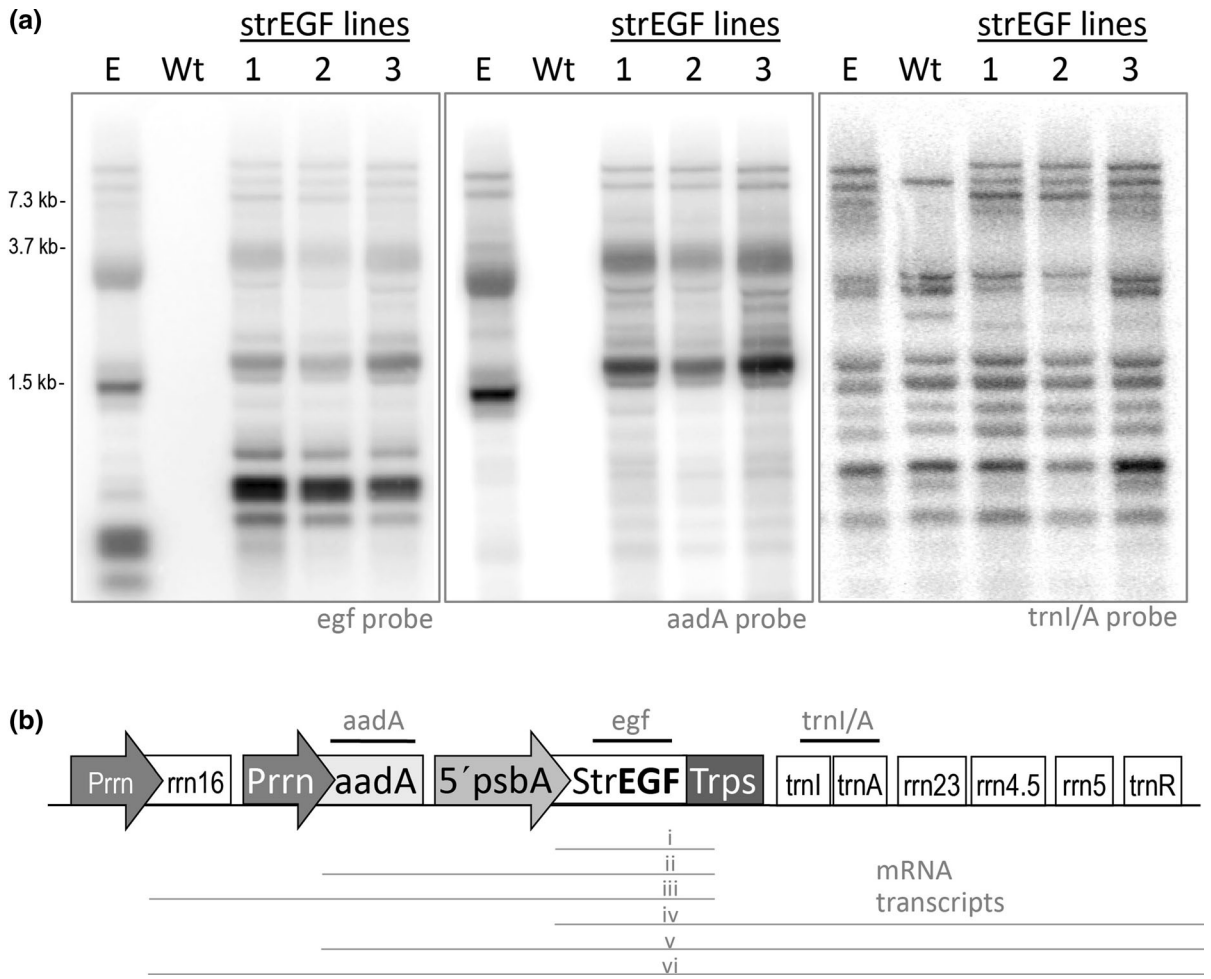

Fig. 3 Characterization of transcripts containing the *aadA* and *hEGF* sequence of transplastomic plants. **a** northern blots using *egf* (left panel), *aadA* (central panel) or *trnI/A* (right panel) ^{32}P-labeled probes. **b** Physical map showing expected transcript sizes related to the RNA operon of the plastoma. (i) Monocistronic transcript (0.6 kb), (ii) bicistronic transcript (1.6 kb). (iii) Polycistronic (3.3 kb), (iv–vi) large RNA precursors without RNA processing (7.2 kb, 8.2 kb and 9.9 kb) transcripted from the same three promoters with the inclusion of six other elements of the RNA operon (*trnI, trnA, rrn23, rrn4.5, rrn5* and *trnR*). E, transplastomic line for unfused EGF; Wt, wild-type; 1-3, transplastomic strEGF lines

Discussion

Transplastomic plants have a great potential for the production of new molecules. However, the accumulation level of recombinant proteins in plastids cannot be predicted beforehand and every new protein is a novel challenge (Bock 2014; Maliga and Bock 2011; Scotti et al. 2012). This is especially relevant when protein degradation by the housekeeping machinery is triggered by misfolding of the polypeptide. An example of this is the recombinant hEGF expressed in the chloroplast stroma. Previous publications (Schlapschy and Skerra 2011; Tong et al. 2001) and our own experience (Wirth et al. 2006) suggest that the acquisition of correct hEGF folding depends on proper disulfide bond formation. In our previous attempts using nuclear transgenic plants, the accumulation of hEGF proved to be unsuccessfull in the reducing cytosolic environment. However, it could be detected in the apoplastic space after its transit through the endoplasmic reticulum which provided a more oxidative environment to catalyze disulfide bond formation. Additionally, when hEGF was expressed in the chloroplast stroma a slight accumulation could only be detected in the dark when the environment became more oxidizing and remained undetectable in the light.

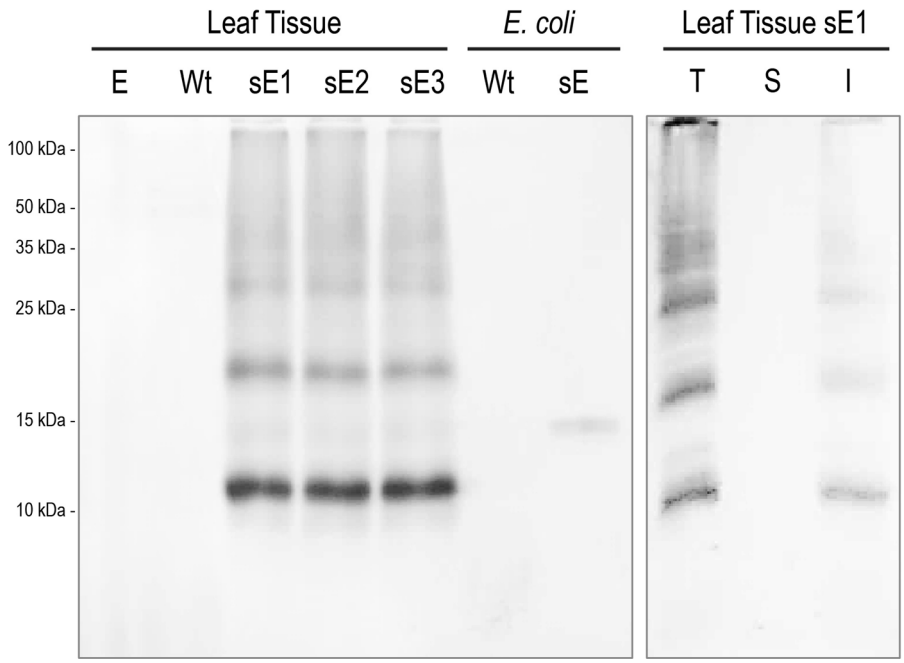

Fig. 4 Expression of hEGF in transplastomic plants. Expression analysis (right panel). Lanes were loaded with total protein extracts obtained from 4 mg of fresh leaf tissue. E, transplastomic line for unfused EGF; Wt, wild-type; sE1-3, transplastomic lines for strEGF. *E. coli* extracts obtained from wild type or transformed bacterial expressing StrEGF (sE) were used as positive controls. Solubility analysis (left panel): Lanes were loaded with total (T), soluble (S) and insoluble (I) protein fractions obtained from 4 mg of fresh leaves from the transplastomic sE1 line

A plausible explanation for this observation is that disulfide bond formation could be hindered in the reducing environment of the plastid stroma. If this was the case, abnormal folding could subsequently target this protein for degradation by chloroplast proteolytic systems.

In bacteria, hEGF does not accumulate in the reducing cytosol and it must be secreted to the periplasm or extracellular space by the addition of a signal peptide (Abdull Razis et al. 2008). The oxidizing periplasm is more suitable for the generation of disulfide bonds, counting with specialized chaperone machinery consisting of the DbsA oxidase and the DsbC isomerase. Overexpression of DsbA in the periplasm has already proven beneficial for the accumulation of unstable recombinant proteins with few disulfide bonds (Gundinger and Spadiut 2017; Kondo et al. 2000; Maskos et al. 2003).

Given the chloroplast evolutionary origin, the stroma and thylakoid lumen are equivalent to the bacterial cytosolic and periplasmic space, respectively. Based on the above observations we hypothesized that redirection of recombinant EGF to the more oxidative environment of thylakoid lumen could facilitate disulfide bond formation allowing for correct folding of the protein. By means of this approach, we tried to prevent protein degradation in order to increase hEGF accumulation in transplastomic plants.

For this purpose, we used the amino-terminal signal peptide from the plastid 23 kDa protein of the oxygen-evolving complex (Str) (Marques et al. 2004). Physiologically, this signal peptide is cleaved off the protein after translocation into the thylakoid lumen. By means of a western blot assay, we observed that StrEGF was processed in the transplastomic plants but not in bacteria, where the signal peptidase is absent. The processing of Str, releasing the peptide of mature hEGF, allows us to conclude that the translocation of hEGF towards thylakoids lumenal space was successful.

Moreover, we observed that directing hEGF to the thylakoid lumen notably improves accumulation levels when compared to the almost undetectable levels observed for stromal hEGF.

These results confirm the potential of lumenal localization to enhance the accumulation of certain

recombinant proteins. They also suggest that hEGF aggregation occurred after import into the thylakoids, where it was not as susceptible to degradation as in the stroma. Because hEGF remains at the insoluble fraction, quantification of the recombinant protein accumulation by ELISA was not possible. Why hEGF accumulates better in the thylakoid lumen compared to the stroma has not been clarified yet. Our hypothesis is that the most oxidizing environment of the lumen favors a better folding of the protein in that compartment. Further studies are required in order to test it.

Recently, a novel plant enzyme localized in the thylakoid membrane was discovered. This protein, called lumen thiol oxidoreductase (LTO1 or AtVKOR-DsbA), exhibits a lumenal domain analogous to the one of bacterial periplasmic DsbA protein which is also capable of catalyzing disulfide bond formation (Du et al. 2015). This domain could be the one responsible for rapidly oxidizing cysteines, favoring incorrect disulfide bonds formation and leading to aggregation of hEGF leaving it insoluble. It was previously shown that accumulation of hEGF in the bacterial periplasm can be improved by overexpression of DsbA, DsbC and two peptidyl-prolyl isomerases with chaperone activity (Schlapschy and Skerra 2011). Moreover, DsbA secreted from Brevibacillus choshinensis was able to promote disulfide bonds formation in vitro, leading to active recombinant hEGF (Tanaka et al. 2001). Another explanation could be the fact that in the lumen of the thylakoids protease diversity is less when compare to other compartments.

Although in most transplastomic plants unintended phenotypes were not observed, sometimes chlorosis, growth retardation, and sterility were reported (Ahmad et al. 2012; Corigliano et al. 2019; Lossl et al. 2003; Rigano et al. 2009; Scotti et al. 2009; Lentz et al. 2012; Scotti and Cardi 2014).

Previously we showed that accumulation of hEGF in the plastid stroma can occurwithout associated penalties (Wirth et al. 2006). However, when the same protein was redirected to the thylakoid lumen, we observed a negative impact on plant growth. Conversely, when we showed that accumulation ofE7CP in the thylakoid lumen, using the same Str signal peptide (StrE7CP) transplastomic plants presented a normal phenotype, suggesting that neither Str signal nor the saturation of the translocation machinery are responsible for the observed phenotype (Morgenfeld et al. 2014). These problems must be addressed before considering Str fusion approach as an effective way of producing functional therapeutic hEGF. In many cases, this can be attenuated by properly agronomic management such as grafting (Oey et al. 2009) and/or the use of fertilizers (Bally et al. 2009). Moreover, molecular approaches could be explored as complementary strategies, including alternative translocation signals and inducible promoters (Bock 2014).

We can conclude that, the strategy explored in this work improved the expression of hEGF in transplastomic plants despite minor alterations observed on transplastomic plants phenotype. Unfortunately, the protein remained insoluble, a limitation that needs to be overcome in order to develop an effective platform to produce recombinant hEGF.

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Author contribution statement FBA and MM conceived and designed research. FBA directed the project. MM, NB, CV, and EFA conducted experiments. FBA and MM analyzed data. FBA and MM wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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