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Tobacco plastidial thioredoxins as modulators of recombinant protein production in transgenic chloroplasts

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Summary

Thioredoxins (Trxs) are small ubiquitous disulphide proteins widely known to enhance expression and solubility of recombinant proteins in microbial expression systems. Given the common evolutionary heritage of chloroplasts and bacteria, we attempted to analyse whether plastid Trxs could also act as modulators of recombinant protein expression in transgenic chloroplasts. For that purpose, two tobacco Trxs (m and f) with different phylogenetic origins were assessed. Using plastid transformation, we assayed two strategies: the fusion and the co-expression of Trxs with human serum albumin (HSA), which was previously observed to form large protein bodies in tobacco chloroplasts. Our results indicate that both Trxs behave similarly as regards HSA accumulation, although they act differently when fused or co-expressed with HSA. Trxs–HSA fusions markedly increased the final yield of HSA (up to 26% of total protein) when compared with control lines that only expressed HSA; this increase was mainly caused by higher HSA stability of the fused proteins. However, the fusion strategy failed to prevent the formation of protein bodies within chloroplasts. On the other hand, the co-expression constructs gave rise to an absence of large protein bodies although no more soluble HSA was accumulated. In these plants, electron micrographs showed HSA and Trxs co-localization in small protein bodies with fibrillar texture, suggesting a possible influence of Trxs on HSA solubilization. Moreover, the *in vitro* chaperone activity of Trx m and f was demonstrated, which supports the hypothesis of a direct relationship between Trx presence and HSA aggregates solubilization in plants co-expressing both proteins.

Introduction

Thioredoxins (Trxs) are small, heat-stable proteins (*c.* 12–14 kDa), found in all free-living organisms, that catalyse redox reactions, promote disulphide bond formation and synergistically work with protein disulphide isomerases and/or chaperones to control a wide range of biochemical pathways (Buchanan and Balmer, 2005; Berndt *et al.*, 2008). Because of their solubility, stability, small size, globular compact structure and high translatability, Trxs have been broadly used in biotechnology. In particular, the *Escherichia coli* TrxA has been employed in microbial systems as a solubility and stability enhancer of recombinant proteins that cannot normally form disulphide bridges (LaVallie *et al.*, 2000; Kolaj *et al.*, 2009). A number of examples of Trx co-expression exist in the literature; this system produces the recombinant protein in a free form and does not require a subsequent processing with specific peptidases. TrxA co-production has been demonstrated to markedly enhance the amount of active proteins (Bessette *et al.*, 1999; Garcia-Ortega *et al.*, 2000; Yuan *et al.*, 2004) and dramatically increase the solubility of proteins that otherwise produce inclusion bodies (IBs) (Yasukawa *et al.*, 1995). TrxA has also been successfully used as a fusion partner to avoid IB formation and increase the level of soluble proteins heterologously expressed in *E. coli* (LaVallie *et al.*, 1993, 2000, 2003; LaVallie and McCoy, 1995; Ribas *et al.*, 2000; Jurado *et al.*, 2006; Xu *et al.*, 2006, 2007; Bogomolovas *et al.*, 2009). Other studies have compared the Trxs behaviour in fusion or co-expression assays and concluded that they have different effects on recombinant protein solubility (Yuan *et al.*, 2004). Although several hypothesis have tried to explain the role of Trxs with recombinant proteins (LaVallie *et al.*, 2000, 2003; Kumar *et al.*, 2004), to date, the mechanisms of how Trx achieves enhanced expression and/or chaperone activity are poorly understood (Bogomolovas *et al.*, 2009).

Because of the endosymbiotic origin of plastids as well as the prokaryotic-like structure and metabolism (McFadden, 2001), such Trx properties in microbial organisms might also be useful for the chloroplast expression system. Chloroplasts are currently known to contain five typical Trx isoforms: f, m, x, y and z. The m- and f-types act as messengers in the ferredoxin/Trx system by transmitting the redox signal to target enzymes. Trx f is mainly implicated in the redox regulation of photosynthetic carbon assimilation, whereas m-type Trxs might also play additional roles (Issakidis-Bourguet *et al.*, 2001; Meyer *et al.*, 2005). The x- and y-types do not appear to primarily be involved in enzyme regulation, but are rather implicated in stress response (reviewed in Schurmann and Buchanan, 2008). The recently reported Trx z seems to have an important role in the regulation of plastid-encoded polymerase-dependent transcription in

chloroplasts (Arsova *et al.*, 2010). Although all plastidial Trxs are codified in the nucleus and their overall structure is similar, phylogenetic studies and structural comparisons have demonstrated that Trx m, x, y and z have a prokaryotic origin (Meyer *et al.*, 2005; Arsova *et al.*, 2010). Trx f, however, displays homology with Trxs of eukaryotic origin (Sahrawy *et al.*, 1996). To study a putative biotechnological application of Trxs in plastids, it would be useful to assay Trxs of both prokaryotic and eukaryotic origin. In this sense, Trx m and f might be good candidates because they are reported to have the widest range of functions and target proteins (Lemaire *et al.*, 2007).

A potentially ideal way to study plastid Trxs m and f as modulators of recombinant protein expression in plants is through plastid transformation technology. As mentioned previously, plastids are prokaryotic-like compartments where a number of homologies with microbial systems have been found. In addition, similar to bacterial fermenter technology, chloroplast transformation allows very high levels of recombinant protein expression. Average accumulation levels are in the range of 4%–20% of total soluble protein (Koop *et al.*, 2007), with maximum values of 70% in the case of lysin expression (Oey *et al.*, 2009). Studies in this field indicate that transgenic plants can produce recombinant proteins 10–100 times cheaper than cell culture systems (Mison and Curling, 2000). Moreover, to our knowledge, no information has yet been reported about how to enhance solubility and proper folding of heterologous proteins expressed in chloroplasts. Even though it has been demonstrated that chloroplasts are able to correctly fold proteins with disulphide bonds (Staub *et al.*, 2000; Daniell *et al.*, 2001; Molina *et al.*, 2004; Ruhlman *et al.*, 2007; Farran *et al.*, 2008), recombinant proteins are not always biologically active nor accumulate in the soluble fraction (Fernandez-San Millan *et al.*, 2003, 2008; Ruhlman *et al.*, 2007, 2010; Daniell *et al.*, 2009). The most useful alternative to increase the level of expression in the chloroplast transformation system is the fusion of the amino-terminus of the coding region with some downstream sequences or even entire proteins, such as GFP or GUS (Ye *et al.*, 2001; Leelavathi and Reddy, 2003; Molina *et al.*, 2004; Herz *et al.*, 2005; Farran *et al.*, 2010; Lee *et al.*, 2010; Lentz *et al.*, 2010). However, it may be necessary to eliminate such fusion partners by *in vitro* protease digestions, which would increase the final cost of production. In these cases, a co-expression system with a protein that enhances and maximizes the active soluble fraction of recombinant proteins accumulated in chloroplasts would be ideal.

We previously showed the plastidial expression of human serum albumin (HSA) (Fernandez-San Millan *et al.*, 2003). Although it was expressed at relatively high levels in tobacco leaves, HSA accumulated as protein IBs within chloroplasts. The formation of IBs might be thought of as 'inappropriate' protein–protein interactions because of the lack of proper polypeptide folding (Mitraki and King, 1989). HSA has a complex globular structure with 17 disulphide bonds, and mismatching of cysteines might cause the insolubility of chloroplast-produced HSA. To obtain HSA with commercial characteristics, it would be necessary to include denaturation and renaturation steps in the production process, which would markedly increase the final cost. In addition, it was observed that, although HSA was present mainly as IBs, its half-life was relatively low, approximately 4 h (Fernandez-San Millan *et al.*, 2007). The lack of stability and solubility of HSA encouraged us to use it as a model protein to investigate whether we could improve its production using a Trx N-terminal protein fusion or co-expression strategy.

In this work, we have explored the possible use of plant Trxs as enhancer elements of recombinant protein accumulation. To date, it has not been demonstrated that Trxs can be used for that purpose in the chloroplast transgenic expression system. Therefore, we have studied the effect of fusion and co-expression of Trx m and f from tobacco plastids as modulators of HSA production.

Results

Design of plasmids for HSA fusion or co-expression with tobacco thioredoxins m and f

Tobacco *Trx m* and *f* genes were selected for the study of improvement in HSA expression within chloroplasts because of their plastidial localization, wide range of functions and different phylogenetic origin. Whereas *Trx f* is of eukaryotic origin, *Trx m* displays homologies with bacterial *Trx A*, which is the preferred partner to increase solubility and expression of recombinant proteins in microbial systems (Hammarstrom *et al.*, 2002; Bogomolovas *et al.*, 2009; Kolaj *et al.*, 2009). To analyse whether plastidial Trxs could modulate the expression of recombinant proteins in chloroplasts, both the fusion and the co-expression of Trx m and f with HSA were assessed. For the fusion constructs, *Trx* sequences corresponding to the mature peptides were translationally fused to the *HSA* sequence (Figure 1a). This fusion included the flexible hinge tetrapeptide GPGP in the middle of both sequences. This tetrapeptide may reduce steric hindrance between Trx and HSA and thus facilitate HSA assembly, as has been previously observed (Molina *et al.*, 2004). The fusions were expressed from the tobacco *psbA* promoter and untranslated region (UTR) and introduced into the chloroplast transformation pLD vector. The pLD vector was previously used for the expression of HSA (hereafter referred to as free-HSA), which was demonstrated to form IBs within chloroplasts in a previous work (Fernandez-San Millan *et al.*, 2003). The pLD vector integrates genes of interest into the plastid genome between the *trnI* and *trnA* sequences (Figure 1a). This vector also includes the aminoglycoside 3′adenylyltransferase (*aadA*) gene from *E. coli*, which confers resistance to both spectinomycin and streptomycin and is driven by the constitutive promoter of the rRNA operon (P*rrn*), and a multiple cloning site between the end of the *aadA* coding sequence and the *psbA* terminator.

For co-expression vectors, *HSA* and *Trxs* were expressed in a free form using different promoters to avoid possible deletions via homologous recombination (Iamtham and Day, 2000) (Figure 1a). The design of co-expression vectors was chosen to minimize changes of the original pLD-psbAHSA (Fernandez-San Millan *et al.*, 2003), where free-HSA accumulates to a level of 7% of total protein (TP) under regular light conditions. *Trx* genes were expressed from the constitutive tobacco *rrn* promoter followed by the T7 phage *gene 10* leader sequence (P*rrnG10*L), which is one of the strongest known expression signals in plastids (Kuroda and Maliga, 2001a). The P*rrnG10*L*Trxm/f* cassette was introduced into the pLD-psbAHSA vector downstream of the *HSA* gene, which was driven by the tobacco *psbA* promoter and 5′-UTR.

The final vectors were sequenced, and Western blot analyses of sonicated *E. coli* extracts showed the expression of both HSA

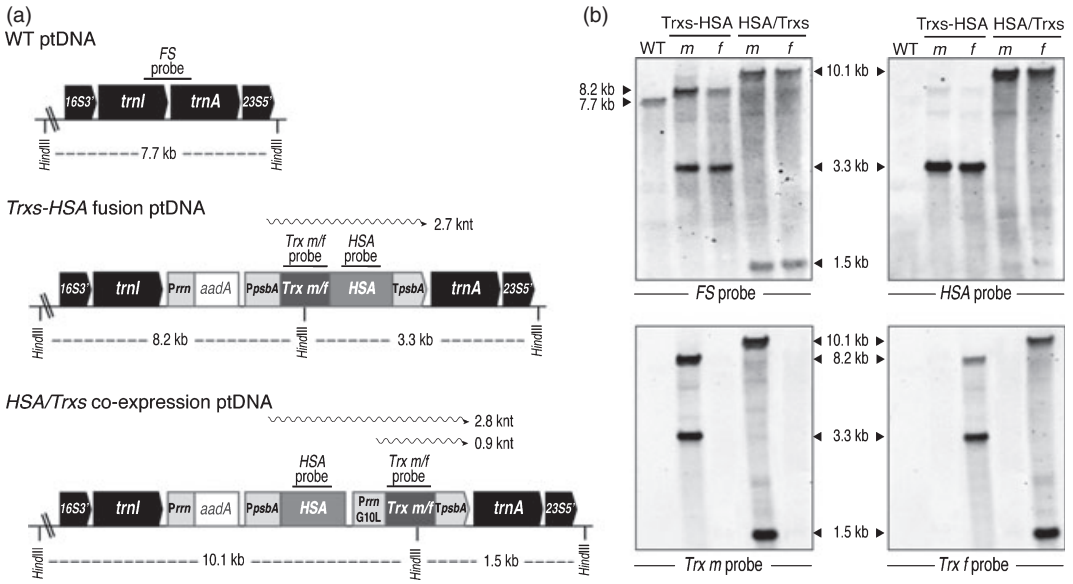

Figure 1 Integration of *Trx m/f* and *HSA* genes by fusion or co-expression into the plastid genome and homoplasmy verification. (a) Map of the WT, *Trxs–HSA* fusion and *HSA/Trxs* co-expression transformed plastid genomes. The transgenes are targeted to the intergenic region between *trnI* and *trnA*. The selectable marker gene *aadA* is driven by the ribosomal RNA operon promoter (P*rrn*). The *Trxs–HSA* fusion is driven by the *psbA* promoter and untranslated region (UTR). In co-expression, *HSA* is also driven by the *psbA* leader region followed by the P*rrnG10*L and the *Trx m or f* sequences. Probes for the Southern blot are shown over the corresponding sequences. Mono- and dicistronic transcripts are denoted by zigzag arrows, and the expected sizes for transcripts are indicated. (b) The Southern blot analysis for the different transgenic lines is shown. The same blot was probed with *FS* probe to study the homoplasmic state and with *HSA*-, *Trx m*- and *Trx f*-specific probes. One line per construct and a WT control are shown. *16S*, *23S*, *trnI*, *trnA*: original sequences of the chloroplast genome; *aadA*: aminoglycoside 3′-adenylyltransferase; P*rrn*: 16S rRNA promoter; P*psbA*: *psbA* promoter and 5′-UTR; P*rrnG10*L: 16S rRNA promoter and the *gene 10* leader from phage T7; *3′psbA*: terminator region of the *psbA* gene; WT: wild-type; Trxs–HSA: fusion plants; HSA/Trxs: co-expression plants. Trx, thioredoxin; HSA, human serum albumin.

and Trxs, demonstrating that all four chloroplast transformation vectors are functional (data not shown).

Tobacco chloroplast transformation and homoplasmic plants selection

Chloroplast transformation by particle bombardment (Daniell, 1997) of tobacco leaves followed by selection through spectinomycin-containing plant regeneration medium produced several fusion and co-expression transformed lines (hereafter referred to as Trx–HSA and HSA/Trx, respectively), as was confirmed by PCR analysis (data not shown). Southern blot analysis was performed to verify site-specific integration and to confirm homoplasmy. Total plant DNA was digested with *Hin*dIII. The flanking sequence probe (*FS* probe) identified a 7.7-kb fragment in the wild-type plant, 8.2- and 3.3-kb fragments in plants expressing the Trxs–HSA fusions and 10.1- plus 1.5-kb fragments in plants co-expressing Trxs and HSA (Figure 1a,b). The absence of 7.7-kb bands in the transformed lines indicated homoplasmy. Absence of residual wild-type copies of the highly polyploidy plastid genome was further confirmed by maternal inheritance of the antibiotic resistance and lack of phenotypic segregation in inheritance assays (data not shown). To confirm that transgenic plants contained the *Trx m/f* and *HSA* sequences, the same membranes were stripped and probed again with the gene-specific probes (Figure 1a). Hybridization was observed in all transgenic lines and was absent in the wild-type control plant (Figure 1b). Under our growth conditions, all transplastomic lines displayed a standard phenotype with the exception of HSA/Trx m co-expressing plants, which exhibited a pale-green coloration but were otherwise normal in growth and development.

Thioredoxin m or f fusions increase HSA accumulation in transgenic chloroplasts

Greenhouse plants from the T_1 generation of transgenic lines and free-HSA expressing plants used as control were analysed for HSA and Trx m/f accumulation in chloroplasts. TP from mature leaves was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and by Western blot with an anti-HSA antibody (Figure 2a). Monomers of HSA protein were detected at 66 kDa in free-HSA and HSA/Trxs co-expressing lines. Likewise, the monomers corresponding to the Trxs–HSA fusion proteins (80 kDa) were also detected. As was observed in free-HSA expressing plants, there were significant amounts of large and small immunoreactive bands in Trxs–HSA fusion-expressing plants. This finding suggests not only the formation of aggregates inside transgenic chloroplasts but also a certain degree of recombinant protein turnover. However, plants co-expressing HSA and either Trx m or Trx f showed mainly the monomeric HSA (Figure 2a). There was neither significant aggregation nor degradation bands, although fivefold more TP was loaded. These results indicate that Trx-fused HSA plants accumulated much higher levels of recombinant protein than did co-expressed HSA plants. To further assess this observation, we analysed electrophoretically separated TP samples by Coomassie staining (Figure 2b). HSA was barely detectable in Trx co-expression lines; however, a clear band was visible in both Trxs–HSA fusion lines, which was even

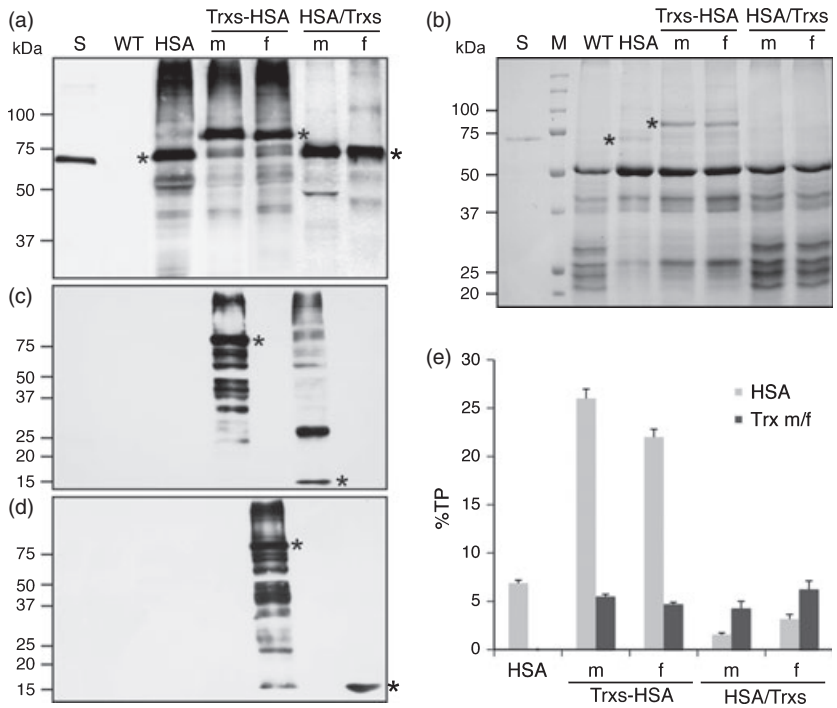

Figure 2 Characterization of HSA and Trx m/f accumulation in fusion or co-expression plants. (a) Immunoblot analysis of protein extracts from mature leaves (fifth from top) of the different transgenic or WT lines with anti-HSA antibody. A total of 50 ng of pure HSA were used as standard. Protein extracts of 45-day-old transgenic and WT plants were loaded onto an 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. For free-HSA control and Trxs–HSA fused lines, proteins extracted from 0.5 and 0.2 mg of leaves were analysed, respectively. For WT and HSA/Trxs co-expression plants, proteins extracted from 5 mg of leaves were loaded. (b) Twenty micrograms of total protein (TP) extracted from WT and transplastomic lines were loaded onto an 8% SDS–PAGE gel and stained with Coomassie Brilliant Blue. (c,d) The same protein extracts as for anti-HSA Western blot were analysed with anti-Trx m and f antibodies respectively in a 13% SDS–PAGE gel. (e) HSA and Trx m/f quantification in mature leaves from transgenic tobacco plants by Western blot analysis. Results are the mean ± SE of two measurements for two independent transgenic lines per construct and are shown as percentage of TP. S: standard; WT: wild-type plant; HSA: free-HSA control plant; Trxs–HSA: fusion plants; HSA/Trxs: co-expression plants; M: molecular weight marker. Monomers of each recombinant protein are denoted by an asterisk. Trx, thioredoxin; HSA, human serum albumin.

more abundant than in free-HSA expressing plants. Transgenic lines were also analysed by Western blot with anti-Trx m/f antibodies (Figure 2c,d). As expected, aggregation and degradation bands were also present in fusion lines. Endogenous Trx m and f were not detected in wild-type plants, which indicated lower expression levels than in transplastomic lines. To corroborate differences in recombinant protein expression among different lines, the levels of HSA and Trxs accumulation in mature leaves (fifth from top) of greenhouse-grown plants 45 days after transplanting were determined by Western blot quantification. This technique allowed us to quantify the aggregates (bands above the expected band size) and discard the degradation bands (below the expected band size). Using serial dilutions of leaf extracts, the intensity of the immunoreacted bands corresponding to recombinant protein monomers plus aggregates was compared with that of commercial HSA standard, as explained in the experimental procedures. Accordingly, transplastomic Trx m-HSA and Trx f-HSA fusion lines accumulated recombinant HSA at levels of 26% and 22% of TP, respectively (Figure 2e). These values include not only the monomeric band detected in the Coomassie stained gel (Figure 2b) but also the abundant aggregates visible in the Western blot (Figure 2c), which explains the differences between band intensities in the Coomassie staining and quantification results. The recombinant protein levels achieved in the Trx–HSA fusion lines are approximately 3.8- and 3.2-fold higher than HSA quantified in free-HSA expressing plants (6.8% of TP). In contrast, HSA/Trxs co-expressing plants accumulated recombinant HSA at 1.5% and 3.1% of TP (Figure 2e) when Trx m or f were used, respectively. The accumulation of Trxs in co-expression lines was also quantified by Western blot (Figure 2e), whereas in fusion lines, Trx accumulation was proportionally estimated from the fusion protein amount, according to its molecular weight (14 kDa for Trxs and 80 kDa for Trxs–HSA fusion). No important differences were found among all transgenic lines, where Trx levels ranged from 4.2% to 6.2% of TP.

Differences in HSA accumulation among transgenic lines are attributed to post-transcriptional factors

Northern blot analysis was performed to determine whether or not differences in *HSA* and *Trxs* mRNA accumulation could account for the remarkable changes in HSA expression among transplastomic lines. The *HSA* coding region probe showed that transcripts from the *psbA* promoter in both fusions (2.7 knt) or co-expressions (2.8 knt) were slightly less abundant than those in free-HSA (2.3 knt) expressing plants (Figure 3a, upper panels), probably because of lower stability. It is interesting to note that such differences are in contrast to the 3.5-fold increase on

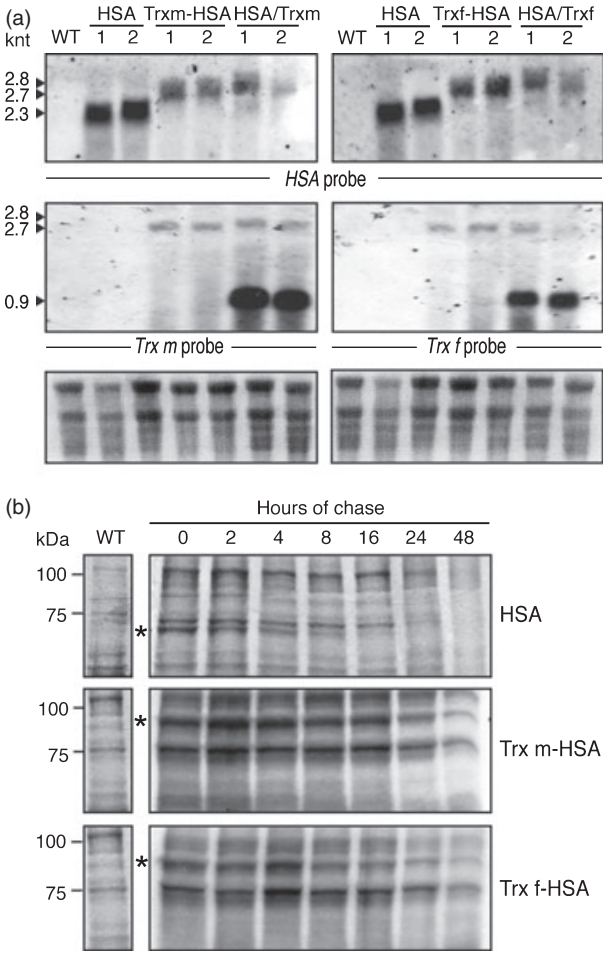

Figure 3 (a) Transcription analysis in transgenic lines. Northern blot analysis of total RNA isolated from transplastomic and WT plants. Ten micrograms of RNA were electrophoresed in denaturing conditions, blotted onto a nylon membrane and hybridized with *HSA*-, *Trx m*- or *Trx f*-specific probes. Two plants per transplastomic line are shown. As load controls, ethidium bromide-stained total leaf RNAs are reported in the lower panels. Mono- and dicistronic transcript sizes are indicated as described in Figure 1a. (b) Pulse-chase analysis of control free-HSA or Trxs–HSA fused proteins. Leaf discs were pulse-labelled for 1 h with ^{35}S-Met and ^{35}S-Cys and chased for 0, 2, 4, 8, 16, 24 and 48 h as indicated. After total protein extraction, analysis was performed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and autoradiography. Bands corresponding to the labelled recombinant proteins are denoted by an asterisk. As negative control, leaf discs from WT plants were also labelled. WT: wild-type plant; HSA: free-HSA control plant; Trxm-HSA and Trxf-HSA: fusion plants; HSA/Trxm and HSA/Trxf: co-expression plants. Trx, thioredoxin; HSA, human serum albumin.

average in HSA accumulation in Trxs–HSA fusion plants. The same blots were hybridized against *Trx m*- and *f*-specific probes (Figure 3a, middle panels). In both cases, the monocistronic *Trx* transcript (0.9 knt) produced from the P*rrnG10*L promoter in co-expression lines was much more abundant than the dicistronic transcript (2.8 knt), which includes *HSA* and *Trx* sequences. The increase in mRNA levels from this promoter may be caused by an increase in either the transcription rate or mRNA stability. However, these higher levels of monocistronic *Trxs* transcripts in co-expression lines did not correlate with an enhancement in Trx accumulation (Figure 2e). In no case, the

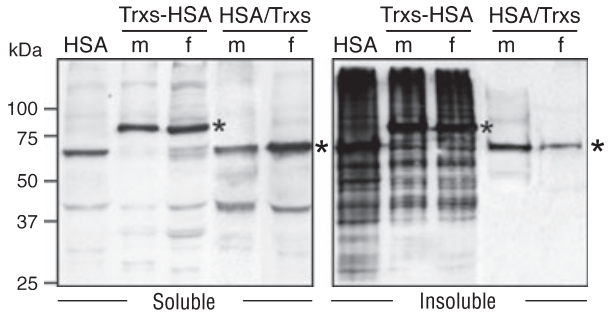

Figure 4 Analysis of soluble and insoluble fractions of transgenic lines. Ten micrograms of soluble and insoluble proteins extracted from mature leaves were loaded onto an 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel. Blots were detected using anti-HSA antibody. HSA: free-HSA control plant; Trxs–HSA: fusion plants; HSA/Trxs: co-expression plants. Monomers of each recombinant protein are denoted by an asterisk. Trx, thioredoxin; HSA, human serum albumin.

presence of polycistronic transcripts arising from genes located upstream was detected.

Considering the Northern blot results, post-transcriptional factors could account for high levels of HSA accumulation. Such levels could be attributed to more favourable translation or higher stability of the Trxs-fused proteins. To evaluate these factors, we performed a pulse-chase analysis of HSA protein in these plants (Figure 3b). Pulse-labelling experiments demonstrated that HSA could barely be detected after 8 h of chase in free-HSA expressing plants; however, in both Trxs–HSA fused lines, the recombinant protein was visible even at 48 h after labelling. In no case, labelled recombinant proteins were detected in untransformed control plants (Figure 3b, WT). Therefore, we concluded that the Trx-fusion strategy considerably increased the HSA half-life in chloroplasts. This result suggests that the enhancement in HSA accumulation levels is, at least to a large extent, caused by higher HSA stability inside chloroplasts.

Co-expression of tobacco Trxs m/f with HSA significantly reduced the accumulation of insoluble HSA

In a previous report, we showed that HSA accumulated in the insoluble fraction as large aggregates or protein IBs within chloroplasts (Fernandez-San Millan *et al.*, 2003). As we saw in Western blots (Figure 2a), Trxs–HSA fusions seem to have the same accumulation pattern as that in the free-HSA form, but a different pattern than co-expressed HSA. Because it is known that bacterial Trxs increase the solubility of fused or co-expressed proteins (Berndt *et al.*, 2008; Kolaj *et al.*, 2009), it might be expected that tobacco Trxs displayed a similar behaviour. Hence, soluble and insoluble protein fractions were separated from all transgenic lines and subjected to Western blot analysis (Figure 4). It was observed that soluble HSA was present in similar amounts when comparing the Trx-fusions and co-expression lines. However, Trx-fused HSA and free-HSA protein accumulated largely in the insoluble fraction. Conversely, a small amount of HSA accumulated in the insoluble fraction of Trx co-expressing lines.

Transmission electron microscopy analysis (Figures 5 and 6) was performed to determine whether the differences observed between solubility and expression among transgenic lines

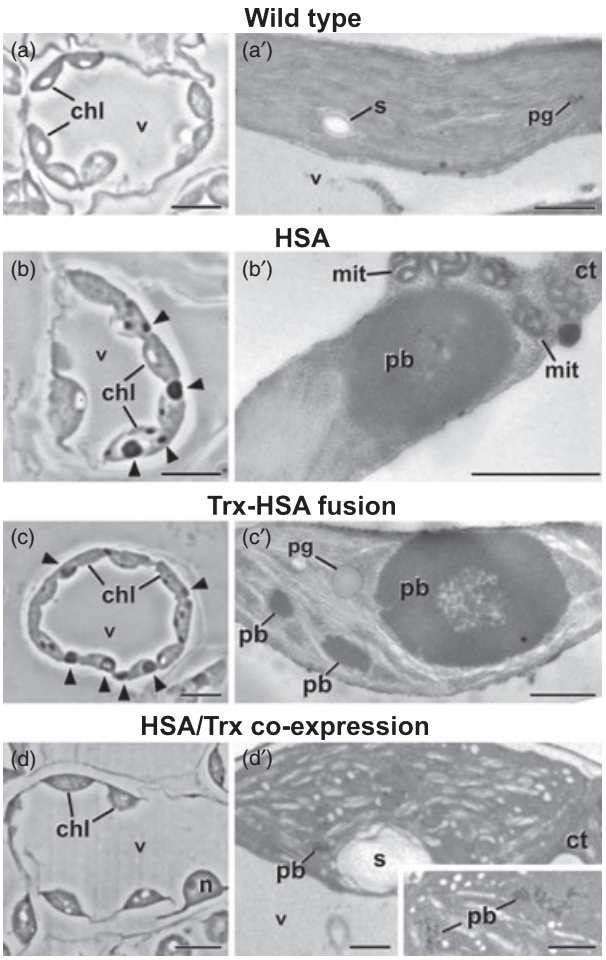

Figure 5 Ultrastructure of wild-type and transgenic HSA-expressing chloroplasts. (a–d) Architecture of leaf mesophyll cells as seen under the light microscope. (a′–d′) Ultrastructure of chloroplasts from the corresponding cells as seen under the transmission electron microscope. (a,a′) Structure of wild-type cells (a) and chloroplasts (a′). (b,b′) Structure of free-HSA expressing transgenic cells (b) and chloroplasts (b′). Note the presence of large and dense protein bodies (pb, arrowheads) in the chloroplasts (chl). (c,c′) Structure of transgenic cells (c) and chloroplasts (c′) expressing a Trx–HSA fusion protein, and exhibiting even larger protein bodies. (d,d′) Structure of transgenic cells (d) and chloroplasts (d′) co-expressing both HSA and Trxs. Note that the size of protein bodies in these chloroplasts is dramatically reduced and display a fibrillar texture (inset), markedly different from that of figures b′ and c′. ct: cytoplasm; mit: mitochondria; n: nucleus; pg: plastoglobule; s: starch; v: vacuole. Bars in a–d: 10 μm; a′–d′: 1 μm. Trx, thioredoxin; HSA, human serum albumin.

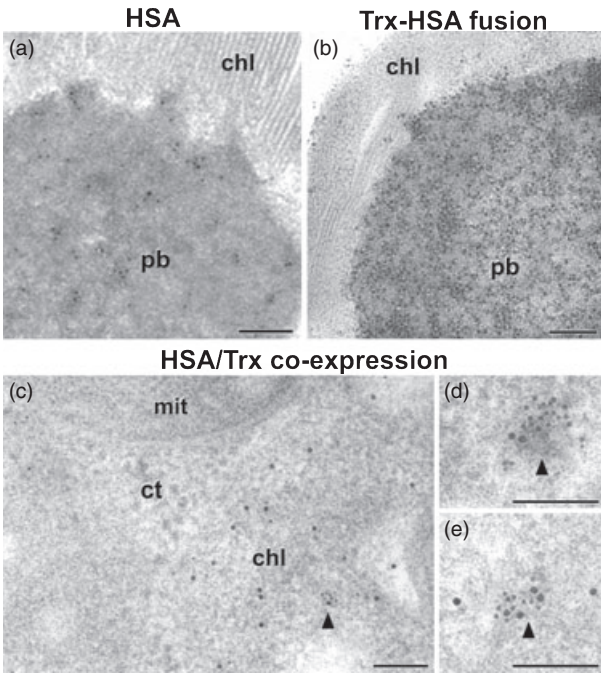

Figure 6 Immunogold labelling of transgenic HSA-expressing chloroplasts. (a) Chloroplast (chl) labelled with anti-HSA antibody, where gold particles concentrate exclusively in the protein body (pb). (b) Anti-HSA immunogold labelling of a chloroplast expressing Trx–HSA fusion. Gold particles decorate exclusively the protein bodies, independently of their size. (c–e) Double immunolocalization of HSA (5 nm gold particles) and Trx (10 nm gold particles) in chloroplasts co-expressing both proteins. The signal corresponding to both proteins is found exclusively within the chloroplast, where it is distributed mostly with a dispersed pattern. However, some clusters are observed (arrowheads) where particles of anti-HSA and anti-Trx can be clearly seen to co-localize (d and e). ct: cytoplasm; mit: mitochondria. Bars in a–b: 200 nm; c–e: 100 nm. Trx, thioredoxin; HSA, human serum albumin.

correlated with structural changes within the chloroplasts. The microscopy study was performed in parallel with ultrastructural analysis and immunogold labelling. Both Trx m and f showed similar results under fusion and co-expression with HSA; therefore, pictures from Trx m and f samples are indistinctively shown. In contrast to wild-type tobacco plants (Figure 5a,a′), transgenic chloroplasts expressing the free-HSA form presented a massive accumulation of a highly packed and electron dense material (Figure 5b,b′), previously described as HSA protein bodies (Fernandez-San Millan *et al.*, 2003). In plants expressing the Trxs–HSA fusion proteins, the number of these bodies increased, and their sizes were larger on average (Figure 5c,c′). However, plants co-expressing the HSA and Trxs (Figure 5d,d′) exhibited a marked reduction in the abundance of HSA protein bodies. Most of the observed protein bodies of plants co-expressing HSA and Trxs presented a fibrillar, coil-like texture (inset within Figure 5d′), in contrast to the solid, dense appearance of the bodies observed in the other transplastomic lines (Figure 5b′,c′). In addition, these bodies were markedly smaller than those observed in the chloroplasts expressing free-HSA or Trxs–HSA fusions.

Immunogold labelling with anti-HSA antibodies on free-HSA expressing chloroplasts showed labelling over the protein bodies (Figure 6a). The labelling pattern consisted of dispersed particles throughout the protein body, together with frequent clusters of 5–15 particles. In chloroplasts expressing Trxs–HSA fusions (Figure 6b), immunogold labelling over protein bodies was remarkably more abundant in both dispersed and clustered patterns. In these samples, clusters were also larger than in the free-HSA chloroplasts, comprising up to 50 particles. This finding is consistent with the higher amount of HSA quantified in these fusion plants. Outside of the protein bodies, occasional gold particles could be observed dispersed in the chloroplast stroma. No particles were identified outside the chloroplast nor in the controls that excluded the primary antibody. In chloroplasts

co-expressing both HSA and Trxs, this scenario changed dramatically. Apart from the anti-HSA labelling found in the small and fibrillar protein bodies, most of the signal appeared dispersed throughout the stroma, with the occasional presence of clusters of about 10 gold particles (data not shown). This result suggests that co-expression of HSA and Trxs somehow affects the distribution pattern of HSA.

A co-localization experiment of Trxs and HSA was performed to shed light on potential interactions between both proteins in HSA co-expressing chloroplasts. As revealed by anti-HSA and anti-Trxs double immunogold labelling (5 and 10 nm gold particles, respectively; Figure 6c), HSA-labelled proteins followed the previously described patterns of distribution: gold particles were mostly dispersed with some occasional clusters. The anti-Trx particles presented a similarly dispersed pattern. Interestingly, in the anti-HSA clusters, the presence of anti-Trx particles was frequently detected as being either included within the cluster or at a distance close enough to be considered co-localizing with the anti-HSA particles (Figure 6d). Some of the dual clusters of anti-HSA and anti-Trx particles were observed to decorate dense stromal structures, very similar to small, growing protein bodies (Figure 6e).

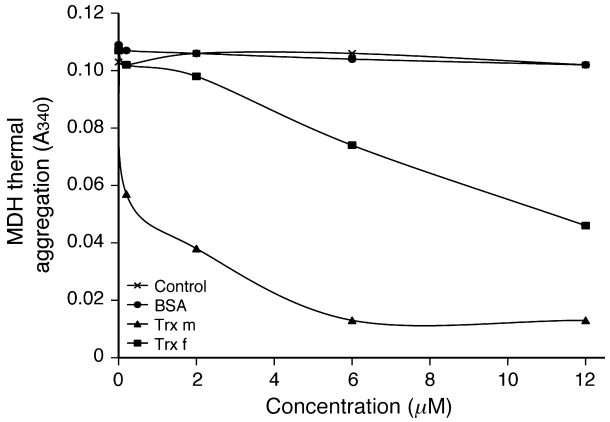

Figure 7 Holdase chaperone activity of tobacco Trx m/f. Thermal aggregation at 43 °C of 2 μM of malate dehydrogenase (MDH) was examined for 60 min in the absence (×) or presence of different concentrations of Trx m (▲) or f (■), by monitoring the turbidity increase at 340 nm. Reactions were also measured using same concentrations of bovine serum albumin (●) instead of Trxs. Representative plot of three independent experiments is shown. Trx, thioredoxin.

Tobacco thioredoxins m and f show holdase activity

Holdase chaperones are proteins that bind to folding intermediates, thereby preventing their nonspecific aggregation (Beissinger and Buchner, 1998). Because it is known that holdase function is a peculiar characteristic of some plant Trxs (Lee *et al.*, 2009; Park *et al.*, 2009), we wanted to study whether the tobacco plastidial Trxs could act as holdase chaperones, which could account to some extent for the absence of large IBs in HSA co-expressing lines. Trx m and f were bacterially expressed and purified to homogeneity. Holdase chaperone activity was analysed by measuring the ability of each Trx to inhibit the thermal aggregation of malate dehydrogenase (MDH), which has often been employed as a model polypeptide substrate to confirm the holdase function of other plant Trxs (Lee *et al.*, 2009; Park *et al.*, 2009). Incubation of 2 μM MDH with increasing amounts of Trxs (2–12 μM) gradually prevented the thermal aggregation of MDH (Figure 7). This aggregation was significantly blocked at a 6-μM concentration of Trx m, which is equivalent to a subunit molar ratio of 1 MDH to 3 Trx m. It appeared that Trx m prevented MDH aggregation more efficiently than Trx f; however, both Trxs act as molecular holdases at high concentrations (12 μM). When Trxs were replaced with bovine serum albumin (BSA), holdase chaperone activity was not detected, suggesting that this activity was derived from the specific function of Trxs. A direct inhibition of HSA thermal aggregation *in vitro* was also attempted, but its physical properties do not allow us to perform this assay as HSA is even more stable at high temperatures than the assayed Trxs.

Discussion

In a previous work (Fernandez-San Millan *et al.*, 2003, 2007), it was demonstrated that HSA, a complex globular protein with 17 disulphide bonds and highly susceptibility to degradation in chloroplasts, accumulated almost completely as protein IBs. It is widely known that protein aggregation into IBs mostly involves intermolecular associations of partially folded intermediates (Mitraki and King, 1989). Therefore, it can be deduced that in this case, HSA most likely did not have a native structure within plastids, although chloroplasts are able to accomplish the correct formation of disulphide bridges and fold foreign proteins in a biologically active form (Staub *et al.*, 2000; Daniell *et al.*, 2001; Molina *et al.*, 2004; Ruhlman *et al.*, 2007; Farran *et al.*, 2008). Production of recombinant protein as insoluble aggregates can offer the advantage of easy purification (Yasukawa *et al.*, 1995; Fernandez-San Millan *et al.*, 2003); however, *in vitro* manipulations must be made to refold the heterologous protein, and further, the native protein conformation is not guaranteed (Yasukawa *et al.*, 1995). Thus, large-scale production of proteins in biologically active soluble form is highly desirable as it facilitates downstream processing and lowers total costs. Fusion or co-expression techniques with proteins such as Trxs are common approaches in microbial systems for the expression of small peptides and unstable or insoluble proteins that accumulate at low levels or in the insoluble fraction (Berndt *et al.*, 2008; Demain and Vaishnav, 2009; Kolaj *et al.*, 2009). Thus, the lack of stability and solubility of HSA expressed in tobacco chloroplasts prompted us to investigate whether we could improve its intraplastidic expression using an N-terminal protein fusion or co-expression strategy. Because of their endosymbiotic origin, chloroplasts harbour genetic, structural and metabolic resemblances with prokaryotic cells (McFadden, 2001). For instance, plant chloroplast Trx m has a prokaryotic origin and strongly resembles bacterial Trxs (Meyer *et al.*, 2005). Thus, it seems conceivable that the transference of bacterial Trxs properties to the chloroplast expression system is possible.

In this context, we have assessed the well-known plastids Trxs m and f as heterologous partners for HSA expression in chloroplasts. In this model system, we hypothesized that the folding and accumulation of HSA in transgenic plastids might also benefit from the biological activities of chloroplast Trxs. To test this hypothesis, we generated four different chloroplast transformation vectors for HSA fusion and co-expression with either Trx m or f. Our results demonstrate an improvement in HSA accumulation mediated by Trx m or f fusions. HSA accumulated between 26% and 22% of the TP, which represents,

on average, a 3.5-fold increase in the expression level achieved in control free-HSA expressing plants (Figure 2e). Expression improvement of other recombinant proteins fused to these tobacco Trxs has been corroborated in our laboratory (unpublished data). In contrast, the presence of Trxs in co-expressing HSA plants resulted in an HSA decrease up to 4.5-fold. Such variability in HSA protein accumulation in transplastomic plants did not appear to depend on differences at the mRNA level (Figure 3a). Although Trxs-fusion plants accumulated the highest levels of HSA, slightly less amount of *HSA* transcripts were detected in fusions or co-expressions when compared to free-HSA control plants. *Trx* transcripts were also analysed by Northern blot, which showed monocistrons for the fusion lines and mono- and dicistrons for the co-expressing lines. However, even though *Trx* monocistrons were significantly more abundant in co-expressing than in fusion plants, this difference did not translate into higher levels of Trxs accumulation, as was observed by protein quantification (Figure 2e). Hence, we can conclude that other post-transcriptional factors must be influencing HSA and Trxs accumulation within chloroplasts.

It is known that plastid protein expression is extensively controlled at the post-translational level, mainly via regulated translation, protein complex assembly and proteolysis (Koop *et al.*, 2007). A more favourable translation or higher degree of protein stability could account for the increase in HSA accumulation levels in the fusion plants. Experiments in plastid expression of recombinant proteins have shown that a crucial factor in obtaining high levels of gene expression is the efficiency of translation initiation (Kuroda and Maliga, 2001b), which is very sensitive to the nucleotide sequence surrounding the initiating methionine codon. For this reason, modifications or extensions of the N-terminus of the desired protein often facilitate protein accumulation (Ye *et al.*, 2001; Herz *et al.*, 2005; Lenzi *et al.*, 2008; Scotti *et al.*, 2009; Farran *et al.*, 2010). However, Trxs could confer stability to the molecule by protecting HSA from proteolytic degradation. As mentioned previously, it is known that stable proteins (e.g. GFP or GUS) in fusion with less stable partners confer a certain degree of stability (Leelavathi and Reddy, 2003; Molina *et al.*, 2004; Lee *et al.*, 2010; Lentz *et al.*, 2010). Pulse-chase analysis of Trxs–HSA fusion proteins demonstrated a marked increase in stability (Figure 3b). We can conclude that higher accumulation levels of the Trxs–HSA fusions are mainly caused by their high stability inside chloroplasts. Similarly, Leelavathi and Reddy (2003) demonstrated a direct relation between stability and recombinant protein yield when IFN-g was expressed alone or translationally fused to the GUS protein.

Escherichia coli TrxA fusion and co-expression is a strategy to increase not only levels of expression but also solubility in microbial systems (Berndt *et al.*, 2008; Kolaj *et al.*, 2009). Knowing these Trx capabilities, the distribution of HSA in soluble and insoluble fractions of plants expressing Trxs–HSA fusions and co-expressions in chloroplasts was analysed and compared with that of free-HSA expressing control line. As demonstrated previously (Fernandez-San Millan *et al.*, 2003), free-HSA was recovered mainly in the insoluble fraction and was detected at very low levels in the soluble fraction, indicating the presence of protein aggregates. The same pattern was observed in the Trxs–HSA fusions. Nevertheless, although similar amounts of HSA were found in soluble fractions of co-expressing plants, low quantities of HSA were found in the insoluble fractions (Figure 4). To further investigate this observation, transmission electron microscopy and immunogold labelling were performed (Figures 5 and 6). As expected, similar to free-HSA expressing control plants, electron micrographs showed the formation of large protein IBs within chloroplasts expressing the Trxs–HSA fusion proteins. The number and size of these bodies increased in fusion plants compared to free-HSA control plants. Furthermore, HSA abundance also increased in protein bodies, as revealed by anti-HSA immunogold labelling, likely as a consequence of an enhancement in the protein stability mediated by Trxs as mentioned earlier. These results indicate that linkage of HSA to Trx m and f failed to increase HSA solubility in chloroplasts. This finding is in contrast to what has been previously observed for a number of insoluble proteins fused to *E. coli* TrxA in bacterial expression systems. However, in some cases, bacterial Trx fusions are not able to inhibit recombinant protein IB accumulation, although the causes remain unclear (Hammarstrom *et al.*, 2002). Dense aggregates practically disappeared when HSA was co-expressed with either Trx m or f. In these plants, electron micrographs showed small protein bodies with a fibrillar texture, suggesting a possible influence of Trxs on HSA solubility. Utilizing the approach previously used (Anderson *et al.*, 2008), we showed that HSA co-localizes with Trxs (Figure 6c–e). Association and interaction *in vivo* is difficult or impossible to prove conclusively; however, our co-localization experiment indicates that Trxs are not randomly distributed with respect to HSA. Thus, it is likely that HSA associates with Trxs *in situ*. In conclusion, it seems that Trx expression in a free or a fused form has different effects on HSA solubility, which would explain the protein IB formation only when HSA is fused to Trx. Different effects of fused or co-expressed *E. coli* TrxA over recombinant proteins have been reported previously (Yuan *et al.*, 2004).

Considering the association observed between Trxs and HSA in co-localization experiments and that chaperones interact preferentially with unfolded proteins (Hartl, 1996), we decided to confirm the putative chaperone activity of tobacco Trxs m and f used in this study. Chaperones have been shown to partially promote the folding of proteins by preventing their aggregation (holdase activity) (Beissinger and Buchner, 1998). This activity has recently been demonstrated *in vitro* for two *Arabidopsis thaliana* proteins: a Trx h (Park *et al.*, 2009) and a Trx-like protein (Lee *et al.*, 2009). In this study, the holdase chaperone activity of Trx m and f was analysed (Figure 7). Although a direct effect of plastid Trxs on HSA aggregation has not been experimentally demonstrated *in vitro*, our results provide biochemical evidence to confirm that tobacco Trx m and f have a holdase-like activity, as both Trxs are able to prevent MDH thermal aggregation. Based on these observations, the hypothetical Trx m and f holdase activity *in vivo* might also explain why the protein bodies practically disappear in chloroplasts of co-expressing lines. In this case, Trxs might increase the solubility by preventing aggregation and precipitation of nascent proteins, giving them an extended opportunity to adopt their correct tertiary folding. We also hypothesize that the lack of large HSA aggregates in these co-expression lines makes HSA more accessible to proteases, thus explaining the low levels of HSA.

Concluding remarks

Four conclusions can be drawn from this work: (i) linkage of Trxs and HSA in chloroplasts significantly increases the final yield of accumulated HSA; (ii) N-terminal Trx fusions confer high

stability to HSA; (iii) co-expression of tobacco Trxs with HSA in chloroplasts restricts large IB formation, although these plants do not accumulate more soluble HSA than control plants; and (iv) a similar behaviour of Trx m and f as modulators of HSA production in chloroplasts is observed despite the fact that their functions and phylogenetic origins have been reported to be different.

Thus, although there is a continuing need for new and easier methods to produce recombinant proteins for use in research, diagnostic and therapeutic applications, there are many problems such as low levels of expression, instability, insolubility and inappropriate folding that need to be addressed. At present, there are some alternatives for increasing the level of expression or stability, but to date, no research has focused on minimizing aggregate formation or improving folding of recombinant proteins produced by the transplastomic system. In this study, we showed that tobacco Trx-fusion or co-expression with HSA is a useful and promising strategy. Our results provide evidence that Trxs might exert an important role in modulating solubility patterns and improving the recombinant protein stability and final yield. To understand in-depth the potential of Trx proteins, other studies are ongoing with structurally simpler proteins than HSA and with a biological function that is easier to measure.

Experimental procedures

Construction of the chloroplast expression vectors

Nicotiana tabacum Trx m and *f* sequences (GenBank accession HQ338525 and HQ338526 respectively) were used for the design of primers to amplify the mature coding region from tobacco cDNA, previously obtained by RT-PCR. For the fusion strategy, *Trx m* and *f* were amplified by PCR with the following primers to introduce an *Nco*I site at the 5′ end and a *Sma*I site at the 3′ end, and to eliminate the TAA termination codon: FusTrxm-For 5′-CCATGGGTGAAGCGCAAA-3′ and FusTrxm-Rev 5′-CCCGGGCAAGAATTTCTCTATGCAGGTGG-3′; FusTrxf-For 5′-CCATGGGTAGCTCCGATGCTACTG-3′ and FusTrxf-Rev 5′-GCGGCCGCACTTGACCGCACATCCTCAATTG-3′. PCR products were cloned into the pGEM-T vector (Promega, Madison, WI). *HSA* was cloned with *Sma*I and *Not*I sites at the 5′ and 3′ ends, respectively, by PCR from the pLD-psbAHSA vector (Fernandez-San Millan *et al.*, 2003) with the following primers: HSA-For 5′-GGGCCAGGGCCAATGAGCCGGAGG-3′ and HSA-Rev 5′-GCGGCCGCTTAGGCCGAGCCCCC-3′. *Trx* and *HSA* sequences were fused together and to the promoter and 5′-UTR of the tobacco *psbA* gene in a pKS intermediate vector (Promega). Finally, the fusion expression cassette was digested by *Eco*RV and *Not*I and introduced into the pLD vector (Daniell *et al.*, 1998; Fernandez-San Millan *et al.*, 2003). The resulting vectors were named pLD-FusTrxm-HSA and pLD-FusTrxf-HSA.

For the co-expression strategy, *Trx m* and *f* were amplified by PCR with the following primers to introduce the *Sca*I and *Not*I sites at the 5′ and 3′ ends, respectively: CoTrxm-For 5′-AGTACTTGAAGCGCAAA-3′ and CoTrxm-Rev 5′-GCGGCCGCTTAC AAGAATTTCTCTATGCAGGTGG-3′; CoTrxf-For 5′-AGTACTTAGCTCCGATGCTACTG-3′ and CoTrxf-Rev 5′-GCGGCCGCTTAACTTGACCGCACATCCTCAATTG-3′ and cloned into the pGEM-T vector (Promega). In this vector, the P*rrnG10*L promoter and leader region (Farran *et al.*, 2008) were fused to *Trx m/f* sequences at the 5′ end. The product P*rrnG10*L-*Trx m/f* was excised with *Not*I and introduced into the pLD-psbAHSA vector (Fernandez-San Millan *et al.*, 2003) downstream of the *HSA* gene. The resulting vectors were named pLD-CoHSA-Trxm and pLD-CoHSA-Trxf.

The final clones were sequenced, and their functionality was tested by Western blot of *E. coli* culture extracts.

Expression and purification of tobacco Trx m/f from *Escherichia coli* and production of polyclonal antibodies

Tobacco Trx m and Trx f were expressed in *E. coli* as His-tagged polypeptides. The coding sequences were amplified by PCR with the same primers as those used for their cloning (FusTrxm/f-For and CoTrxm/f-Rev); the resulting PCR fragment was digested with *Nco*I and *Not*I and cloned in a pET 28a(+) vector (Novagene; Merck KGaA, Darmstadt, Germany). The resulting plasmids, termed pET Trxm and pET Trxf, were introduced into *E. coli* (BLR DE3, Novagene). Overexpressed proteins were purified by chromatography in affinity columns packed with Ni-NTA agarose (Qiagen, Hilden, Germany). Specific anti-Trx m and anti-Trx f antibodies were raised by immunizing rabbits with purified His-tagged proteins (Abyntek, Bilbao, Spain). Antibody cross-reactivity was eliminated by affinity purification (exclusion) of anti-Trx m IgG with the Trx f antigen and *vice versa*.

Bombardment and regeneration of chloroplast transgenic plants

Gold microprojectiles (0.6 μm) coated with plasmid DNA (pLD-FusTrxm-HSA, pLD-FusTrxf-HSA; pLD-CoHSA-Trxm and pLD-CoHSA-Trxf) were bombarded into *in vitro*-grown tobacco leaves (*Nicotiana tabacum* cv. Petite Havana SR1; National Germplasm Resources Laboratory, Beltsville, MD) using the biolistic device PDS1000/He (Bio-Rad, Hercules, CA) as previously described (Daniell, 1997). After bombardment of the abaxial leaf side, leaves were cut into small pieces (c. 5 × 5 mm) and placed adaxial side up on Magenta vessels (Sigma-Aldrich, St Louis, MO) containing 500 mg/L spectinomycin dihydrochloride as a selecting agent. Resistant shoots were subjected to a second round of selection under the same conditions. PCR was used to analyse integration of different cassettes in the transformed plants with the following primers: primer 3P 5′-AAAACCCG-TCCTCAGTTCGGATTGC-3′, which binds on the chloroplast genome upstream of the *trnI* gene outside the vector integration site, and primer 3M 5′-CCGCGTTGTTTCATCAAGCCT-TACG-3′, which binds to the *aadA* gene.

Southern and Northern blot analyses

Total plant DNA was extracted from leaves using the cetyltrimethylammonium bromide procedure. Ten micrograms of total DNA were digested with *Hin*dIII, separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane. The digestion by *Bgl*II and *Bam*HI of the pFS vector generated a 0.8-kb probe (*FS*) homologous to the flanking sequences. A 0.75-kb *HSA* probe was obtained by *Nco*I–*Not*I digestion of pLD-psbAHSA vector (Fernandez-San Millan *et al.*, 2003). A 0.37-kb fragment (*Trxm* probe) of the *Trx m* gene and a 0.39-kb fragment (*Trxf* probe) of the *Trx f* gene were generated by PCR with the same primers used for their cloning. Hybridization was performed using the chemiluminescent AlkPhos direct labelling-detection system (GE Healthcare, Buckinghamshire, UK). After Southern blot confirmation, plants were transferred to soil. Seeds from the T_0 generation were germinated *in vitro* on the

spectinomycin selection medium. The T_1 seedlings were isolated and cultured for 4 weeks in Magenta vessels. Finally, plants were transferred to pots. Plants from the T_0 and T_1 generations were analysed for homoplasmy.

Total RNA was extracted (Ultraspec RNA; Biotecx Laboratories, Houston, TX) from leaves of transformed and untransformed plants. RNA (10 μg) was electrophoresed on 1.5% agarose/formaldehyde gels and then transferred to a nylon membrane. The previously described HSA- and Trx-specific probes were used. Labelling and hybridization were performed using the chemiluminescent detection system mentioned earlier. Ethidium bromide-stained total leaf RNA was used to assess loading.

Western blot analysis

Transformed and untransformed leaves of plants grown in a greenhouse were ground in liquid nitrogen. Leaves (100 mg) were homogenized in 600 mL of Laemmli buffer (0.5 M Tris–HCl pH 6.5, 4% SDS, 20% glycerol and 10% β-mercaptoethanol) and heated at 95 °C for 5 min. After 5 min of centrifugation at 20 000 ***g***, the supernatant was considered the TP. TP was measured using the RC-DC protein assay (Bio-Rad) with BSA as a standard, according to the manufacturer's instructions. For soluble extractions, 100 mg of leaves were homogenized in 600 μL of buffer (Ouerghi *et al.*, 2000). After centrifugation at 20 000 ***g*** for 5 min, the supernatant was considered the soluble fraction. The pellet was re-suspended in 200 μL of Laemmli buffer and boiled for 5 min. After centrifugation at 20 000 ***g*** for 5 min, the supernatant was considered the insoluble fraction. Soluble and insoluble proteins were measured using the Bradford or RC-DC protein assay (Bio-Rad), respectively. Proteins were separated by SDS–PAGE on 8%–13% polyacrylamide gels and transferred to a nitrocellulose membrane for immunoblotting. Anti-HSA (Nordic Immunology, Tilburg, the Netherlands) at 1 : 10 000 dilution or anti-Trx m/f antibodies (Abyntek) at 1 : 5000/1 : 7500 dilution were used as primary antibodies. Peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma-Aldrich) was used as the secondary antibody at 1 : 10 000 dilution. Detection was performed using the chemiluminescence ECL Western blotting system (GE Healthcare).

Quantification of HSA and Trxs was performed by comparing dilution series of TP from transgenic plants with dilution series of purified HSA (Sigma-Aldrich) or Trxs (purified from *E. coli*). For each protein, adequate amounts were loaded on an SDS–PAGE gel, electrophoretically separated and then analysed by Western blot. Immunoblots were quantified using the GeneTools analyzer software (SynGene, Cambridge, UK).

Pulse-chase analysis

Leaf discs (0.97 cm^2) from young, fully expanded leaves of transformed and nontransformed plants growing in a greenhouse were sampled, layered immediately on 0.5 mL of Murashige and Skoog (Duchefa, Haarlem, the Netherlands) liquid medium, pH 5.8 containing 40 μCi of a ^{35}S-methionine and ^{35}-S-cysteine (Perkin Elmer, Boston, MA) mixture per leaf disc and vacuum infiltrated for 3 min. Leaf discs were then illuminated for 1 h at 25 °C, blotted briefly with blotting paper, rinsed by vacuum infiltration with 2.5 mL of water per disc, blotted again and vacuum infiltrated with Murashige and Skoog liquid medium containing cold 10 mM methionine and 5 mM cysteine. Samples were stored in a culture chamber under continuous light for the different chase periods (up to 48 h). Three leaf discs were harvested and frozen in liquid nitrogen per time-point. Leaf samples were ground in 150 μL of Laemmli buffer and boiled for 5 min. Protein samples were separated by 8% SDS–PAGE. The resulting gels were dried and exposed to film for 4 days.

Light and electron microscopy and immunogold labelling

Leaf samples from plants grown in a growth chamber were fixed in Karnovsky fixative (4% formaldehyde and 5% glutaraldehyde in 0.025 M cacodylate buffer, pH 6.7), post-fixed in 2% OsO_4, dehydrated in methanol series for 3 days and slowly embedded in Epon resin for 2 days. Epon blocks were polymerized at 60 °C for 2 days. For light microscopy, semi-thin (1 μm) sections were obtained, stained with toluidine blue, mounted and observed in a Nikon Eclipse E1000 microscope. For ultrastructural observations, thin sections (*c.* 80 nm) were collected on 100-mesh copper grids, counterstained with uranyl acetate and lead citrate, and observed using a Philips CM10 transmission electron microscope (Philips, Hillsboro, OR) operating at 100 kV.

For single immunogold labelling, leaf samples were fixed in 4% formaldehyde in phosphate buffer saline (PBS) and dehydrated in methanol by the 'Progressive Lowering of Temperature' (PLT) method in a Leica AFS automated system (Leica, Wetzlar, Germany). Then, samples were infiltrated in Lowicryl K4M resin and polymerized at −30 °C under ultraviolet light. For immunolabelling, Lowicryl sections (thickness, *c.* 80 nm) were deposited on Formvar- and carbon-coated nickel grids. Immunogold labelling was performed as described previously (Segui-Simarro *et al.*, 2003). Briefly, sections were hydrated, floated in PBS, blocked with 5% BSA in PBS and incubated with goat anti-HSA (Bethyl Laboratories Inc., Montgomery, TX) or rabbit anti-Trx m/f (Abyntek) antibodies diluted 1 : 2 and 1 : 5, respectively, in 1% BSA for 1 h at room temperature. Sections were then incubated with an anti-goat IgG antibody for the anti-HSA detection, or with an anti-rabbit IgG antibody for the anti-Trxs detection, both from British Biocell International (Cardiff, UK) and conjugated to 10-nm gold particles, diluted 1 : 25 in 1% BSA for 45 min. Finally, sections were washed, air dried, counterstained with uranyl acetate and lead citrate and observed in a Philips CM10 transmission electron microscope operating at 100 kV. Controls were performed excluding the corresponding primary antibody from the incubation buffer.

For co-localization experiments, double immunogold labelling was performed essentially following the protocol described earlier, but a mixture of anti-HSA and anti-Trx m or f primary antibodies diluted as previously mentioned was used instead. Then, sections were incubated with a mixture of an anti-goat IgG antibody conjugated to 5-nm gold particles (British Biocell International) for the specific detection of the anti-HSA antibody and with the previously described anti-rabbit IgG antibody conjugated to 10-nm gold particles for the detection of the anti-Trx m/f antibodies, both diluted 1 : 25 in 1% BSA for 45 min. Controls were performed by excluding both primary antibodies from the incubation buffer.

Holdase activity assay

Thermal aggregation of MDH was used as holdase activity assay (Park *et al.*, 2009). MDH from porcine heart (Sigma-Aldrich) was incubated at 2 μM in 50 mM HEPES-KOH (pH 8.0) buffer at

43 °C with various concentrations of Trxs (1 : 1, 3 : 1 and 6 : 1 Trx : MDH molar ratio). Thermal aggregation of MDH was determined in a microtiter plate by monitoring the turbidity increase at 340 nm in a temperature-controlled spectrophotometer (Spectra Max 340PC; Molecular Devices, Sunnyvale, CA). BSA was used as control.

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