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Overexpression of plastidial thioredoxin f leads to enhanced starch accumulation in tobacco leaves

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Summary

Starch, the most abundant storage carbohydrate in plants, has been a major feedstock for first-generation biofuels. Growing fuel demands require, however, that the starch yields of energy crops be improved. Leaf starch is synthesised during the day and degraded at night to power nonphotosynthetic metabolism. Redox regulation has been associated with the coordination of the enzymes involved in starch metabolism, but neither the signals nor mechanisms that regulate this metabolism are entirely clear. In this work, the thioredoxin (Trx) *f* and *m* genes, which code for key enzymes in plastid redox regulation, were overexpressed from the plastid genome. Tobacco plants overexpressing *Trx f,* but not *Trx m*, showed an increase of up to 700% in leaf starch accumulation, accompanied by an increase in leaf sugars, specific leaf weight (SLW), and leaf biomass yield. To test the potential of these plants as a nonfood energy crop, tobacco leaves overexpressing *Trx f* were subjected to enzymatic hydrolysis, and around a 500% increase in the release of fermentable sugars was recorded. The results show that Trx f is a more effective regulator of photosynthetic carbon metabolism *in planta* than Trx m. The overexpression of *Trx f* might therefore provide a means of increasing the carbohydrate content of plants destined for use in biofuel production. It might also provide a means of improving the nutritional properties of staple food crops.

Introduction

Biofuels have the potential to reduce our dependence on fossil fuels as well as reduce the environmental damage caused by their extraction and use. A search for nonfood energy crops for the economically viable production of environmentally friendly biofuels is therefore underway. Starch has been a major feedstock for first-generation biofuel production as it is relatively easy to convert into fermentable sugars (Smith, 2008). Improving our knowledge of how carbohydrates are metabolised in plants could help in the development of crops with increased starch and sugar contents, improving the efficiency of biofuel production.

Starch is an insoluble glucan composed of two glucose polymers: amylose and amylopectin. It is synthesised in the plastids of both photosynthetic and nonphotosynthetic cells. In leaves, a portion of the photosynthetically fixed carbon is retained in the chloroplasts to synthesise starch as a transient carbon store; this is remobilised during the night to support nonphotosynthetic leaf metabolism and the export of sucrose. Both the synthesis and degradation of starch in chloroplasts has been studied, and the pathways and enzymes involved have been characterised. However, the signals and mechanisms that regulate starch metabolism in response to environmental and metabolic signals remain unclear. Diurnal starch accumulation appears to involve several regulatory mechanisms, including allosteric regulation by metabolites, reversible phosphorylation, and redox regulation (Buchanan and Balmer, 2005; Kötting *et al.*, 2010). Reversible glucan phosphorylation is essential in the control of starch degradation (Stitt *et al.*, 2010), and the number of enzymes involved in starch metabolism and believed to be subjected to phosphorylation has increased in recent years (Kötting *et al.*, 2010). Redox regulation, which has been widely studied in the context of starch synthesis, has also been associated with the coordination of enzymes involved in starch degradation (Kötting *et al.*, 2010). The redox regulation of the activity of ADP-glucose pyrophosphorylase (AGPase) (Geigenberger, 2011) and β-amylase 1 (BAM1) (Sparla *et al.*, 2006; Valerio *et al.*, 2010), and the redox regulation of the activity and the starch granule-binding capacities of α-glucan, water dikinase (GWD or SEX1) (Mikkelsen *et al.*, 2005) and phosphoglucan phosphatase (DSP4 or SEX4) (Silver *et al.*, 2013; Sokolov *et al.*, 2006) are all well known. Proteomic techniques have identified other potential redox-regulated starch-related enzymes including a major membrane transporter (Brittle-1 or ADP-glucose transporter) and starch-branching enzyme IIa (SBE IIa) (Balmer *et al.*, 2006). Recently, the activities of the isoamylase complex (ISA1/ISA2), limit dextrinase (LDA), and starch synthases (SS1 and SS3) have been reported increased by their reduction at physiological redox potentials (Glaring *et al.*, 2012). The plastid thioredoxins (Trxs) f and m have been shown to act *in vitro* as reductants for many of the redox-regulated enzymes involved in starch metabolism, suggesting that the redox regulation that occurs in response to light signals is similar to that controlling other photosynthetic enzymes (Schürmann and Buchanan, 2008). The f- and m-type Trxs transfer reducing power from photosynthetically reduced ferredoxin (Fd) to target enzymes through the action of ferredoxin–thioredoxin reductase, thus increasing or reducing their activity. As the discovery of Trx f and m in chloroplasts, a growing number of putative target enzymes have been identified, and the molecular mechanisms underlying Trx-dependent regulation, including those in which Trxs interacts

with other redox regulators such as glutaredoxins (Grxs) or glutathione (Lemaire *et al.*, 2007; Meyer *et al.*, 2009), have been elucidated. The f- and m-type Trxs differ in primary structure and show *in vitro* specificity for some Calvin cycle enzymes (Schürmann and Buchanan, 2008).

Based on the notably influence of redox regulation on photosynthetic and carbon metabolism enzymes, the possibility that the overexpression of chloroplastic Trx f or Trx m might increase the rate of carbohydrate synthesis in plants was explored. Previously isolated and characterised f- and m-type tobacco Trxs (Sanz-Barrio *et al.*, 2012) were overexpressed in the tobacco plastid genome. Plants overexpressing Trx f, but not Trx m, showed a striking increase in leaf starch accumulation, showing that Trx f is more effective at regulating leaf carbon metabolism than Trx m. However, the post-translational redox activation of AGPase, which other authors report is increased *in vitro* by Trx f and Trx m (Ballicora *et al.*, 2000; Geigenberger *et al.*, 2005), remained unaffected in the present plants. Enzyme hydrolysis assays revealed the potential of Trx f-overexpression in the development of high starch-accumulating plants for use as alternative energy crop.

Results

Generation of transplastomic tobacco plants overexpressing Trx f and Trx m

Previously isolated tobacco *Trx f*- or *Trx m*-coding sequences (Sanz-Barrio *et al.*, 2012) were inserted into the chloroplast genome under the control of three different 5′-regulatory sequences—P*rrn*, P*rrnG10*L and P*psbA*—associated with different protein expression levels (Farran *et al.*, 2008) (Figure 1a). The chimeric *Trx* genes were cloned into the chloroplast transformation pL3 vector containing the selectable spectinomycin resistance gene (*aadA*), flanked by two *loxP* sequences suitable for CRE-lox-mediated marker gene excision (Corneille *et al.*, 2001). The transformation vector targeted the insertion of the foreign genes into the plastid genome between the *16S3′/trnV* and 3′*rps12* genes in the duplicated inverted repeat region. The resulting tobacco transformants, termed *rrnf*—*rrnm*, *G10Lf*—*G10Lm*, and *psbAf*—*psbAm*, expressed *Trx f* and *Trx m* under the control of the P*rrn*, P*rrnG10L* and P*psbA* promoters, respectively.

Total cellular DNA from independent transformants was digested with *Bgl*II, electrophoresed, transferred to nylon membranes, and probed with a ptDNA flanking region fragment (P1 probe, Figure 1a). Southern blot analyses confirmed the stable integration of the foreign genes into the transplastomic tobacco chloroplasts; it also confirmed homoplasmy via the absence of detectable wild-type genomes (4.5 kb, Figure 1b).

Transplastomic lines show different Trx accumulation levels

Trx f and Trx m overexpression was confirmed by immunodetection (Figure 2a). Different degrees of accumulation were seen in the transplastomic lines. The immunoreactive signal was only detected in the *G10Lf/m* and *psbAf/m* lines when 10 μg of total protein (tp) was loaded per lane, with the Trx levels in the *psbAf/m* lines higher than in *G10Lf/m* lines. Overexpressed Trx in the *rrnf/m* lines was undetectable in these blots (Figure 2a). It was visible, however, when 50 μg was loaded (data not shown), and the expression level was analogous to the wild-type endogenous protein. The accumulation of Trx in transplastomic plants was quantified by Western blotting using serial dilutions of Trx f and

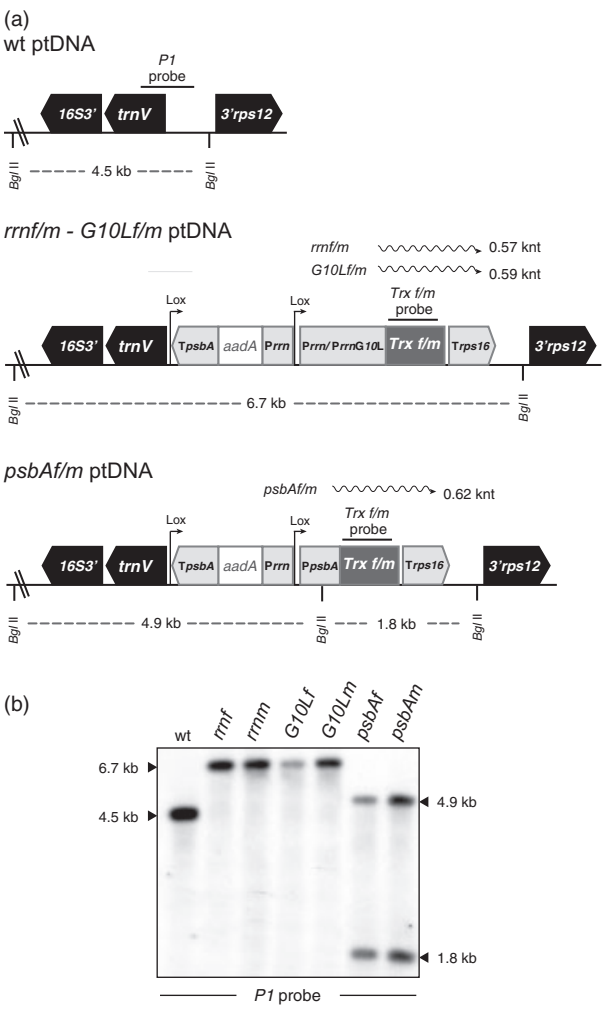

Figure 1 Integration of *Trx f/m* genes into the plastid genome and homoplasmy. (a) Map of the wild-type (wt) and transformed plastid genomes. P1 and Trxf/m probes are shown over the corresponding sequence. Transcripts are denoted by zigzag arrows, and the expected sizes are indicated. *16S3′*, *trnV*, *3′rps12*: original sequences of the chloroplast genome; *aadA*: aminoglycoside 3′-adenylyltransferase gene; P*rrn*: *16S* rRNA promoter and 5′-untranslated region; P*psbA*: *psbA* promoter and 5′-untranslated region; P*rrnG10*L: *16S* rRNA promoter fused to the leader region of the bacteriophage T7 gene *10*; Trps16: terminator region from the chloroplast *rps16* gene. (b) Southern blot analysis. Total DNA (10 μg) was digested with *Bgl*II and probed with P1. One line per construct and a wt control is shown.

Trx m proteins produced in *E. coli* as standards (Sanz-Barrio *et al.*, 2011). The transplastomic *G10Lf* line accumulated Trx f at approximately 0.4% tp in mature leaves, whereas the *psbAf* line accumulated tenfold more Trx f protein (~ 4% tp, Figure 2b), which represent an over 20-fold and over 200-fold increase compared with the endogenous protein level. The *G10Lm* and *psbAm* lines accumulated approximately 0.6% and 1% tp, values 15 and 30 times the Trx m expressed in the wild type, respectively.

Northern blot analysis of total mRNA revealed no significant differences in the *Trx* transcript content among the *rrnf/m* and *G10Lf/m* lines (Figure 2c), suggesting that differences in protein accumulation are most likely due to enhanced translation by

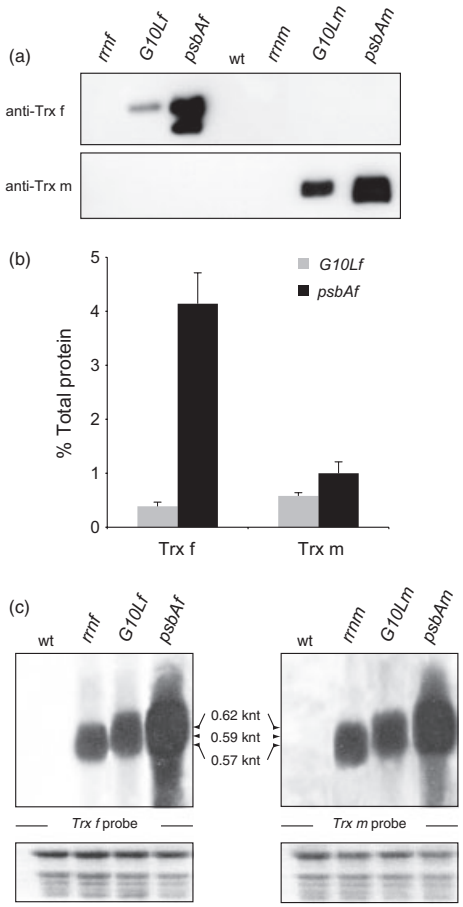

Figure 2 Analysis of Trx f and Trx m expression in transplastomic plants. (a) Immunoblot analysis of protein extracts from mature leaves (fourth from the top) of the wild-type (wt) and transgenic lines. 10 μg of total protein is loaded per lane. Specific anti-Trx f and anti-Trx m antibodies were used. (b) Trx f and Trx m quantification in leaves from transgenic tobacco plants by Western blot analysis shown as percentage of total protein (tp). Results are the mean ± SE (bars) of two measurements for three independent transgenic plants per construct. (c) Northern blot analysis of total RNA isolated from transplastomic and wt control plants. 10 μg RNA was electrophoresed in denaturing conditions, blotted onto a nylon membrane and hybridised with Trxf/m-specific probes (Figure 1a). As load controls, EtBr-stained total leaf RNAs are reported. The expected transcript sizes are indicated as described in Figure 1a.

the presence of the G*10*L sequence (Staub *et al.*, 2000). However, *Trx* transcripts from the P*psbA* promoter were much more abundant than those from the P*rrn* or P*rrn*G10L promoters (Figure 2c), a consequence of either the strength of the promoter or the stability of the transcript afforded by the *psbA* 5′UTR sequence (Zou *et al.*, 2003). The increased mRNA levels in the *psbAf/m* lines may be responsible to some extent for the increased amount of protein observed in these lines.

Phenotypic analysis of Trx-overexpressing plants

T_1 generation transformants were used for phenotypic analysis. No differences in germination were observed between Trx f-overexpressing plants, the *rrn*m line and the wild type, whereas a slight delay (2–3 days) was observed in the *G10Lm* and *psbAm* lines. *G10Lm* and *psbAm* plants also showed a pale-green phenotype throughout the entire life cycle [a reduction in chlorophyll content was confirmed (Table 1)] and a slight delay in flowering. However, all of the transplastomic lines were fertile, produced mature seeds, and did not differ from the wild type with respect to plant size and leaf number (Table 1). The *G10Lf* and *psbAf* lines showed an increase in specific leaf weight (SLW) of more than 20% and 27%, respectively, compared with wild-type plants (Table 1). Similar carbon assimilation rates were observed in the wild type and transformants under ambient CO_2 concentrations (360 ppm) and under saturating (1000 μmol/m^2/s) and low light intensities (200 μmol/m^2/s) (Table 1). The lower chlorophyll content detected in the Trx m-overexpressing lines did not affect photosynthetic efficiency. These results show that the overexpression of plastid Trx f or m in tobacco chloroplasts was not accompanied by any significant change in photosynthesis or growth under the present assay conditions, except for the increase in SLW observed in plants overexpressing the f-type Trx.

Plants overexpressing Trx f, but not Trx m, produced large quantities of leaf starch

Plants overexpressing Trx f (*G10Lf* and *psbAf*) that were grown in a greenhouse under natural sunlight accumulated exceptionally higher levels of leaf starch than wild-type plants (Figure 3a), which could likely account for the increased SLW observed in these plants (Table 1). The Trx m-overexpressing plants, in contrast, produced the same amounts of starch as the wild-type plants, or even less. As expected, the *rrnf* plants, with expression levels analogous to the wild-type endogenous Trx f, did not differ from the wild type with respect to starch production (Figure 3a). However, no clear relationship was detected between Trx f and starch accumulation in the *G10Lf* and *psbAf* lines (Figure 2b and 3a). In fact, the greatest increase in starch accumulation was observed in the *G10Lf* line, which had a lower Trx f content than the *psbAf* plants. Moreover, the daily starch turnover shows more starch accumulation in the Trx f-overexpressing plants (Figure 3b), with no apparent differences in the rate of starch synthesis and degradation between the transgenic and control plants. Potassium iodide-stained leaf discs verified the striking difference in starch accumulation between the transgenic and wild-type plants (Figure 3c). Similar behaviour was observed in the plants cultured in the phytotron under supplemental lighting (200 μmol/m^2/s). However, the differences in starch accumulation between the wild-type and transformed plants were smaller (Figure S1). All subsequent analyses were therefore performed using the greenhouse-grown plants (unless otherwise specified). These findings suggest that light intensity plays a crucial role in the starchy phenotype caused by Trx f overexpression.

The presence of starch in transgenic chloroplasts was also monitored by ultrastructural analysis in the *G10Lf*, *G10Lm* and wild-type plants (Figure 3d–h). The *G10Lm* chloroplasts showed starch granules (Figure 3e and e′) similar to those of wild-type plants (Figure 3d and d′). In contrast, the leaf mesophyll cells of the *G10Lf* plants had substantially enlarged chloroplasts, a consequence of massive starch accumulation (Figure 3f and f′). This accumulation was observed in all chloroplasts of the mesophyll cells in both transverse (Figure 3f) and longitudinal section (Figure 3g). Interestingly, the grana and individual thylakoids (the ultrastructural compartments involved in photosynthetic reactions) appeared unaltered (Figure 3h) despite the abnormal size of the starch deposits. These results confirm that

Table 1 Phenotypic analysis of Trx f and Trx m-overexpressing tobacco plants

Phenotypic character	wt	*rrnf*	*G10Lf*	*psbAf*	*rrnm*	*G10Lm*	*psbAm*
Height (cm)	165.4 ± 6.5	163.0 ± 8.3	178.6 ± 6.3	167.0 ± 6.1	169.0 ± 9.8	178.8 ± 2.1	163.0 ± 9.6
Leaf number	13.7 ± 0.1	13.5 ± 0.2	13.3 ± 0.1	13.3 ± 0.3	14.1 ± 0.1	14.0 ± 0.0	13.7 ± 0.3
Specific Leaf Weight (SLW, mg/cm^2)	2.51 ± 0.13	n.d	3.02 ± 0.10*	3.19 ± 0.17*	n.d	2.83 ± 0.15	2.71 ± 0.10
Chlorophyll (SPAD)	39.4 ± 1.1	39.6 ± 0.7	41.6 ± 1.1	40.1 ± 0.9	38.0 ± 0.7	31.6 ± 0.6*	31.6 ± 0.8*
Photosinthesis (μmol·CO_2/m^2/s)							
1000 μmol/m^2/s	13.10 ± 0.73	13.10 ± 1.53	12.67 ± 1.52	11.26 ± 0.75	13.35 ± 1.46	10.97 ± 0.98	11.59 ± 0.23
200 μmol/m^2/s	7.02 ± 0.72	7.04 ± 0.21	8.17 ± 0.35	7.46 ± 0.75	7.52 ± 0.27	7.81 ± 0.37	6.87 ± 0.75

Phenotypic traits were analysed over greenhouse growing plants at the beginning of ripening state. Values are the mean ± SE of six individual plants. n.d: not determined. *$P \leq 0.05$ (*t*-test) compared with control plants.

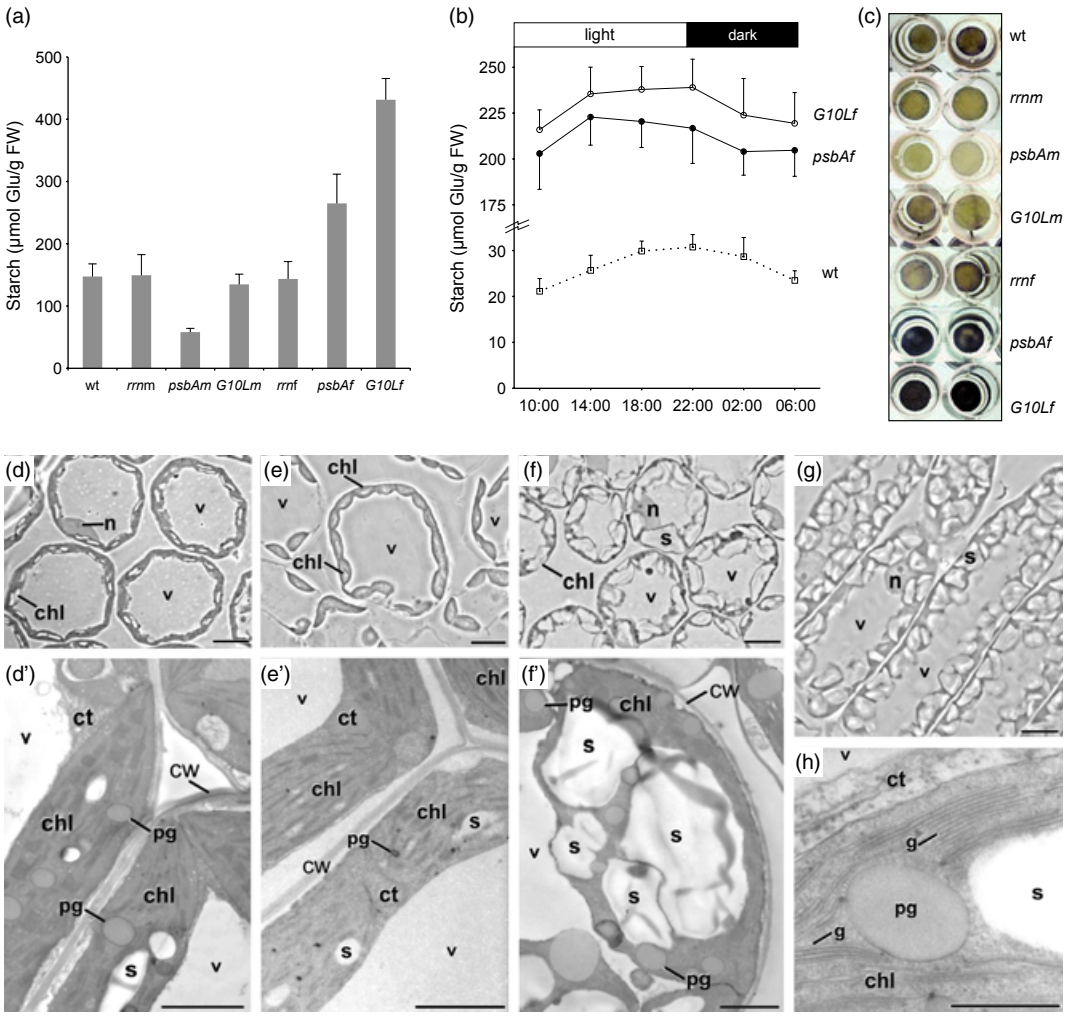

Figure 3 Trx f overexpression promotes accumulation of starch in tobacco leaves. (a) Starch levels from mature leaves (fourth from the top) of greenhouse growing plants harvested at the ripening stage with first capsules darkened, after 12 h in the light period. Each value is the mean ± SE (bars) of six individual plants. (b) Characterisation of the starch accumulation pattern throughout the diurnal cycle from mature leaves (fourth from the top) of greenhouse-grown plants harvested at ripening stage. The open bar atop the figure represents the light period, the filled bar indicates time in darkness. Each data point is the mean ± SE (bars) of two independent samples from six individual plants. (c) Iodine staining of leaf discs from the same leaves used in (a). (d–h) Ultrastructural examination of chloroplasts overexpressing Trx. (d–g) Leaf mesophyll cells under light microscope from wild type (wt) (d), *G10Lm* (e), and *G10Lf* (f and g). (d'–f', h) Ultrastructure of chloroplasts from wt (d'), *G10Lm* (e'), and *G10Lf* (f'). (h) Detail of a chloroplast from *G10Lf* with typical grana. chl, chloroplast; n, nucleus; v, vacuole; ct, cytoplasm; cw, cell wall; g, grana; pg, plastoglobule; s, starch. Scale bars in (d–g): 10 μm; (d'–f'): 2 μm; (h): 500 nm.

the increased starch accumulation observed in tobacco leaves is due only to the overexpression of the Trx f gene and that Trx m is less effective in the activation of photosynthetic carbohydrate metabolism.

Overexpression of Trx f in chloroplasts does not affect light redox activation of AGPase in tobacco leaves

AGPase in higher plants is a heterotetramer consisting of two large (AGPS) and two small (AGPB) subunits, which catalyses the first committed step of starch synthesis. AGPase is sensitive to allosteric regulation, being activated by glycerate-3-phosphate (3PGA) and inhibited by Pi (Preiss *et al.*, 1991), as well as to post-translational redox regulation (Fu *et al.*, 1998). *In vitro* experiments using heterologously expressed potato tuber AGPase and pea leaf chloroplasts have shown that Trx f and Trx m mediate the change in the redox status of the small subunit, leading to an increase in AGPB monomerisation (Ballicora *et al.*, 2000; Geigenberger *et al.*, 2005). Because the overexpressed Trx f was mostly reduced in the light (Figure S2) and presumably was able to transfer the reducing equivalents from photosynthesis to their target enzymes, we investigated whether the starchy phenotype observed in the Trx f-overexpressing plants was due to an increase in AGPase redox activation by means of the Trx reducing power. For that purpose, the degree of AGPB monomerisation was analysed in leaf samples collected at the end of the light (12 h) and dark (9 h) periods. AGPB was partially converted to monomer form in the light and appeared mostly dimerised in the dark in wild-type and Trx f-overexpressing leaves (Figure 4a). A densitometric quantification of Western blot protein bands of the oxidised (100 kDa) and reduced (50 kDa) forms of AGPB revealed a doubling of AGPB monomerisation in the light, with no significant differences between Trx f-overexpressing lines and wild-type plants (Figure 4b). The starch content was measured in the same leaf material to confirm a starchy phenotype (Figure 4c). When the extracts were treated with a reductant (DTT) or an oxidant (diamide) before loading onto the gel, the immunosignal was detected only at 50 kD or 100 kD, respectively (Figure 4a), showing that the AGPB intermolecular bridge is indeed redox regulated, but that its reduction is not affected by enhanced levels of either Trx f or Trx m (Figure S3) *in planta*. These results suggest that the high starch accumulation levels observed in Trx f-overexpressing plants are unlikely due to an increase in AGPase redox activation.

Carbohydrate accumulation pattern in Trx f-overexpressing plants

The optimum time (from a biofuel production point of view) for harvesting the Trx f-overexpressing plants was determined by evaluating the accumulation of carbohydrate over crop development. Maximum starch accumulation in these plants occurred at the end of the growth cycle, when all the seed capsules were darkened (Figure 5a). At this stage, the starch content in *psbAf* and *G10Lf* plant leaves was up by 500% and 700%, respectively. Additionally, hexoses and sucrose were measured at the ripening stage. The sucrose content was increased by up to 100% in the leaves (Figure 5b). Moreover, a significant increase in glucose and fructose levels was observed in the *G10Lf* line, which showed the greatest starch accumulation. These data show that the overexpression of Trx f in tobacco chloroplasts provides not only good starch raw material, but also increases the soluble sugar content of tobacco leaves.

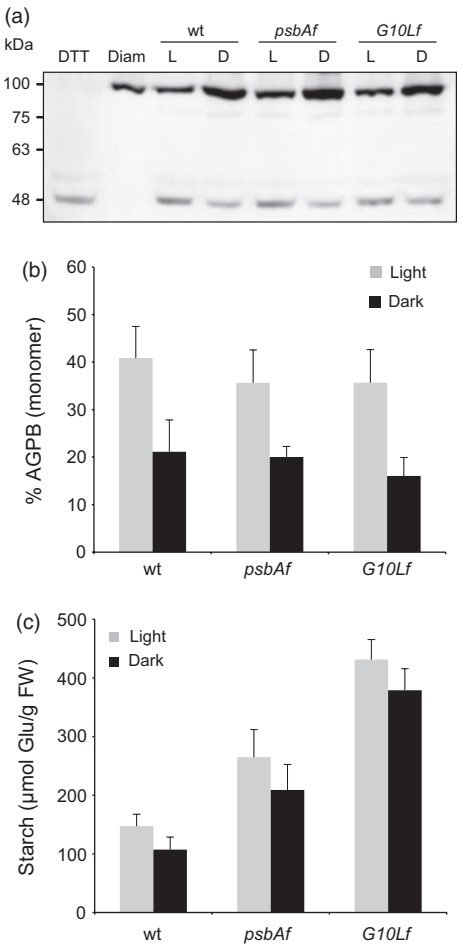

Figure 4 Redox activation of ADP-glucose pyrophosphorylase (AGPase) in Trx f-overexpressing tobacco leaves. (a) Redox status of AGPase of mature leaves (fourth from the top) from wild type (wt) and transformants grown in greenhouse sampled at the end of the light (L, 12 h) or dark (D, 9 h) periods at the ripening stage with first capsules darkened. Wild-type samples incubated with 50 mM DTT (DTT) or 5 mM diamide (Diam) were undertaken as reduced and oxidised controls, respectively. Specific AGPB antibody was used. (b) Quantification of AGPB monomerisation by Western blot analysis shown as the percentage of the 50-kDa monomer relative to the total amount of AGPB. Results are the mean ± SE (bars) of two measurements for three independent transgenic plants per construct. (c) Starch levels from the same leaves used in (b). Results are the mean ± SE (bars) of six individual plants.

Trx f overexpression leads to a large increase in fermentable sugars

To assess the potential of the *G10Lf* tobacco plants for biofuel production, enzymatic hydrolysis assays were performed using enzyme cocktails that co-hydrolyse both starch and structural carbohydrates. The analysis of the sugars released from *G10Lf* and wild-type leaves after enzymatic hydrolysis showed glucose (Glu) and fructose (Fru) to be the most abundant monosaccharides, but to be in significantly greater quantities in the *G10Lf* leaves (Figure 6). The proportion of glucose to fructose was appreciably higher in the *G10Lf* hydrolysates compared with those of the wild type, likely due to the extremely high starch content present in these plants. Indeed, 5.5 times more

fermentable sugars were detected in the G10Lf hydrolysates than in those of the wild-type plants (Table 2). The *G10Lf* plants also showed an increased biomass yield [up to 1.7-fold in dry weight (DW)] (Table 2); this is consistent with the enhanced SLW values observed for Trx f-overexpressing plants (Table 1). Considering a specific ethanol conversion rate of 0.64 L/kg sugars and a fermentation yield of 95% [data provided by CENER (Spanish National Centre of Renewable Energy)], up to 40 L of bioethanol per fresh ton of *G10Lf* tobacco leaves could be obtained. These values are 10 times those achieved by wild-type leaves (Table 2). The present data show that *G10Lf* tobacco plants could be an excellent alternative feedstock for biofuel production.

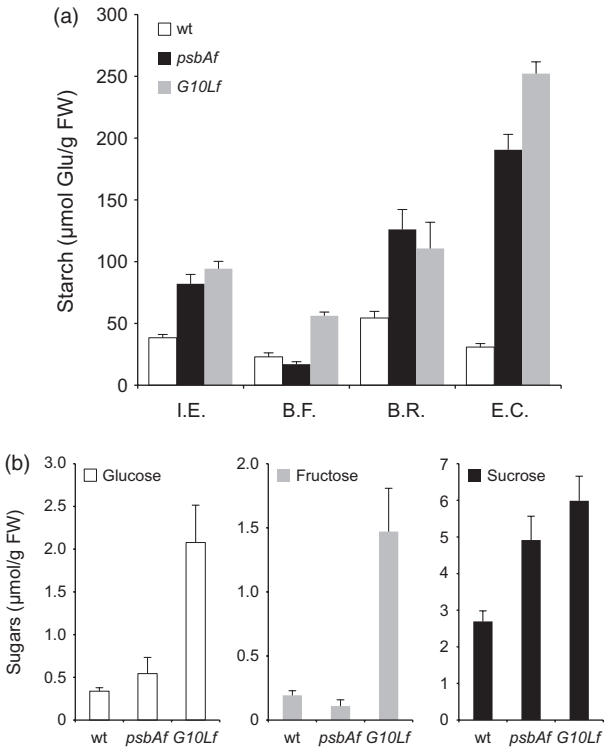

Figure 5 Characterisation of the carbohydrate accumulation pattern in Trx f-overexpressing tobacco plants grown in greenhouse. (a) Starch content from whole plants throughout the crop development. Results are the mean ± SE (bars) of three leaves (young, mature, and old) from four individual plants harvested 12 h after the start of the light period. I.E., inflorescence emergence; B.F., beginning of flowering; B.R., beginning of ripening; E.C., end of crop. (b) Soluble sugar content from same plants from (a) harvested at ripening stage 12 h after the beginning of light period. Results are the mean ± SE of six individual plants (fourth leaf from the top).

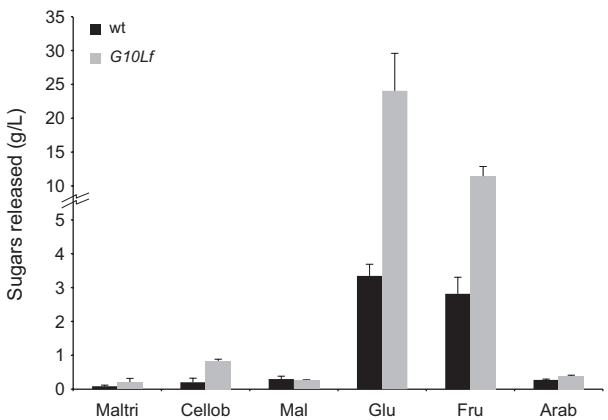

Figure 6 Analysis of sugars released after enzymatic hydrolysis of *G10Lf* and wild-type (wt) tobacco leaves. Maltri: maltotriose; Cellob: celobiose; Mal: maltose; Glu: glucose; Fru: fructose; Arab: arabinose. Results are the mean ± SE (bars) of three individual plants.

Discussion

Starch is the major dietary source of carbohydrates and the most abundant storage polysaccharide in plants. It has a broad range of biotechnological applications and has been a major feedstock for first-generation biofuels. The improvement of starch and sucrose yields has been the target of plant breeding and plant biotechnology for many years. The first studies focused on increasing the starch content of storage organs by manipulating the enzymes involved in starch synthesis, particularly AGPase. However, this failed to provide increased starch contents; the quantitative importance of AGPase in controlling the rate-limiting step in starch synthesis was less than expected. These findings led to a growing interest in discovering other enzymes and proteins that might exert greater control over the flux of fixed carbon towards starch (Smith, 2008). The most promising approaches have focused on the manipulation of ATP availability for starch synthesis (Geigenberger, 2011) and on the control of starch degradation (Smith *et al.*, 2005; Zeeman *et al.*, 2007). Recent studies have shown that starch metabolism is subject to post-translational redox regulation at the level of synthesis (AGPase, SS, BE, ISA) and degradation (GWD, DSP4, BAM1, LDA), sugg esting the complexity of the plastid redox network (Glaring *et al.*, 2012; Kötting *et al.*, 2010). Further, all evidence regarding starch yield improvement suggests that if large changes in carbon allocation are to be secured, the properties and expression levels of several enzymes need to be modified. However, despite the importance of the chloroplast Fd/Trx system in modulating a number of enzymes associated with carbon fixation and metabolism, it remains unknown how an increase in the chloroplast redox potential *in planta* would affect the carbon status of the cell.

The present study shows that Trx f, a member of the Fd/Trx system, but not its counterpart Trx m, plays an important role in photosynthetic carbohydrate metabolism. To our knowledge, this study is the first to distinguish between the two types of Trxs belonging to the Fd/Trx system *in vivo*. Trx f overexpression led to a large increase in starch accumulation, although no

Table 2 Evaluation of tobacco traits for bioethanol feedstock

Trait	wt	*G10Lf*
Fermentable sugars (%)*	7.1 ± 1.0	38.8 ± 6.9
Biomass yield (%)†	9.6 ± 0.4	16.6 ± 1.5
Ethanol yield (l/t FW)‡	4.2 ± 0.7	39.8 ± 9.2

Values are the mean ± SE of three individual plants.

*Fermentable sugar content as percentage over dry matter.

†Expressed as percentage of dry weight over fresh weight (FW).

‡Estimated data considering a specific ethanol conversion rate of 0.64 L/kg sugars and a fermentation yield of 95% (data provided by CENER).

dose-dependent effect was observed. The greatest starch leaf content was achieved at Trx f levels of 0.4% tp; further increases in Trx f (*psbAf* line) did not lead to further increases in the starch content. This might be explained by the extremely large amounts of Trx f in this line having nonspecific effects in the plant, as previously reported for cyanobacterial flavodoxin expressed in chloroplasts (Ceccoli *et al.*, 2012). Alternatively, the lack of a dose-response might be explained by the ability of Trx f to form different homo-oligomers (Peltier *et al.*, 2006; Sanz-Barrio *et al.*, 2012), leaving them without a starch production function.

The mechanism by which the Trx f-overexpressing plants come to show their markedly starchy phenotype remains to be elucidated. AGPase, the enzyme that catalyses the production of ADP-glucose linked to starch biosynthesis, was found to be subject to post-translational redox modification, making it more sensitive to fine control through allosteric regulation (Geigenberger, 2011). It has been suggested that AGPAse redox activation is mediated by Trx (Ballicora *et al.*, 2000; Geigenberger *et al.*, 2005) or by a plastid-localised NADP-thioredoxin reductase C (NTRC) (Michalska *et al.*, 2009), although activation can also occur in response to sugars, even at night (Geigenberger, 2011). Recently, light-dependent activation in *Arabidopsis* leaves has been shown dependent on both Trx f1 and NTRC, whereas sugar-dependent activation in the dark relies essentially on NTRC (Michalska *et al.*, 2009; Thormahlen *et al.*, 2013). The present results show, however, that AGPase redox activation in Trx f-overexpressing tobacco leaves did not differ from that of wild-type plants (Figure 4), despite enhanced levels of the reduced form of Trx f (Figure S2) and sugars (Figure 5b) in these plants. Therefore, it seems like the putative redox activation of AGPase by Trx f might have reached its maximum level in the wild-type plants, making AGPase redox-insensitive to the overexpressed Trx f in the transplastomic tobacco plants. Alternatively, AGPase redox sensitivity might be affected by other factors, such as the presence of allosteric regulators or inhibitors/activators, as a consequence of the great starch accumulation in the chloroplasts of Trx f-overexpressing plants. The redox regulation of AGPase is reported to be influenced by environmental conditions (Li *et al.*, 2012), showing moderate NTRC-dependent AGPB monomerisation in response to light only when *Arabidopsis* plants are grown under photo-oxidative conditions. Thus, it cannot be ruled out that the large amount of Trx f in the transplastomic plants might alter the chloroplast photosynthetic metabolism modifying its regulation pattern, which could affect to enzymes such as AGPase whose regulation seems to be determined by environmental conditions. Nevertheless, the poor relationship between AGPase redox activation and starch content revealed from our data (Figures 3 and 4) has also been reported recently by other authors. No change was observed in the pattern of starch accumulation (Li *et al.*, 2012), or only slightly enhanced starch levels (Hädrich *et al.*, 2012), in *Arabidopsis* leaves expressing a redox-insensitive AGPB mutant. Exceptionally high levels of starch are also reported in leaves of an AGPB-antisense mutant and in wild-type potato plants after fungal volatile compound treatment, but with no increase in AGPase redox activation (Ezquer *et al.*, 2010). Further, the starch accumulation pattern in the leaves of two *Arabidopsis* Trx f1 mutants showing a strong reduction in AGPase redox activation (up to 70%) was very similar to that seen in their wild-type counterparts during the light period (a slight reduction in starch content only towards the end of the day) (Thormahlen *et al.*, 2013).

Starch synthesis and degradation relies on an intricate network of different interacting factors, thus, control over starch hyperaccumulation in tobacco Trx f-overexpressing leaves is likely due to more than just AGPase redox activation. It is well known that photosynthetic carbon metabolism falls under the influence of light and sugars (Stitt *et al.*, 2010). A number of studies have shown that starch synthesis is strongly stimulated when leaf sugar levels are increased by the interruption of phloem transport (Geigenberger *et al.*, 1996; Krapp and Stitt, 1995) or by external sugar supplies (Krapp *et al.*, 1991; Sokolov *et al.*, 1998). Some authors suggest that DTT-dependent activation of Trxs leads to the inhibition of sucrose transport functions (Thormahlen *et al.*, 2013). Thus, impaired sucrose export exerted by overexpressed Trx f might contribute towards an increase in the sucrose content of the leaves (Figure 5b), which could in turn exert feedback control over carbon metabolism and contribute, at least in part, to the starch-excess phenotype. Additionally, the Trx f-overexpressing lines contained far more starch than the wild-type throughout the day/night cycle (Figure 3b), with comparable ratios of starch synthesis and degradation. Differences observed in the basal level of starch show an accumulative effect on the starch steady-state level caused by the Trx f overexpression. However, whether this accumulative effect is due to an impaired degradation or an increased synthesis remains unclear. The starch-excess phenotype observed in Trx f-overexpressing plants resembles that of *Arabidopsis* mutants lacking the plastid SEX1 or SEX4 proteins, which show reduced rates of starch degradation and retain more starch at the end of the night (Kotting *et al.*, 2005, 2009). Interestingly, both enzymes are redox regulated, but how their activity is regulated in the chloroplast stroma is not well understood (Mikkelsen *et al.*, 2005; Silver *et al.*, 2013; Sokolov *et al.*, 2006; Valerio *et al.*, 2010). Several other enzymes involved in the starch synthesis–degradation pathways are known to be redox regulated (Glaring *et al.*, 2012; Kötting *et al.*, 2010). Among these, post-translational activation of starch synthase III (SSIII) has been proposed an important determinant for starch overaccumulation in potato and *Arabidopsis* leaves after fungal volatile treatment (Ezquer *et al.*, 2010; Li *et al.*, 2011). Thus, the starchy phenotype observed in Trx f-overexpressing leaves could be ascribed, at least in part, to a putative redox activation of SSIII. As the complexity in the preservation of the redox state of enzymes during extraction, redox proteomic studies might be performed to shed more light on the changes in starch-related enzymes that occur as a response to altered redox conditions *in planta*. Finally, it cannot be ruled out that enhanced starch accumulation in tobacco leaves is a consequence of an indirect effect of overexpressed Trx f on the activity of starch biosynthesis enzymes. Both protein phosphorylation and protein complex formation may play roles in controlling starch biosynthesis (Kötting *et al.*, 2010), where the controlling factors themselves (i.e. kinases, phosphatases and chaperones) might also be subject to redox regulation.

Trx f has been described as the main redox regulator of several enzymes in the Calvin cycle (Schürmann and Buchanan, 2008). However, Trx f overexpression in the present tobacco chloroplasts was accompanied by no significant change in photosynthesis or growth. Similar findings have been reported for Trx f1 *Arabidopsis* mutants with more than a 90% reduction in Trx f protein levels (Thormahlen *et al.*, 2013) and Trx f-silenced pea plants (Luo *et al.*, 2012). Thus, surprisingly, neither overexpression nor repression of Trx f seems to affect photosynthesis or the

phenotype of tobacco, *Arabidopsis*, or pea plants. The latter two sets of authors suggest that other plastid Trxs can compensate for the lack of Trx f in *Arabidopsis* and pea plants, although this could not be so in Trx f-overexpressing tobacco plants. Traditionally, a number of enzymes belonging to the Calvin cycle such as, sedoheptulose-1,7-bisphosphatase (SBPase), fructose-1, 6-bisphosphatase (FBPase), and phosphoribulokinase (PRKase), which are efficiently activated by Trx f (Schürmann and Buchanan, 2008), have been thought to exert great control over the rate of CO_2 fixation. However, *in vivo* studies have provided evidence that the activity of some of these enzymes (FBPasa and PRKase) have little impact on the control of carbon fixation (Raines, 2003). The present results show that Trx f overexpression was not accompanied by any significant change in the rate of CO_2 fixation, but did lead to starch hyperaccumulation. Similarly, a change in the carbon flux towards the starch synthesis pathway without any enhancement of photosynthetic activity has been observed in tobacco plants showing increased FBPase activity (Tamoi *et al.*, 2006). The fact that the overexpressed Trx f in the transgenic chloroplasts seems to be mostly in the reduced form (Figure S2) suggests that reducing power coming from photosynthesis is not a limiting factor in these plants. Thus, the potential increase in the activity of redox-regulated Calvin cycle enzymes due to Trx f overexpression in tobacco plants could change the carbon flux towards starch synthesis in chloroplasts, without increasing carbon fixation. Finally, despite the large quantities of reduced Trx f in these plants could act as an additional sink of reducing power coming from photosynthesis, which was unaltered in these plants, no limitations has been observed in plant growth and development in the assayed conditions. However, it cannot be dismissed that a limitation in the redox system could be harmful to plant growth under different growth conditions.

There is a growing interest in the discovery of new, inexpensive, nonfood carbohydrate sources for biofuel production. In this context, tobacco is an excellent, industrially proven candidate for ethanol production (Andrianov *et al.*, 2009). It supplies up to 170 t per hectare of green tissue when grown for biomass (Schillberg *et al.*, 2003) and is suitable for coppicing, allowing multiple harvests per year and potentially very high yields. The present work shows that the starch produced by *G10Lf* tobacco plants could be an excellent alternative feedstock for biofuel production. Trx f overexpression results in an around 500% increase in fermentable sugars and also in biomass yields (Table 2) with no need for further inputs into the crop, leading to a theoretical ethanol yield of up to 40 L/ha. Based on field experiments, commercial cultivars yield up to 50 t/ha of fresh leaves. Thus, the bioethanol yield of a *G10Lf* commercial cultivar could reach 2000 L/ha, similar to the annual yield (l/ha) associated with potato, wheat, and sorghum crops (Mojović *et al.*, 2009). Contemplating three potential harvests of tobacco per year (Verma *et al.*, 2010), 6000 L/ha of bioethanol could be produced annually, a yield similar to that achieved with maize, sugar beet, or sugar cane (Mojović *et al.*, 2009). The potential use of tobacco stalks as raw material for bioethanol production has also been shown (Martín *et al.*, 2002); the entire aerial plant might therefore be used. Field experiments with commercial cultivars are needed to test the practicality of Trx f overexpression for energy crop improvement. Also, this technology has the potential to be transferred to other starch-based food crops to improve their biomass and carbohydrate yield.

Experimental procedures

Plant transformation and DNA analysis

Tobacco *Trx f*- and *Trx m*-coding sequences (GenBank Acc. No. HQ338526 and HQ338525 respectively) were generated by PCR (Sanz-Barrio *et al.*, 2012) and cloned into the pL3 chloroplast transformation vector under the control of three different promoters (Farran *et al.*, 2008)—P*rrn*, P*rrnG10*L, and P*psbA* –to generate the expression vectors pL3-PrrnTrxf/m, pL3-PrrnG10LTrxf/m, and pL3-PpsbATrxf/m, respectively.

Nicotiana tabacum (cv. Petite Havana SR1) plastid transformation was performed using a Bio-Rad PDS-1000/He biolistic device as previously described (Daniell, 1997). Bombarded leaves were subjected to three rounds of selection on RMOP medium containing 500 mg/L of spectinomycin to regenerate transformants. Resistant shoots were rooted on P3 medium containing 500 mg/L of spectinomycin and transferred into soil.

Southern and Northern blot analyses

For Southern blot analysis, 10 μg of genomic DNA was digested with *Bgl*II, separated on a 0.8% (w/v) agarose gel, and transferred to a nylon membrane. The membrane was hybridised with a 0.8 kb probe (P1) homologous to the flanking sequences, obtained as previously described (Farran *et al.*, 2008). For Northern blot analyses, total RNA (10 μg) was separated on a 1.5% agarose/formaldehyde gel and transferred to a nylon membrane. The blot was hybridised with Trx-specific probes obtained as previously described (Sanz-Barrio *et al.*, 2011). Probe labelling and hybridisation were performed using the chemiluminiscent AlkPhos Direct Labeling and Detection System (GE Healthcare, Buckinghamshire, UK). Ethidium bromide-stained rRNA bands were used as loading controls.

Plant growth and characterisation

Transformed and wild-type seeds were germinated on P3 medium supplemented with or without 500 mg/L of spectinomycin, respectively. After 4–6 weeks, the seedlings were transferred to soil and grown in a phytotron with supplemental lighting (16-h light/8-h dark; 200 μmol/m^2/s) and a day/night temperature regime of 28º/25 °C or in a greenhouse (natural sunlight). Experiments were performed in summer season, with a light photoperiod of 15 h and light intensities reaching up to 1500 μmol/m^2/s. The relative leaf chlorophyll concentration was measured using a Minolta® SPAD 502 chlorophyll metre (Konica Minolta Optics Inc, Tokyo, Japan), examining five expanded leaves per plant from the top of greenhouse-grown plants at the beginning of the ripening stage. Photosynthetic activity was measured using a portable photosynthesis metre (LCpro-SD; ADC BioScientific Ltd, Herts, UK). Net CO_2 assimilation rates were measured over the third and fourth leaf form the top at the beginning of the flowering stage.

Protein analyses

For immunoblot analysis, proteins from leaf samples were extracted and blotted using specific anti-Trxf and anti-Trxm antibodies as previously described (Sanz-Barrio *et al.*, 2012). Total protein was extracted in Laemmli buffer and quantified using the RC-DC protein assay (Bio-Rad, Hercules, CA) with BSA as a standard, according to the manufacturer's instructions. Overexpressed Trxs in leaf extracts were quantified by Western blotting using serial dilution of Trx f and Trx m

proteins produced in *E. coli* as standards (Sanz-Barrio *et al.*, 2011).

Soluble sugars and starch determination

Soluble sugar contents were determined in ethanol extracts using high-performance liquid chromatography (HPLC) as previously described (Baroja-Fernandez *et al.*, 2009). The pellets left over after ethanol extraction were used for starch determination using an amyloglucosidase-based test kit (R-Biopharm AG, Darmstadt, Germany).

Starch staining and microscopy

Leaf samples were collected at the middle of the light period and fixed in a 3 : 1 ethanol/acetic acid solution. After the removal of the pigments, these samples were re-hydrated, stained in iodine solution for 10 min, rinsed in deionised water and photographed. For light and electron microscopy analysis, leaf samples were processed as previously described (Sanz-Barrio *et al.*, 2011).

AGPB redox status

The AGPase redox status of the tobacco leaves was determined by immunoblotting, analysing the degree of AGPB monomerisation in leaf samples collected at the end of the light (12 h) and dark (9 h) periods. Protein extraction was performed as previously described (Hendriks *et al.*, 2003). Samples incubated for 15 min on ice with 50 mM of DTT (+DTT) or 5 mM of diamide (+Diam) were used as controls. Proteins from 1 mg of fresh weight were subjected to 10% nonreducing SDS-PAGE and transferred to a nitro-cellulose membrane for immunoblotting. The membrane was incubated with a rabbit polyclonal antibody raised against *Arabidopsis* AGPase (Agrisera AB, Vännäs, Sweden) at a dilution of 1 : 1 000, and a peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich, St Louis, MO) at a 1 : 20 000 dilution.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed at CENER (Pamplona, Spain). Assays were performed using a two-step process consisting of a liquefaction and a saccharification stage. Greenhouse-grown tobacco leaves were harvested at the end of crop growth, air-oven-dried and ground. For liquefaction, 1 g of leaf material was dissolved in 10 mL of 100 mM citrate buffer (pH 4.8) supplemented with 10% (w/w) α-amylases (Liquozyme; Novozymes A/S, Bagsvaerd, Denmark) and treated for 1 h at 98–100 °C. For saccharification, a mixture (1 : 1 w/w) of amyloglucosidase and carbohydrases [Spirizyme Ultra and Viscozyme (Novozymes A/S)] was added to the sample in the ratio of 1 : 20 (w/w) and incubated for 42 h at 60 °C with constant agitation (180 rpm). After centrifugation, the supernatant was analysed for its sugar content by HPLC using an ICSep ION-300 column (7.8 x 300 mm, Transgenomic, Glasgow, UK), a mobile phase consisting of Milli Q water with 8.5 mM H_2SO_4, and an Agilent 1200 refractive index detector.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Starch content in tobacco leaves is influenced by growth conditions.

Figure S2 Redox state of endogenous and overexpressed Trx f in tobacco chloroplasts.

Figure S3 Redox activation of AGPase in Trx m-overexpressing leaves.