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Research Article

Post-harvest light treatment increases expression levels of recombinant proteins in transformed plastids of potato tuber

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Abbreviations: TSP, total soluble protein

Abstract

Plastid genetic engineering represents an attractive system for the production of foreign proteins in plants. Although high expression levels can be achieved in leaf chloroplasts, the results for non-photosynthetic plastids are generally discouraging, mainly due to low transcriptional and translational rates in comparison with chloroplasts. Here, we report the expression of two thioredoxin genes (*trx f* and *m*) from the potato plastid genome to study transgene expression in amyloplasts. As expected, the highest transgene expression was detected in the leaf (up to 4.2% of TSP). The Trx protein content in the tuber was approximately 2-3 orders of magnitude lower than in the leaf. However, we demonstrate that a simple post-harvest light treatment of microtubers developed in vitro or soil-grown tubers induces up to 55 times higher accumulation of the recombinant protein in just 7-10 days. The promoter and 5'-UTR of the *psbA* gene displayed higher light induction than the *rrn* promoter. After the applied treatment, the Trx f levels in microtubers and soil-grown tubers increased to 0.14% and 0.11% of TSP, respectively. Moreover, tubers stored for 8 months maintained the capacity of increasing the foreign protein levels after the light treatment. Post-harvest cold induction (up to 5 times) at 4 ºC was also detected in microtubers. We conclude that plastid transformation and post-harvest light treatment could be an interesting approach for the production of foreign proteins in potato.

1 Introduction

Plastid genetic engineering is an attractive technology for the development of plants as bioreactors for the production of recombinant proteins to be used as biopharmaceuticals or in the chemical industry. Plastid transformation often allows high levels of foreign protein accumulation to be achieved, representing up to 70% of total soluble protein (TSP) [1], mainly because of the high copy number of the transgene in each cell [2].

However, there are large differences in gene expression in different types of plastids. Several studies have shown that foreign genes are expressed at high levels in chloroplasts but are substantially downregulated in non-photosynthetic plastids such as tomato chromoplasts and potato amyloplasts [3-5]. Moreover, data on the signals and factors that govern gene expression in chloroplasts cannot be directly applied to non-green plastids [6]. Plastid transcriptomic and translatomic analyses of potato tubers and tomato fruits have highlighted common mechanisms in amyloplasts and chromoplasts. In general, RNA accumulation is much lower in potato tubers [7] and in tomato fruits [8] than in leaves. However, some transcriptional activity was detected in tuber amyloplasts only for the *psbA* and *rrn* genes. The ribosome association of transcripts is generally low, in both tubers and fruits, indicating low-level translational activity. Therefore, increasing plastid gene expression levels in non-green edible organs, such as fruits, roots and tubers, is one of the challenges of plastid biotechnology, especially for the high-level production of recombinant proteins.

Potato is the fourth most important food crop worldwide, after wheat, rice and maize, due to its high yield production and high nutritive value [9]. Conventional breeding programs involving crossings, primarily directed at increasing yields, are mainly hampered by the tetraploid nature of cultivated potato cultivars. Currently, genetic engineering is being used as a tool to achieve resistance to biotic stresses, such as viruses, nematodes and fungi, as well as improved storage or processing characteristics. In addition, the advantage of the tuber as a storage organ would facilitate the use of potatoes as a biofactory for the production of new compounds [10]. So far, stable plastid transformation of potato has only been performed with the *gfp* and *uida* reporter genes [4, 5, 11, 12]. GFP accumulation corresponding to 0.05% of TSP in microtubers was reported. Valkov et al. (2011)

tested several promoters, terminators and 5'-UTRs to increase the expression of the *gfp* gene in tubers. The best results (0.02% TSP) were achieved with the *rrn* promoter and the *rbcL* 5'-UTR and with the promoter and 5'-UTR of the plastid *clpP* gene. In the case of the *uidA* gene, it was shown that despite low GUS accumulation in soil-grown tubers, microtubers developed in vitro exhibited higher levels of β-glucuronidase (9.8% TSP). This effect was most likely due to the transformation of amyloplasts to chloroplasts, as in vitro microtuberisation was performed in the light (F. Bravo-Almonacid, personal communication).

Thioredoxins (Trxs) are small ubiquitous thiol-disulphide oxidoreductases that play important roles in the regulation of many cellular processes by causing post-translational redox modifications in target proteins. The chloroplast Trx system includes five different types: f, m, x, y, and z [13]. Recently, *trx f* and *m* were overexpressed in the plastid genome of tobacco [14]. Overexpression of *trx f* induced an increase of up to 700% in leaf starch accumulation, accompanied by an increase in leaf sugars and leaf biomass yield. In contrast, plants overexpressing *trx m* specifically displayed increased methionine sulfoxide reductase activity, conferring consequently tolerance to oxidative treatments [15].

In the present study, we report the overexpression of *trx f* and *m* from the plastid genome of potato with two objectives. First, we wanted to elucidate whether a starch-storing organ, such as the tuber, is affected by the altered redox status due to high levels of Trx present in the amyloplast. To this end, the *trx f* or *m* gene was expressed from the potato plastid genome. Second, we explored how post-harvest environmental conditions (e.g., light or temperature) could affect the accumulation of the foreign protein in plant storage organs. For this purpose, the *trx f* gene was expressed under two of the most promising promoters used in previous works: those of the ribosomal *rrn* operon and *psbA*. Both soil-grown tubers and in vitro-developed microtubers are suitable materials to answer this question.

2 Materials and methods

2.1 Construction of potato plastid expression vectors

Previously cloned tobacco *trx f* or *trx m* coding sequences (GENBANK Accessions HQ338526 and HQ338525) were inserted into the chloroplast genome under the control of different tobacco 5'-regulatory sequences: the 16S ribosomal RNA promoter fused to the *gene 10* 5'-UTR from phage T7 (*PrrnG10L*) or the *psbA* promoter and 5'-UTR (*PpsbA*). The *trx* genes with their corresponding promoters were cloned into the potato chloroplast transformation pLD-St vector between the *aadA* selectable spectinomycin resistance gene (driven by the tobacco *rrn* promoter) and the *psbA* 3'-UTR sequence of petunia, which acts as terminator of both transgenes. The transformation vector includes potato homologous recombination sequences (103,146–105,346 of GENBANK Accession DQ386163) and targets the insertion of the foreign genes into the plastid genome between the *trnI* and *trnA* genes in the duplicated inverted repeat region. The pLD-St vector was kindly provided by Dr. H. Daniell (University of Pennsylvania, USA). The final clones were sequenced, and potato plants were transformed with the corresponding vectors.

2.2 Bombardment and regeneration of transplastomic plants

Potato plants (*Solanum tuberosum* L. cv. Désirée) were cultured in vitro on Murashige and Skoog (MS) salts and vitamins (Duchefa, Haarlem, the Netherlands) containing 20 g L^{-1} sucrose and solidified with 0.8% (w/v) bacto-agar (Becton-Dickinson, Le Pont de Claix, France) under a 16 h photoperiod at 25 ºC. The two-step tissue culture protocol that is generally employed for nuclear transformation in potato was used [16]. Six to eight fully expanded dark green leaves were placed, abaxial side up, on callus induction medium (CIM) (MS salts and vitamins, 16 g L^{-1} glucose, 5 mg L^{-1} α-naphtalenacetic acid, 0.1 mg L^{-1} 6-benzylaminopurine and 0.8% (w/v) bacto-agar). The plates were kept for 24 h under a 16 h photoperiod at 20 ºC prior to bombardment. Leaves were bombarded with 0.6 μm gold particles coated with plasmid DNA. Bombardment was performed with the PDS-1000/He biolistic particle delivery system (BioRad, Hercules, USA), using a rupture disc pressure of 1,100 psi, a partial vacuum pressure of 27 in Hg and a target distance of 9 cm. The bombarded plates were kept for 2 days in the dark, after which the leaves were cut into 4 x 4 mm pieces and placed, abaxial side down, on CIM containing 300 mg L^{-1} spectinomycin and

incubated at 20 ºC under a 16 h photoperiod for 2 weeks. The leaf explants were then transferred to shoot induction medium (SIM) (MS salts and vitamins, 16 g L^{-1} glucose, 2 mg L^{-1} zeatin riboside, 0.02 mg L^{-1} gibberellic acid and 0.8% (w/v) bacto-agar), containing 300 mg L^{-1} spectinomycin and subcultured in the same selective medium every 3 weeks. The first spectinomycin-resistant shoots were identified after 8-10 weeks of selection in SIM. The shoots were excised and subcultured in hormone-free MS medium containing 300 mg L^{-1} spectinomycin for root development. The rooted plants were transplanted to pots. Culture was always performed in perlite, and the plants were grown hydroponically to maturity in a growth room or in the greenhouse. Additionally, plantlets were maintained in vitro for subsequent microtuberisation experiments. The resulting potato transformants, designated PrrnG10L-Trxf, PrrnG10L-Trxm and PpsbA-Trxf, expressed the *trx f* and *trx m* genes under the control of either *PrrnG10L* or *PpsbA* regulatory sequences.

2.3 Southern and northern blot analysis

Southern and northern blot experiments were performed as previously described [14].

2.4 Western blot analysis

Leaf, stem, root, microtuber and tuber samples (100 mg) from plants grown hydroponically in the greenhouse were ground in liquid nitrogen and resuspended in 200 μL of total soluble protein extraction buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 0.1% Triton X-100) and incubated for 30 min on ice. The supernatant obtained after 5 min of centrifugation at 20 800 ***g*** contained the total soluble protein fraction. After boiling with 2X loading buffer (0.5 M Tris–HCl pH 6.5, 4% SDS, 20% glycerol and 10% β-mercaptoethanol), the protein extracts were electrophoresed in SDS-PAGE gels and blotted using specific anti-Trxf and anti-Trxm antibodies as previously described [17]. Several western blots with 3 different dilutions of each sample were produced and analysed for quantifications. Different dilutions of purified Trx f or Trx m protein produced in *E.*

coli were used as standards. Finally, the resultant bands were densitometrically quantified (Gene-Tools, Syngene, Cambridge, UK).

2.5 In vitro microtuberisation assays and post-harvest treatments

In vitro-developed shoots, 4-6 cm in length (including approximately 6 buds), were excised. The shoot apex and roots were discarded. Three shoots per flask were cultured in liquid MS medium without hormones (20 mL of medium in a 250 mL Erlenmeyer flask). The flasks were incubated for 2 weeks on an orbital shaker at 90 rpm (Innova 4340, New Brunswick Scientific, Edison, USA) at 21 ºC under a 16 h photoperiod. Subsequently, the composition of the medium in each flask was modified for tuber induction by adding 5 mg L^{-1} kinetin and 500 mg L^{-1} chlorocoline chloride and increasing the sucrose concentration from 2 to 8% (w/v). The flasks were alternatively kept stationary in the dark for 4 weeks or maintained for one week in the dark, followed by 3 weeks under a 16 h photoperiod. Microtubers were harvested weekly for gene expression analysis.

To study the effect of post-harvest conditions on gene expression, detached microtubers developed after 2 weeks of culture in darkness were subjected to the following treatments in empty Petri dishes sealed with Parafilm: 4 ºC in the dark, 24 ºC in the dark or 24 ºC under a 16 h photoperiod. Microtubers were sampled at days 0, 7, 18 and 28 for gene expression analysis.

Soil-developed tubers (5-10 g/tuber) from different plants were subjected to the same 3 treatments mentioned above. The samples were collected at days 0 and 10. To avoid the differences that are usually observed between tubers, samples from each tuber were harvested at both time points for subsequent fine comparisons. To this end, transverse, symmetrical samples through the whole depth of the middle part of the tuber were obtained with the aid of a cork-borer (5 mm diameter).

Soil-developed tubers stored for 8 months at 4ºC in the dark were subjected to the 24 ºC/16 h photoperiod treatment during 10 days. Outer and inner layers of the tubers were sampled at days 0 and 10.

Three independent experiments were performed in all cases and the results of western blots were quantified as indicated in section 2.4.

2.6 Semi-quantitative RT-PCR

Total RNA was extracted from microtubers using the Ultraspec RNA kit (Biotecx Laboratories, Houston, USA) and was employed to perform semi-quantitative RT-PCR analysis of *trx* genes. The cDNA was obtained following the manufacturer's instructions (PCR SuperScript III reverse transcription kit, Invitrogen, Carlsbad, USA). Semi-quantitative PCR was performed with specific primers for the *trx f* and *18S ribosomal RNA* genes, designed with Primer Express Software (Applied Biosystems, Life Technologies, Grand Island, NY, USA): *trx f*, qRTf_F (5′-AGTGATCGCTCCAAAGTTTCAAG-3′) and qRTf_R (5′-TT GTCCTGGTTACAGTCCAGCTT-3′); *18S rRNA*, 18S_F (5'-ACAAACCCCGACTTCTGGAA-3') and 18S_R (5′-CATGAATCATCGCAGCAACG-3′). The number of cycles and amount of cDNA (1 µg or 250 ng for *trxf* and *18S rRNA*, respectively) were optimised to work in the exponential range. The reactions were performed in a PCR Mastercycler (Eppendorf, Hamburg, Germany) with Taq polymerase (Biotools B&M Labs, Madrid, Spain), using the following PCR profile: 5 min at 94°C, followed by 28 (*18S rRNA*) or 40 (*trx f*) cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C, with a final extension period of 72°C for 5 min to complete the reaction. Each amplification reaction was verified in 3 to 5 replicate experiments, and *18S rRNA* was used as an internal standard to normalise the relative amount of the *trx f* transcript in each reaction. The amplifications were performed in a final volume of 30 µl. The PCR products were separated in agarose gels (2%), and the intensities of the bands were quantified using Gene-Tools analysis software. Relative ratios were calculated by dividing the intensity values of *trx f* by the *18S rRNA* products.

3 Results

3.1 Production of transplastomic potato plants and phenotypic analysis

Five plates were bombarded with each construct (PrrnG10L-Trxf, PpsbA-Trxf and PrrnG10L-Trxm), and finally, up to 7 shoots were regenerated per plate. The first spectinomycin-resistant shoots were identified 10-12 weeks after bombardment.

The integration of the foreign genes into the chloroplast genome and the analysis of homoplasmy were confirmed via Southern blotting by using a 0.8 kb probe homologous to the flanking regions *trnI* and *trnA* (Fig. 1A). Total plant DNA was digested with *BamH*I. The probe identified a 1.2 kb fragment in the wild-type plant and a 3.0 kb fragment in the transformed plants (Fig. 1B). The absence of the 1.2 kb band in the transformed lines indicated homoplasmy.

Phenotypic analysis was performed in plants grown in the greenhouse. The PrrnG10L-Trxm plants were smaller than the control plants (Supporting information, Fig. S1A). Chlorophyll levels and photosynthesis rates were significantly lower in all transgenic plants, with the effect being more severe in PrrnG10L-Trxm plants (Supporting information, Table S1). The starch content in tubers was lower in the three transgenic lines; however, in the leaves, despite the observation of a downward trend, only the PrrnG10L-Trxm plants showed significant differences. The tuber yield was lower in all of the transgenic plants, considering both the tuber number and tuber weight per plant (Supporting information, Fig. S1B). Again, the effect was more severe in PrrnG10L-Trxm plants.

3.2 Analysis of transgene expression

The transcripts of the *trx f* and *m* genes were analysed via northern blotting (Fig. 1). In the majority of analysed tissues, the presence of monocistrons from the *PrrnG10L* or *PpsbA* promoter (~1 kb), dicistrons from the *Prrn* promoter of the *aadA* gene (~2 kb) and polycistrons from the endogenous *rrn* promoter (~4.6 kb) were detected. The *trx f* gene was expressed at approximately equal levels from both the *PrrnG10L* and *PpsbA* promoters (Fig. 1C). The *psbA* promoter rendered a transcript that was 50 bp longer than the *rrnG10L* promoter, but this difference was barely detectable in northern blots. The highest expression level of the transgene was observed in the leaf, followed by the stem. The major signal detected in the leaf corresponded to monocistrons, but dicistrons and polycistrons were also observed (Fig. 1C). Transgene expression in the tubers was weak (but all cistrons were present), and it was nearly undetectable in the roots. The endogenous level of the *trx f* transcript in all tissues of the wild-type control plants was under the detection limit of this technique. The expression

pattern of the *trx m* transcript in PrrnG10L-Trxm transgenic plants was similar to that indicated above for PrrnG10L-Trxf plants (Fig. 1D). However, polycistronic mRNA was barely visible in stem and tuber samples, and no transcripts were detected in the roots by this method.

Immunoblot analyses were performed with specific antibodies against tobacco Trx f and Trx m to confirm the presence of both Trx proteins in different potato tissues (Fig. 2). The two detected bands probably correspond to different levels of Trx oxidation [18]. As indicated for the *trx* transcripts, the highest contents of Trx f and Trx m proteins were detected in the leaves. The accumulation of Trx in the stem was up to 160 times lower than in the leaf (Table 1). The levels of Trx proteins in non-photosynthetic tissues (tubers and roots) were even lower. The Trx protein content in the tubers of transplastomic plants was approximately 2-3 orders of magnitude lower than in the leaves. The lowest Trx protein levels were measured in the roots, being undetectable in PrnG10L-Trxm plants (Fig. 2B), in line with the very low transcript content observed (Fig. 1C, D). The endogenous Trx protein levels in the tissues of wild-type plants were under the detection limit of this technique.

The effect of different translational regulatory elements (the *G10L* 5'-UTR from phage T7 and the plastidial *psbA* gene 5'-UTR) was analysed by western blotting in Trx f-overexpressing plants. After quantitation (mean values of several experiments with 3 different dilutions of samples), the accumulation of Trx f protein in the leaves was observed to be higher (4.2% of TSP) when the 5'-UTR of the *psbA* gene was used instead of that of *G10L* (2.6% of TSP) (Table 1). In contrast, in non-photosynthetic tissues such us roots and tubers, the inverse tendency occurred. The content of Trx f protein in the tuber was notably higher when using *G10L* (0.011% of TSP) than *psbA* (0.002% of TSP). The same trend was observed in the roots, which showed the lowest levels of Trx f (3-4 orders of magnitude lower in comparison with the leaves). In general, Trx protein levels in PrrnG10L-Trxm plants were much lower than in Trx f-overexpressing plants (Table 1).

3.3 Transcript and protein levels of *trx f* during microtuber development

We used an in vitro microtuberisation system to study the evolution of *trx f* gene expression during tuber induction and development (Fig. 3A). Potato microtubers developed through tissue culture are a suitable system for studying physiological and metabolic processes [19]. Tuberisation was synchronically induced in shoots grown in darkness in liquid medium by the addition of kinetin, chlorocoline chloride and additional sucrose, and *trx f* gene expression was monitored for 4 weeks (Fig. 3B). Microtubers appeared 7 days post-induction, in which the expression level of Trx f was higher when using the *rrnG10L* promoter and 5'-UTR than the *psbA* regulatory sequences (0.0085±0.0008% and 0.003±0.0004% of TSP, respectively). The Trx f protein content declined during the subsequent 21-day period of tuber growth in darkness to 30% of the TSP in day 7. Light induction of Trx f expression when the flasks were transferred to a 16 h photoperiod was observed for both promoters after only 1 week of treatment and was maintained during the whole period of microtuber development. The *psbA* promoter and 5'-UTR favoured stronger induction (up to 22 times the level of Trx f protein relative to day 7) than the *rrnG10L* (up to 10.5 times). However, final Trx f accumulation was slightly greater in PrrnG10L-Trxf (0.088±0.012% of TSP) than in PpsbA-Trxf microtubers (0.067±0.012% of TSP) (Fig. 3B). Semi-quantitative RT-PCR was performed to analyse whether Trx f protein accumulation after the light treatment was due to transcriptional activation. The content of *trx f* transcripts was slightly higher (twice) in microtubers developed under light conditions than in those developed in continuous darkness (Fig. 3C). This effect was observed after only 1 week of light treatment and was maintained during the 3 weeks of microtuber growth. A similar transcriptional activation response was observed with both promoters.

3.4 Increased expression levels of *trx f* under different post-harvest storage conditions of tubers

To determine whether exposure to different temperature and light regimes could influence recombinant protein accumulation, detached PrrnG10L-Trxf and PpsbA-Trxf microtubers were incubated in Petri dishes for 4 weeks under different conditions: 4 ºC/dark, 24 ºC/dark and 24 ºC/16 h photoperiod. Clear induction was observed under the light treatment, with up to 46.7 times (PpsbA)

and 9 times (PrrnG10L) higher expression being detected than at the initiation of post-harvest conditions (day 0, Fig. 4A). The final accumulation of Trx f was 0.062±0.007% and 0.14±0.02% of TSP in PrrnG10L-Trxf and PpsbA-Trxf microtubers, respectively. Cold treatment at 4 ºC also induced Trx f accumulation in PrrnG10L-Trxf microtubers (up to 5 times), but not in PpsbA-Trxf microtubers. This effect was specific to the low temperature as microtubers kept at 24 ºC in the dark showed no induction and instead presented a progressive decline in Trx f protein levels (Fig. 4A). The transcript levels of *trx f*, determined via semi-quantitative RT-PCR, increased 3-4 times under the 4 ºC/dark and 24 ºC/16 h photoperiod treatments in PrrnG10L-Trxf microtubers, whereas no effect was detected in the 24 ºC/dark treatment (Fig. 4B). In contrast, a 2-fold increase of *trx f* transcription was detected in PpsbA-Trxf microtubers only under the 24 ºC/16 h photoperiod treatment.

Enhanced Trx f accumulation was also detected when soil-grown tubers were subjected to light treatment for 10 days, with up to 55 times (PpsbA) and 8 times (PrrnG10L) higher levels of Trx f being observed (Fig. 4C), reaching 0.082±0.008% and 0.11±0.02% of TSP in PrrnG10L-Trxf and PpsbA-Trxf tubers, respectively. However, contrary to the result obtained in microtubers, no induction was detected under cold treatment at 4 ºC in the assayed condition.

Soil-grown tubers stored at 4 ºC for 8 months maintained the capacity to light-induce the accumulation of Trx f, with up to 41 times (PpsbA) and 6 times (PrrnG10L) increase in the outer layer of tubers (Fig. 4D). As expected, no induction was observed in the inner part of tubers.

4 Discussion

In this work, we overexpressed the *trx f* and *m* genes from the potato plastid genome and conducted a detailed study of transgene expression in amyloplasts, primarily during tuber development and under different post-harvest storage conditions. By using the potato-specific flanking sequences *trnI* and *trnA* for transgene integration via homologous recombination in the plastome, we obtained transplastomic plants with a high efficiency. The importance of using potato flanking sequences in the transformation vector for improving

transformation efficiency was previously highlighted [5, 20]. For use as regulatory sequences in the transformation vector, we selected two different promoters, from the *rrn* and *psbA* genes. The *rrn* operon promoter was chosen due to the high transcription level of the *rrn16* gene in tubers [7]. In addition, the analysis of different regulatory sequences showed that the *rrn* promoter generates a high level of *gfp* expression in tomato fruits [21] and produces the best results in potato tubers [5]. This promoter was fused to the leader region of *gene 10* of the bacteriophage T7 (G10L). The PrrnG10L combination resulted in very high levels of foreign protein accumulation in chloroplasts [1, 22]. In the case of the *psbA* gene, the promoter shows a moderate transcription level in amyloplasts [7] but favours the accumulation of GFP in tomato chromoplasts [21]. In addition, there are several studies showing that the *psbA* 5′-UTR is responsible for the light-dependent translation of the endogenous gene [23] and of foreign genes, such as *hsa* [24], *cardiotrophin* [25] and *uidA* [12, 26, 27]. Therefore, we selected the *psbA* promoter and 5′-UTR because this feature could be of major interest in the post-harvest treatment of detached tubers.

Overexpression of the *trx f* or *m* gene from the plastid genome of potato had a negative impact on photosynthesis and tuber yields, with this effect being much more pronounced in PrrnG10L-Trxm plants. Although less marked than in potato, the overexpression of *trx m* in tobacco plants also showed a negative impact on the chlorophyll content, photosynthetic linear electron transfer, plant growth and leaf starch accumulation [14, 15]. However, in the case of *trx f*-overexpressing plants, results in potato contrast with those previously observed in tobacco plants, in which no phenotypic differences were observed between transplastomic and wild-type plants, but the starch content was significantly increased in leaves [14, 28]. Although both species belong to the same family, potato plants, unlike tobacco, have a natural storage organ (tuber) and this may impede leaves to accumulate high amounts of starch. Nevertheless, the starch levels in Trx f-overexpressing potato leaves were statistically indistinguishable from those of wild-type plants, despite their lower photosynthetic rate, implying a putative role of Trx f in the maintenance of starch levels. The main increase of the starch content in *trx f*-overexpressing tobacco leaves was reached at maturity [14], when tobacco leaves may act as temporary storage organs [29]. However, *trx f* overexpression failed to increase the starch content in potato tubers, suggesting

that redox mechanisms controlling starch metabolism are different between autotrophic and heterotrophic tissues. Furthermore, the physiological impairment in the transplastomic potato plants could also be due to pleiotropic effects caused by high endogenous levels of Trx in these plants. Aided by the advancement of techniques of proteomic analysis, approximately 400 potential targets of Trxs, related to a great variety of cellular processes, have been identified [30]. Therefore, the interactions of various cellular processes regulated by a given oxidoreductase in species with strong sink organs, such as potato, could be very different from that in the tobacco model plant. Additional investigations must be performed to elucidate the effect of *trx* overexpression in potato plants.

Northern and western blot analyses of different tissues revealed the highest transgene expression in the leaves, independent of the promoter (*rrn* or *psbA*) or the gene sequence (*trx f* or *m*) used. The transcript and protein levels in non-green plastids were dramatically reduced, especially in the roots. This is in agreement with previous observations made through genome-wide analysis of plastid gene expression in potato, where transcript accumulation was found to be much lower in tuber amyloplasts than in leaf chloroplasts [7, 31]. Transcriptomic analysis of tomato plants also revealed that most plastid genes are downregulated in fruits compared with leaves [8]. PrrnG10L is one of the best regulatory sequences conferring high-level gene activity in chromoplasts, resulting in the accumulation of GFP at levels up to 1% of total protein [21]. In contrast, the level of Trx f in potato tubers under the control of PrrnG10L is approximately two orders of magnitude lower than that of GFP in chromoplasts. This divergence points to basic differences between gene expression in chromoplasts and amyloplasts, assuming that differences in protein translatability and stability between GFP and Trx f are not an issue.

In vitro-grown microtubers offer some considerable advantages over soil-grown tubers, most notably in terms of the speed, synchronicity and reproducibility of the process [19]. By using a 4-week tuber induction protocol, it was possible to monitor the evolution of recombinant protein levels during microtuber development. Trx f levels decreased during the period of microtuber growth in the dark, irrespective of the promoter used. This was an expected result because swelling begins on axillary buds of shoots that were previously grown in the light. During the first week in the dark, the plastids of the axillary bud retain some their

original gene expression activity, but it declines rapidly with increased time in darkness. This pattern can be reversed by developing microtubers in the light. Very strong induction of *trxf* expression (up to 22 times) was observed in PpsbA-Trxf microtubers and was maintained throughout the period of growth in light. This induction was moderate (up to 11 times) in the case of PrrnG10L-Trxf microtubers. The analysis of *trxf* transcript levels via semi-quantitative PCR revealed increased transcript accumulation due to the light regime for both constructs. This suggests that the induction of gene expression by light is at least partially due to transcriptional activation. However, due to the discrepancy between the slight increment in transcript levels and the large increase in protein levels (up to 22 times), changes in translation rates are probably more important. The potato tuber is a storage organ that is suitable for physical treatments once it is detached from the mother plant. We investigated whether post-harvest cold or light treatments affected the accumulation of the recombinant protein. Similarly to the results observed in attached microtubers, strong induction of *trx f* gene expression (up to 47 times) was observed under the 16 h photoperiod regime in microtubers from both PrrnG10L-Trxf and PpsbA-Trxf plants. The regulatory sequences of the *psbA* gene triggered more potent stimulation of *trx f* expression. Induction was detected after only 7 days of light treatment. The same positive effect on gene expression (up to a 55-fold increase) was observed in soil-grown tubers. Moreover, stored tubers for 8 months maintained the capacity of increasing the accumulation of Trx f protein after the light treatment, being this effect specific of the outer layer of the tuber. Previously, in another study using the *psbA* promoter and 5'-UTR, an 18-fold increase in β-glucuronidase activity in potato tubers was observed after exposure to light for 8 days [12]. However, this light treatment was applied to tuber slices cultured in vitro, rather than using intact tubers. Taken together, these results are most likely due to the phenomenon of amyloplast-to-chloroplast transformation of potato tubers exposed to light. This is a fast process, especially in microtubers, where an increase in 16S and 23S rRNA is detectable after 2 d of illumination, whereas chlorophylls *a* and *b* can be detected after just 12 h of light exposure [32]. The final accumulation of the foreign protein was higher in microtubers than in soil-grown tubers. This is likely due to the higher greening capacity of microtubers [32] and to the difference in size, causing the strongest greening in the layers just below the periderm of soil-

grown tubers, with a steep gradient being observed inwards [33]. Our results are also consistent with the high expression levels of *rrn* genes reported in potato chloroplasts [7] and with the light-dependent translation conferred by the *psbA* 5′-UTR, as mentioned above.

Interestingly, cold induction (up to 5 times) of transgene expression was also detected, but only in the case of PrrnG10L-Trxf microtubers. Up to a 3-fold increase in transcript levels was measured, showing that the increase in protein accumulation was dependent, at least in part, on transcriptional activation. This response was independent of the gene sequence involved as it has also been detected in potato transplastomic plants overexpressing other transgenes (unpublished results). Additionally, this effect was tuber-specific because the other tissues analysed (leaf, stem and root) showed unaltered levels of Trx f after the cold treatment (data not shown). The observed cold induction was weaker than the induction under the light treatment. This result could not be due to the conversion of amyloplasts to chloroplasts because microtubers grown and stored in the dark are white, thus lacking chlorophyll, and protein synthesis occurs at very low levels [32, 34]. In addition, electron microscopy analysis showed that only amyloplasts and leucoplasts were visible. Recently, it has been demonstrated that all three plastidial RNA polymerases are active at the low temperature of 4 ºC during the stratification step of Arabidopsis seed germination, with up to a 10-fold increase in mRNA levels being observed relative to resting seeds [35]. During the stratification period, seed eoplasts have not been transformed into chloroplasts and show similarities to the experiment we performed with microtubers kept at 4 ºC in darkness. Plastid transcriptional activation of the *rrn* operon and most of the genes encoding ribosomal proteins was observed during seed stratification [35, 36]. Stratification at low temperature is usually performed to break seed dormancy, but it is also used to speed up the release of the dormant period of potato tubers. It is plausible that similar mechanisms responsible for transcriptional activation of the *rrn* operon in Arabidopsis could operate in potato microtubers under cold treatment. Further investigations are needed to address this hypothesis. On the other hand, we did not observe cold induction of transgene expression in the microtubers of PpsbA-Trxf plants. This is in line with the *psbA* transcript being downregulated after the stratification of Arabidopsis seeds [35]. Surprisingly, Trx f protein levels in soil-

grown tubers did not increase after cold treatment for 10 days. The reason for this result is unknown, but it could be that the time period at 4 ºC was not sufficiently long to trigger the response. Physiological processes appear to develop more rapidly in microtubers than in soil-grown tubers [32].

5 Concluding remarks

In this work, we demonstrate that simple post-harvest light treatment (16 h photoperiod) of whole microtubers or soil-grown tubers induces up to 55 times higher accumulation of the recombinant protein just in 7-10 days. Under continuous light exposure, this period of time could most likely be shortened. Potato offers several advantages as a bioreactor for foreign protein production: high biomass, ease of storage and transport of tubers, and stability of the recombinant protein in tubers stored at 4 ºC over time [37, 38]. Therefore, the light-induction method described in this article could be an attractive biotechnological approach for the production of recombinant proteins via soil-grown tubers, particularly for minitubers with a high surface-to-volume ratio, followed by post-harvest light treatment prior to the extraction and purification of foreign proteins.

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

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Table 1 Thioredoxin expression levels (expressed as the percentage of TSP) in transplastomic potato plants. Plants were grown in the greenhouse for the analysis of different tissues. The presented values are the mean±SE of 3 independent experiments. ND, not detected.

	Leaf	**Stem**	**Root**	**Tuber**
PrrnG10L-Trxf	2.6 ± 0.37	0.043 ± 0.001	0.0016 ± 0.000	0.011 ± 0.001
PpsbA-Trxf	4.2 ± 0.49	0.065 ±0.001	0.0008 ± 0.000	0.002 ± 0.000
PrrnG10L-Trxm	1.0 ±0.10	0.006 ±0.000	ND	0.0008 ± 0.000

Figure 1

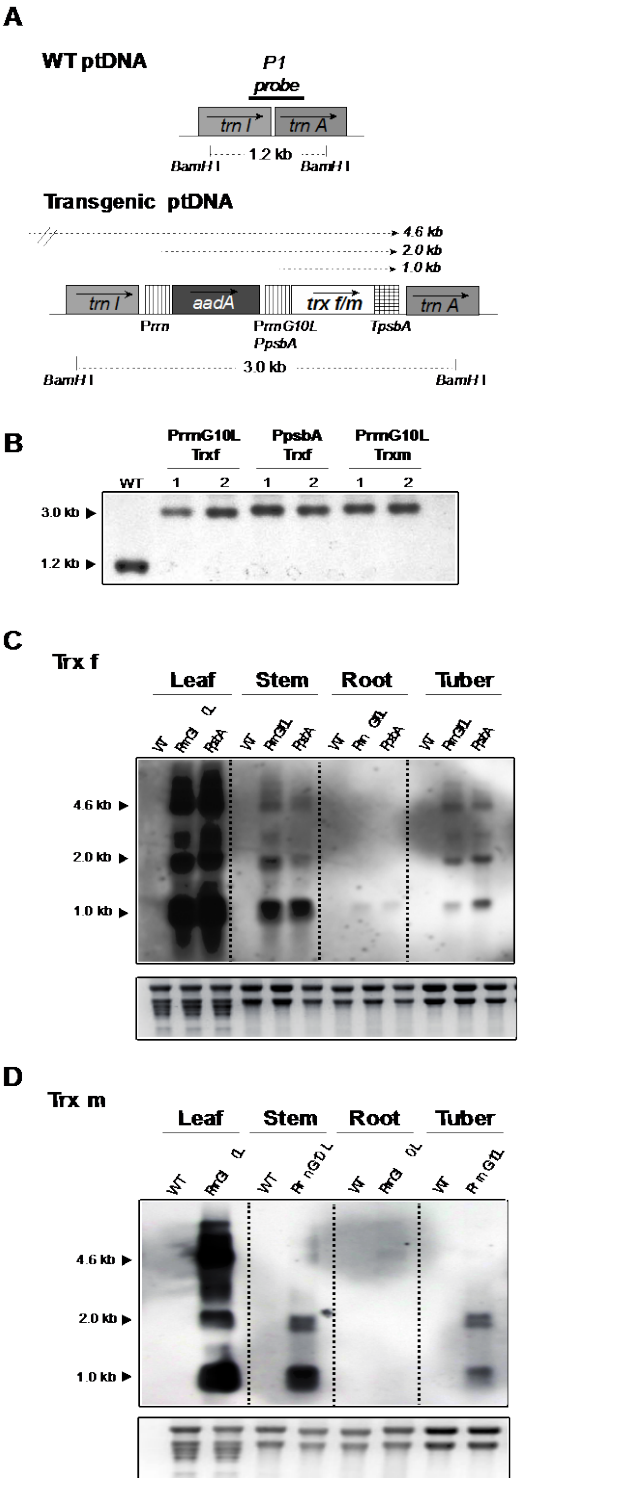


Fig. 1 Integration of *trx f/m* genes into the potato plastid genome. (**A**) Map of the WT and *trxs* transformed plastid genomes. The transgenes are targeted to the intergenic region between *trnI* and *trnA*. The selectable marker gene *aadA* is driven by the 16S ribosomal RNA operon promoter (*Prrn*). *trx f* is driven by the

rrn promoter fused to the 5'-untranslated region (UTR) of phage T7 *gene 10* (*PrrnG10L*) or the *psbA* promoter and UTR (*PpsbA*). *trx m* is driven by *PrrnG10L.* Arrows within boxes show the direction of transcription. Numbers below ptDNA indicate the predicted hybridising fragments when total DNA was digested with *BamH*I. A 0.8-kb fragment of the targeting region for homologous recombination was used as a probe (P1) for the Southern blot analysis. (**B**) Southern blot analysis of two independent lines (1, 2) for each transformation cassette. (**C, D**) Analysis of *trx f* and *trx m* transcript expression in different tissues of transplastomic plants. Ethidium bromide-stained rRNA was used to assess loading. The expected transcript sizes of mono, di- and polycistrons originating from different promoters are indicated in (A). *trnI, trnA*: original sequences of the potato plastid genome; *aadA*: aminoglycoside 3'-adenylyltransferase; *ptDNA*: plastid DNA; *TpsbA*: terminator region of the *psbA* gene; WT: wild type; *trx*, thioredoxin.

Figure 2

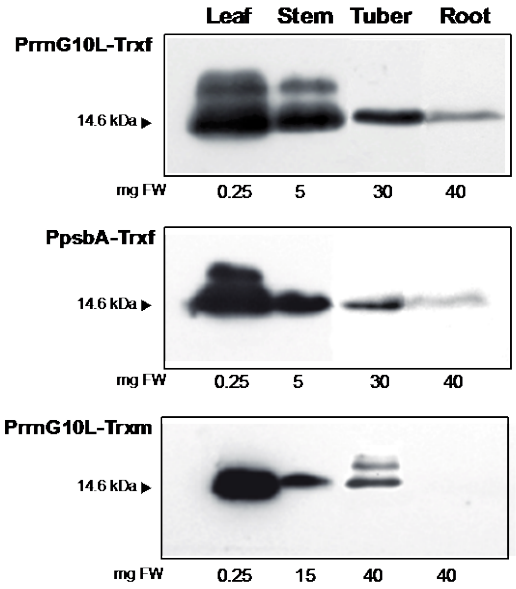


Fig. 2 Immunoblot analysis of Trx f and Trx m protein expression in different tissues of transplastomic plants that were hydroponically grown in the greenhouse. Trx f protein expression in PrrnG10L-Trxf and PpsbA-Trxf plants and Trx m protein expression in PrrnG10L-Trxm plants. The amount of fresh tissue (mg of fresh weight, mg FW) from which total protein extract was obtained is indicated below each panel. Specific anti-Trx f and anti-Trx m antibodies were used.

Figure 3

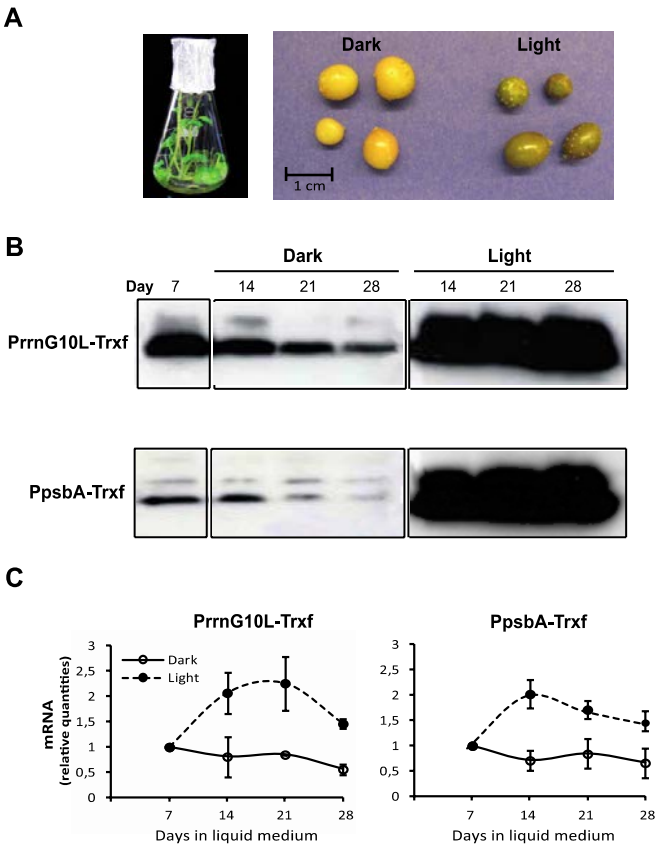
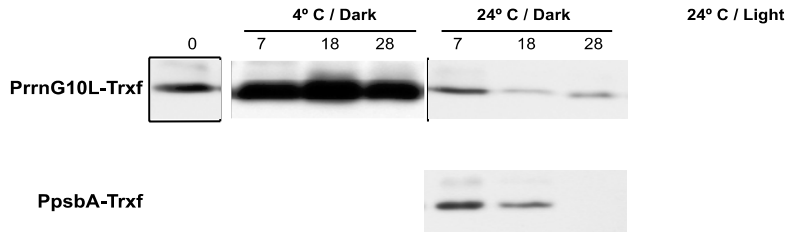


Fig. 3 Influence of *PrrnG10L* or *PpsbA* regulatory sequences on *trx f* gene expression in microtubers developed in vitro. (**A**) Microtubers were induced on shoots grown in liquid medium and were harvested after 7-28 days of continuous darkness or 14-28 days under a 16 h photoperiod (first week in darkness). Average weight of microtubers after 28 days: 300-500 mg. (**B**) Trx f protein expression during microtuber development. The protein extract from a 15 mg microtuber sample was loaded in each lane. (**C**) Transcription rates during microtuber development. *trx f* transcripts were estimated via semi-quantitative RT-PCR using specific *trx f* and *18S rRNA* primers to normalise the relative amount of *trxf* transcripts in each reaction. The transcript levels were also related to values obtained from microtubers harvested after 7 days. Means and standard errors are shown (n=3-5).



C

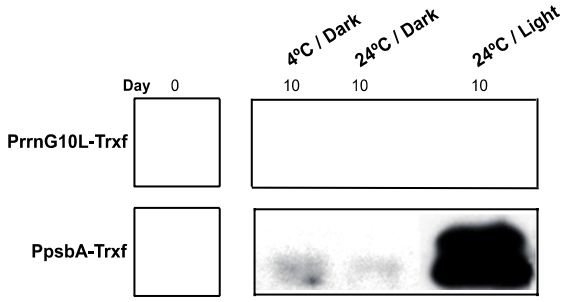


Fig. 4 Influence of post-harvest storage conditions on *trx f* gene expression in microtubers and soil-grown tubers. (**A**) Immunoblot analysis of microtubers from PrrnG10L-Trxf and PpsbA-Trxf plants. Microtubers that developed for 28 days in darkness (day 0) were subjected to three different combinations of environmental conditions for 7, 18 and 28 days: 4ºC/darkness, 24ºC/darkness and 24ºC/16 h photoperiod. The protein extract from a 15 mg microtuber sample was loaded in each lane. (**B**) Transcription rates during microtuber storage. *trx f* transcript levels were estimated via semi-quantitative RT-PCR using specific *trx f* and *18S rRNA* primers to normalise the relative amount of *trxf* transcripts in each reaction. Transcript levels were related to the values obtained from microtubers harvested on day 0. Means and standard errors are shown (n=3-5). (**C**) Immunoblot analysis of soil-grown tubers subjected to the same environmental conditions indicated

above for 10 days. The protein extract from a 10 mg tuber sample was loaded in each lane.

Supplementary information

Table S1 Physiological and biochemical analysis of *trx f* and *trx m*-overexpressing transplastomic potato plants. The presented values are the mean±SE from 12 individual plants (except for the tuber starch content, which was obtained from 6 plants). Different letters indicate significant differences ($P<0.05$) using the Tukey-Kramer test.

	WT	PrrnG10L-Trxf	PpsbA-Trxf	PrrnG10L-Trxm
Chlorophyll (SPAD)[a]	37,3 ± 0,4 **a**	33,8 ± 0,3 **b**	35,1 ± 0,4 **b**	21,1 ± 0,3 **c**
Photosynthesis (μmol·CO_2/m^2/s)[b]				
200 μmol/m^2/s	8,8 ± 0,2 **a**	6,7 ± 0,2 **b**	6,2 ± 0,2 **b**	4,0 ± 0,3 **c**
1000 μmol/m^2/s	20,3 ± 0,6 **a**	15,1 ± 0,6 **b**	14,1 ± 0,5 **b**	6,0 ± 0,5 **c**
Starch content (μmol Glu/g FW)[c]				
Leaf	36,9 ± 2,2 **a**	32,8 ± 4,2 **a**	32,5 ± 2,9 **a**	12,4 ± 0,7 **b**
Tuber	847,8 ± 43,9 **a**	658,1 ± 50,7 **b**	676,0 ± 44,9 **b**	648,5 ± 21,6 **b**

a) Chlorophyll concentration was measured using a Minolta SPAD 502 chlorophyll metre. b) Photosynthesis was quantified using a portable photosynthesis measurement system (LCpro-SD, ADC BioScientific). c) starch was quantified using an amyloglucosidase-based test kit (R-Biopharm).

Figure S1

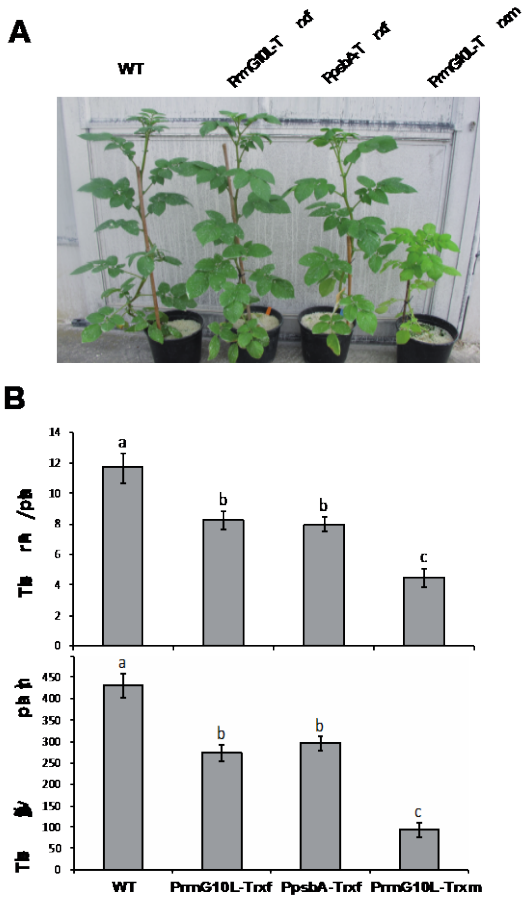


Fig. S1 Analysis of tuber yields in Trx f and m transplastomic plants. (**A**) Phenotypic appearance of potato plants at the time of harvest. (**B**) The tuber number and total tuber weight per plant were quantified in plants that were hydroponically grown in the greenhouse for 16 weeks. Mean and standard errors are shown (n=13-15). Different letters above columns indicate significant differences among lines (P<0.05) using the Tukey-Kramer test.