Type-f thioredoxins have a role in the short-term activation of carbon metabolism and their loss affects growth under short-day conditions in Arabidopsis thaliana

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Abstract

Redox regulation plays a central role in the adaptation of chloroplast metabolism to light. Extensive biochemical analyses in vitro have identified f-type thioredoxins (Trxs) as the most important catalysts for light-dependent reduction and activation of the enzymes of the Calvin–Benson cycle. However, the precise function of type f Trxs in vivo and their impact on plant growth are still poorly known. To address this issue we have generated an Arabidopsis thaliana double knock-out mutant, termed trxf1f2, devoid of both f1 and f2 Trxs. Despite the essential function previously proposed for f-type Trxs, the visible phenotype of the trxf1f2 double mutant was virtually indistinguishable from the wild type when grown under a long-day photoperiod. However, the Trx f-deficient plants showed growth inhibition under a short-day photoperiod which was not rescued at high light intensity. The absence of f-type Trxs led to significantly lower photosynthetic electron transport rates and higher levels of non-photochemical energy quenching. Notably, the Trx f null mutant suffered from a shortage of photosystem I electron acceptors and delayed activation of carbon dioxide fixation following a dark–light transition. Two redox-regulated Calvin–Benson cycle enzymes, fructose 1,6-bisphosphatase (FBPase) and Rubisco activase, showed retarded and incomplete reduction in the double mutant upon illumination, compared with wild-type plants. These results show that the function of f-type Trxs in the rapid activation of carbon metabolism in response to light is not entirely compensated for by additional plastid redox systems, and suggest that these Trxs have an important role in the light adjustment of photosynthetic metabolism.

Key words: Carbon assimilation, chloroplast, fructose 1,6 bisphosphatase, photosynthesis, redox regulation, thioredoxin.

Introduction

Chloroplasts are essential for plant life because these organelles perform photosynthesis, the process that allows the conversion of light energy into biomass with the concomitant production of molecular oxygen. In addition, chloroplasts act as sensors of environmental conditions, particularly light quantity and quality, thus playing an important role in harmonizing the growth of plant photosynthetic and non-photosynthetic tissues as well as the adaptation of plants to the environment (Jarvis and López-Juez, 2013). To meet these requirements, chloroplast metabolism needs to respond rapidly to external and internal signals, redox regulation being an important aspect of this adaptability. Redox regulation is a post-translational modification consisting of the dithiol-disulphide interchange of selected and well-conserved cysteine residues of proteins. It is thus a reversible mechanism that allows the rapid
regulation of metabolic pathways (Buchanan et al., 2012). Protein disulphide reductases such as thioredoxins (Trxs), small polypeptides of 12–14 kDa with a highly conserved active site (WCGPC) and a characteristic structure, the so-called Trx-fold, play a central role in redox regulation. Trxs catalyse the reduction of disulphide bridges in target proteins and, as a consequence, the two cysteine residues at the Trx active site become oxidized as a disulphide bridge. Therefore, for a new catalytic cycle this disulphide needs to be reduced, reducing power being provided by NADPH in a reaction catalysed by NADPH-dependent Trx reductase (NTR). This two-component redox system formed by NTR and Trx is universally distributed in all kinds of organisms from bacteria to fungi, animals, and plants (Jacquot et al., 2009).

In contrast to heterotrophic organisms and non-photosynthetic plant tissues, redox regulation in chloroplasts has unique features. These organelles harbour a complex set of Trxs and Trx-like proteins (Lemaire et al., 2007; Chibani et al., 2009; Meyer et al., 2012; Balsera et al., 2014), which are not reduced by NADPH, but by ferredoxin (Fdx) reduced by photosynthetic electron transport in a process catalysed by a Fdx-dependent Trx reductase (FTR) found exclusively in plastids and cyanobacteria (Dai et al., 2007). Therefore, chloroplast redox regulation is mediated by the Fdx/FTR/Trx system and, thus, is dependent on light. Initial biochemical analyses in vitro led to the identification of two types of Trxs in chloroplasts, termed f and m, based on their ability to reduce and activate fructose-1,6-bisphosphate phosphatase (FBPase) and NADP-malic dehydrogenase (NADP-MDH), respectively (Wolosiuk et al., 1979). The availability of genome sequences from different plants has uncovered the complex set of chloroplastic Trxs, which in Arabidopsis thaliana include two isoforms of f-type, four isoforms of m-type, two isoforms of the y-type, and an x-type Trx (Lemaire et al., 2007; Meyer et al., 2012; Balsera et al., 2014). Trxs f and m were proposed to play a predominant role in the redox regulation of the central biosynthetic pathways, such as the Calvin–Benson cycle, whereas Trxs x and y show a capacity to reduce peroxiredoxins (Prxs) and so were considered to have an antioxidant function (Collin et al., 2003, 2004). In addition, a novel Trx, type-z, was identified in Arabidopsis, which is involved in the redox regulation of plastid transcription (Arsova et al., 2010; Schröter et al., 2010; Steiner et al., 2011; Wimmelbacher and Börnke, 2014). Beside these canonical Trxs, several atypical Trxs have been described in the chloroplast. This is the case of HCF164, which is localized in the thylakoid membrane facing the lumen (Motahashi and Hisabori, 2006), and the small family of atypical Trxs identified in Arabidopsis termed AtACHTs (for atypical Cys His-rich Trxs) (Dangoor et al., 2009). Finally, different Trx-like proteins were identified in chloroplasts, among which the so-called CDSP32 is the best characterized (Broin et al., 2002).

In addition, chloroplasts harbour an NADPH-dependent redox system based on the activity of a bimodular enzyme consisting of an NTR with a joint Trx domain at the C-terminus, termed NTRC (Serrato et al., 2004; Kirchsteiger et al., 2012). NTRC is able to conjugate both NTR and Trx activities for the efficient reduction of 2-Cys Prxs (Pérez-Ruiz et al., 2006; Moon et al., 2006; Alkhalfioui et al., 2007), thus suggesting an antioxidant function for this enzyme (Puerto-Galán et al., 2013). Additional reports indicate a function of NTRC in the redox regulation of starch synthesis (Michalska et al., 2009; Lepistó et al., 2013) and in different reactions of the biosynthesis of tetrapyrroles (Richter et al., 2013; Pérez-Ruiz et al., 2014). The high affinity of NTRC for NADPH introduces the notion that redox regulation in chloroplasts relies not only on photosynthetically reduced Fdx but also on NADPH which can be produced during the night from sugars by the oxidative pentose phosphate pathway (Spinola et al., 2008; Cejudo et al., 2012).

In parallel with the knowledge of the increasing complexity of the plastidial Trxs, the advance in proteomics has allowed the identification of a large number of putative targets of Trxs (Balmer et al., 2003; Buchanan and Balmer, 2005; Montrichard et al., 2009), which indicates that redox regulation is important for virtually any process taking place in the chloroplast. However, the question of the level of specificity or redundancy of the different Trxs in redox regulation in this organelle is still poorly understood. An Arabidopsis knockout mutant deficient in Trx z showed a severe phenotype indicating that the function of this Trx in chloroplast transcription is not redundant with other plastidial Trxs (Arsova et al., 2010). By contrast, an Arabidopsis knockout mutant lacking Trx x shows almost a wild-type phenotype, indicating that this deficiency is compensated for by other plastidial redox systems (Pulido et al., 2010). More uncertain is the specificity of Trxs with several isoforms, such as those of type m or f, which are considered to play a relevant role in the redox regulation of photosynthetic metabolism. Trx m4 is involved in alternative photosynthetic electron transport (Courteille et al., 2013), and the simultaneous deficiency of Trxs m1, m2, and m4 resulted in impaired photosystem II biogenesis (Wang et al., 2013).

Proteomic and biochemical studies in vitro have shown the relevant function of type-f Trxs in the redox regulation of most of the enzymes of the Calvin–Benson cycle (Michelut et al., 2013). In addition, an Arabidopsis mutant lacking Trx f1 shows impaired light-dependent reductive activation of ADP-glucose pyrophosphorylase (AGPase) and starch turnover (Thormählen et al., 2013). Surprisingly, despite the key role proposed for type-f Trxs in the redox regulation of chloroplast metabolism, the Trx f1-deficient mutant shows a wild-type phenotype (Thormählen et al., 2013). Moreover, a double mutant devoid of both Trx f1 and Trx f2 showed a visible phenotype indistinguishable from wild-type plants (Yoshida et al., 2015), suggesting that other plastidial redox systems are able to compensate for the deficiency of f-type Trxs. Therefore, with the aim of establishing the role of type f Trxs in chloroplast performance and plant growth, we generated a double knockout mutant of Arabidopsis devoid of Trx f1 and Trx f2, and have performed the analysis of its phenotype under different growth conditions. The double mutant shows no visible phenotype, compared with wild-type plants,
When grown under long-day conditions, however, it shows retarded growth under short-day conditions which is not rescued by high light intensity. Analysis of photosynthetic parameters and changes in the redox status of FBPase and Rubisco activase in response to light showed that type-f Trxs are required for the rapid reduction of the Calvin–Benson cycle enzymes in response to light, a function not compensated for by other plastidial redox systems.

Materials and methods

Growth conditions and plant material

Arabidopsis thaliana wild-type (ecotype Columbia) and mutant plants were grown in soil in growth chambers under long-day (16/8 h light/dark) or short-day (8/16 h light/dark) conditions at 22 °C during the light and 20 °C during the dark periods and a light intensity of 125 μE m⁻² s⁻¹. For experiments addressing the effects of irradiance, plants were grown under short-day conditions at 125, 350, and 950 μE m⁻² s⁻¹ light intensity. A homozygous line, GK-020E05-013161, with a T-DNA insertion in the TRX f2 gene (see Supplementary Fig. S1 at JXB online) from Arabidopsis, termed the trxf2 mutant, was selected by PCR analysis with the oligonucleotides described in Supplementary Table S1. This mutant was manually crossed with the trxf1 mutant, which was previously reported by Pérez-Ruiz et al. (2014). Seeds resulting from this cross were tested for heterozygosity of the T-DNA insertions in the TRX f1 and TRX f2 genes. Plants were then self-crossed and double homozygous lines were identified in the progeny by PCR analysis of genomic DNA using the oligonucleotides described in Supplementary Table S1.

RNA extraction and qRT-PCR analysis

Total RNA was extracted using the TRIzol RNA extraction reagent (BIONE) and cDNA synthesis was performed with 1 μg of total RNA using the Maximax first strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer’s instructions. The content of Trx f1 and Trx f2 transcripts was determined by real-time quantitative PCR (qRT-PCR) with RNA samples extracted from seedlings. qRT-PCR was performed with oligonucleotides shown in Supplementary Table S2 in an IQ5 real-time PCR detection system (Bio-Rad) following a standard thermal profile (95 °C, 3 min, 40 cycles of 95 °C for 10 s, and 60 °C for 30 s). The relative level of each transcript was referred to the level of the UBQUITIN transcript.

Protein extraction, alkylation assays, and Western blot analysis

For protein extraction, leaves were ground in liquid nitrogen and 10% (v/v) trichloroacetic acid (TCA) was immediately added to quench thiol oxidation. Samples were incubated on ice for 20 min and then centrifuged at 16 200 g at 4 °C for 10 min. The pellets were washed with acetone, resuspended in alkylation buffer (2% SDS, 50 mM TRIS–HCl pH 7.8, 2.5% glycerol, and 4 M urea) with 10 mM methyl-maleimide polyethylene glycol (MM-PEG₉₅) and incubated for protein thiol alkylation for 20 min at room temperature. Samples were subjected to SDS-PAGE (9.5% polyacrylamide), transferred on to nitrocellulose membranes, and probed with an anti-FBPase antibody which was kindly provided by Dr M Sahrawy, Estación Experimental del Zaidín, Granada, Spain, or with an anti-Rubisco activase antibody which was kindly provided by Dr AR Portis, USDA, Urbana, USA. The anti-Trx f antibody was raised by rabbit immunization with purified recombinant Trx f1 from Arabidopsis at the Servicio de Producción Animal, University of Seville, Spain. This antibody also detected Trx f2 although with somewhat lower efficiency (see Supplementary Fig. S2).

Measurements of chlorophyll a fluorescence and P₇₀₀ absorbance

Room temperature chlorophyll fluorescence was measured using a pulse-amplitude modulation fluorometer (DUAL-PAM-100, Walz, Effeltrich, Germany). The maximum PSII quantum yield, determined as variable fluorescence (Fᵥ) to maximal fluorescence (Fₚ), Fᵥ/Fₚ was measured after the dark adaptation of the plants for 30 min and a single saturating pulse of red (635 nm) light at 10 000 μE m⁻² s⁻¹ was applied. Induction-recovery curves were performed using red (635 nm) actinic light at 75 μE m⁻² s⁻¹ for 8 min. Saturating pulses of red light at 10 000 μE m⁻² s⁻¹ intensity and 0.6 s duration were applied every 60 s and recovery in darkness was recorded for up to 10 min. The parameters Y(I) and Y(NPQ) corresponding to the respective quantum yields of PSII photochemistry and non-photochemical quenching (NPQ) were calculated by the DUAL-PAM-100 software according to the equations in Kramer et al. (2004). Relative linear electron transport rates were measured in leaves of pre-illuminated plants by applying stepwise increasing actinic light intensities up to 2 000 μE m⁻² s⁻¹. The redox state of photosystem I P₇₀₀ was monitored by following the changes in absorbance at 830 nm versus 875 nm using the DUAL-PAM-100. Plants were kept in the dark for 30 min and then, to probe the maximum extent of P₇₀₀ oxidation, leaves were illuminated with far red (730 nm) light superimposed on the actinic light. Thereafter, absorbance traces were recorded during a 5 min illumination with 126 μE m⁻² s⁻¹ red (635 nm) actinic light followed by 5 min darkness. Saturating pulses of red light at 10 000 μE m⁻² s⁻¹ were applied every 20 s. The amount of PSI photochemistry Y(I), donor side limitations Y(ND) and acceptor side limitations Y(NA) were based on saturating pulse analysis and calculated by the DUAL-PAM-100 software.

Determination of the rate of carbon assimilation Aᵦ

Net CO₂ assimilation rate (Aᵦ) was measured using an open gas exchange system Li-6400 equipped with the chamber head (Li-6400–40). All measurements were conducted in dark-adapted leaves of short-day grown plants (50 d-old) at 500 μmol mol⁻¹ of CO₂, a constant leaf temperature of 20 °C, and a vapour pressure deficit between leaf and air of below 1 kPa. Before the light, at an intensity of 70 μE m⁻² s⁻¹, was turned on in the leaf chamber, Aᵦ was recorded for 30 min every 5 s, then recording continued until equilibrium was reached. Six leaves were measured per line.

Determination of chlorophylls

Leaf discs were weighed and incubated in 1 ml methanol overnight at 4 °C. After extraction, chlorophyll levels were measured spectrophotometrically, as described in Porra et al. (1989), and normalized to fresh weight or leaf area. The values were compared with a Tukey Test (Anova) using a confidence interval of 99%.

Determination of starch content

Starch content was determined in leaves of wild-type and the Trx f-deficient mutant grown under long-day conditions harvested before flowering (22–26 d-old), essentially as described in Lin et al. (1988). For starch extraction, leaves (100–200 mg fresh weight) harvested at the end of the day were ground in liquid nitrogen and washed with 80% (v/v) ethanol in 10 mM HEPES pH 7.6, for 2 h at 80 °C before being washed with the same solution at room temperature until the tissue was free of pigments. Dry pellets, after centrifugation, were resuspended in 1 ml of 0.2 N KOH and heated at 100 °C for 30 min. After cooling, samples were centrifuged at 17 000 g for 10 min and the supernatant was adjusted to pH 5.0 with 1 N acetic acid. An aliquot of 200 μl of this solution was used to determine the amount of starch using the enzymatic method described by Lin et al. (1988). The values were compared with a Tukey Test (Anova) using a confidence interval of 99%.
Results

Type-f Trxs are dispensable for plant growth

The Arabidopsis genome encodes two isoforms of f-type Trxs, f1 and f2. It was recently reported that Arabidopsis knockout mutants lacking either Trx f1 (Thormählen et al., 2013) or both Trx f1 and Trx f2 (Yoshida et al., 2015) show a wild-type phenotype with respect to growth and pigmentation. These results suggest that the loss of f-type Trxs might be compensated for by other plastidial redox systems. To address this issue in more detail, we have generated a double knockout mutant of Arabidopsis devoid of both Trx f1 and Trx f2 and have analysed its growth under different conditions. To that end, the Arabidopsis line (GK-020E05-013161) with a T-DNA insertion at the AT5G16400.1 locus encoding Trx f2 was isolated (Supplementary Fig. S1). This line was manually crossed with the Trx f1 knockout mutant (SALK_128365.45.75.x) previously reported by our group (Pérez-Ruiz et al., 2014), which was also characterized by Thormählen et al. (2013). Plants homozygous for both T-DNAs were selected by PCR analysis of genomic DNA (Supplementary Fig. S1). The double mutant, here termed trx f1f2, was effectively devoid of both Trx f1 and Trx f2 as shown by the lack of transcripts of the two genes, based on qRT-PCR (Fig. 1A), and the corresponding polypeptides, as shown by Western blots probed with an antibody raised against Trx f1 (Fig. 1B), which also cross-reacted with Trx f2 (Supplementary Fig. S2). These results confirmed that Trx f1 is much more abundant than Trx f2 in wild-type plants. In addition, we tested whether the absence of type-f Trxs had any effect on the expression of other plastidial redox systems, but only minor differences in the levels of the transcripts of genes encoding NTRC, type-m and x Trxs were detected (see Supplementary Fig. S3).

The trx f1f2 double mutant, like the single mutants trx f1 and trx f2, showed the wild-type phenotype when grown under the long-day photoperiod (Fig. 2A), as confirmed by the weight of the rosette leaves (Fig. 2B) and leaf chlorophyll content (Fig. 2C) of mature plants immediately before bolting. To analyse in more detail the effect of Trx f deficiency on plant growth, the different lines were grown under short-day conditions. Wild-type and Trx f-deficient mutants showed an indistinguishable growth rate up to the stage of young rosette leaves (34 d of growth), as determined by fresh weight (Fig. 3A, B). However, after 53 d of growth, the double mutant displayed a significant growth inhibition, as shown by the lower weight of rosette leaves (Fig. 3B), although the chlorophyll content was unaffected (Fig. 3C). Data of the long growth period, 53 d, are only shown for short-day (Fig. 3) and not for long-day conditions (Fig. 2), because the plant developmental stages, adult plants before bolting and advanced leaf senescence, respectively, are not comparable. We then analysed the effect of different light intensities on these mutants grown under short-day conditions. While at 125 and 350 μE m−2 s−1 light intensity no difference was observed between the wild type and the mutant lines after 34 d of growth, Trx f-deficient mutants grown at 950 μE m−2 s−1 light intensity displayed lower rosette weights than the wild-type plants (Fig. 4A, B). At high light intensity the leaf chlorophyll content was decreased, but no significant differences were observed between wild-type and mutant plants (Fig. 4C). Therefore, despite the central function previously attributed to type-f Trxs in the redox regulation of chloroplast metabolism, these Trxs are dispensable for plant growth under the long-day photoperiod. Nevertheless, the retarded growth of the trx f1f2 double mutant under the short-day photoperiod at adult plant stages, even under high light conditions, confirms the light-dependent participation of f-type Trxs in chloroplast photosynthetic metabolism.

Activation of photosynthesis upon a dark–light transition is retarded in plants lacking type f Trxs

To explore the function of f-type Trxs in chloroplast metabolism, different photosynthetic parameters of plants lacking either or both of the Trx f enzymes were first examined...
using chlorophyll fluorescence. The integrity of PSII, determined as the ratio of variable to maximal fluorescence in dark-adapted leaves, was not affected in the single or double trxf1f2 mutants (Table 1). Non-photochemical quenching (NPQ) is a loss of chlorophyll fluorescence in the light which is not due to photochemistry and reflects adaptation mechanisms regulating the fraction of absorbed light that reaches the PSII reaction centre (Szabó et al., 2005; Baker, 2008). The energy-dependent quenching, $q_E$, which involves thermal dissipation of the absorbed light energy, is the main component of NPQ in plants under moderate light intensities and depends on the proton gradient across the thylakoid membrane (Szabó et al., 2005; Baker, 2008; Ruban et al., ...
Following the onset of actinic light at low or moderate intensity, a brief peak of NPQ is normally observed in wild-type plants due to transient acidification of the thylakoid lumen before activation of photosynthesis (Kalituho et al., 2007). Such an initial peak of NPQ was also found in the \textit{trxf2} mutant and a somewhat broader peak in the \textit{trxf1} mutant (Fig. 5A; see Supplementary Fig. S4). By contrast, the double mutant displayed an extensive NPQ which did not relax completely even after 8 min of illumination at 75 μE m⁻² s⁻¹ (Fig. 5A). As a result, the PSII effective quantum yield, \( Y_{\text{II}} \), increased more slowly in the light and remained lower in the \textit{trxf1f2} double mutant (Fig. 5B). In agreement with these results, the relative linear photosynthetic electron transport rates were considerably lower in the double mutant at all light intensities examined (Fig. 6A). By contrast, only a small but significant reduction in photosynthetic electron transport rates was observed in the \textit{trxf1} mutant, whereas the \textit{trxf2} mutant displayed wild-type rates (Fig. 6A). Notably, the yields of NPQ were higher in \textit{trxf1f2} plants, particularly at low light intensities (Fig. 6B). These measurements were performed using plants grown under short-day conditions and very similar results were obtained when these photosynthetic parameters were analysed in plants grown under the long-day photoperiod (see Supplementary Figs S5 and S6).

The effect of the type \( f \) Trxs on the control of energy quenching and photosynthetic yield might be through direct interaction with the photosynthetic apparatus or an indirect consequence of limited biosynthesis and demand for ATP, leading to lower luminal pH. The latter case should be revealed by a lower rate of consumption of electrons from photosynthetic electron transport. Therefore, we measured the PSI quantum yield, \( Y(I) \), based on \( P_{700} \) absorbance changes, in mutant and wild-type plants (Fig. 7; see Supplementary Fig. S7). Indeed, the double mutant had a lower PSI activity due to prolonged limitations on the acceptor side, \( Y(\text{NA}) \), after turning on the light (Fig. 7D). This indicates retardation of the activation of the biosynthetic processes which consume reducing equivalents derived from photosynthetic electron transport.

The response of the rate of CO₂ fixation (\( \Delta A \)) to illumination was more retarded in the \textit{trxf1} than in the \textit{trxf2} single mutant while the \textit{trxf1f2} double mutant displayed even slower

Table 1. \( F_v/F_m \) and kinetics of response of net CO₂ assimilation rate to light

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>\textit{trxf1}</th>
<th>\textit{trxf2}</th>
<th>\textit{trxf1f2}</th>
</tr>
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<tbody>
<tr>
<td>( F_v/F_m )</td>
<td>0.81 ± 0.01</td>
<td>0.82 ± 0.01</td>
<td>0.81 ± 0.01</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>( t_{1/2} ) (s)</td>
<td>106.0 ± 2.3 a</td>
<td>187.9 ± 7.5 b</td>
<td>134.3 ± 9.5 a</td>
<td>237.2 ± 10.1 c</td>
</tr>
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The maximum PSII quantum yield was determined as variable fluorescence (\( F_v \)) to maximal fluorescence (\( F_m \)), \( F_v/F_m \), in dark-adapted leaves of plants grown under short-day conditions. The \( F_v/F_m \) values (±SD) are the average of 12 measurements. \( t_{1/2} \) represents the time to achieve 50% of the final rate of CO₂ assimilation, as shown in the data of Supplementary Fig. S5. Data presented are the means (±SE; \( n=6 \)). The differences between mutants and the wild type, when significant, are indicated by different letters (\( P < 0.01 \); Anova, Tukey test).

Fig. 4. Effect of light intensity on growth of \textit{Arabidopsis} lines lacking type-\( f \) Trxs under a short-day photoperiod. (A) Representative plants of the wild type and mutant lines grown under short-day conditions (8/16 h light/dark) for 34 d at increased light intensities, as indicated. (B) The weight of the rosette leaves from all lines was determined from 12 plants. (C) Chlorophyll content was determined from leaf discs (\( n=6 \)). Average values ±SD are represented. Letters indicate significant differences with the Tukey Test and a confidence interval of 99%.
Light-dependent reduction of FBPase and Rubisco activase is impaired in the trxf1f2 double mutant

There is extensive evidence in vitro supporting the relevant function of f-type Trxs in light-dependent reductive activation of different chloroplast enzymes including those of the Calvin–Benson cycle (recently reviewed by Michelet et al., 2013). To analyse further the function of f-type Trxs in vivo, we examined the change of the redox status of two well-known in vitro targets of type f Trxs, FBPase and Rubisco activase, in response to light in the mutant lines. To this end, samples were taken at the end of the night period from plants that had been grown at a light intensity of 125 μE m⁻² s⁻¹ and then subjected to illumination with the same or higher (500 μE m⁻² s⁻¹) light intensity. Short-term changes of the redox status of these enzymes were analysed with the aid of the thiol-alkylating agent methyl maleimide-polyethylene glycol₂₄ (MM-PEG₂₄), which adds 1.24 kDa per thiol group, thus producing a shift in the electrophoretic mobility of the labelled proteins that reflects their redox status. In dark-adapted plants FBPase was detected as a single band indicating that, as expected, the enzyme was fully oxidized under these conditions (Fig. 8). In wild-type plants, FBPase becomes rapidly reduced in response to light within seconds after illumination (Fig. 8). The level of reduction of FBPase proved to be dependent on light intensity since the enzyme became fully reduced after 10 min at 500 μE m⁻² s⁻¹, whereas the growth light intensity of 125 μE m⁻² s⁻¹ only promoted a partial reduction, approximately 50%, of the enzyme (Fig. 8). While the deficiency of Trx f₂ exerted almost no effect on FBPase reduction in response to light, the deficiency of Trx f₁ significantly impaired the level of reduction of the enzyme at both light intensities (Fig. 8). The trxf1f2 double mutant showed not only a pronounced delay of FBPase reduction in response to light but also a lower level of reduction of the enzyme after 10 min of illumination, even at the higher light intensity tested here (Fig. 8). A similar pattern of reduction in response to light was observed for another prominent redox-regulated enzyme of the Calvin–Benson cycle, Rubisco activase (Fig. 9). In dark-adapted samples Rubisco activase was detected as a double band corresponding to isoforms of 46 and 43 kDa, respectively, due to alternative splicing. The long isoform has a C-terminal extension that includes two cysteines which may form an intramolecular disulphide that renders the enzyme inactive (Zhang and Portis, 1999).

Quantification of the reduction of FBPase and Rubisco activase from a series of experiments (such as those presented in Figs 8 and 9), revealed that the levels of reduction of both enzymes remained at 50% of the wild-type level in the trxf1f2 double mutant after 10 min illumination at the growth light intensity (see Supplementary Fig. S9A, B). Furthermore, we analysed the change of the redox status of FBPase following a light–dark transition. Re-oxidation of FBPase in the dark was faster in all three Trx f deficient mutants than in wild-type plants (Fig. 10). However, after 5 min darkness, FBPase was also nearly completely oxidized in the wild type (Fig. 10). Finally, we compared the starch content in leaves which was significantly lower in the trxf1f2 double mutant than in the wild type and the single mutants (Fig. 11).

Discussion

Following the demonstration of the function of Trxs in the light-dependent regulation of chloroplast photosynthetic...
metabolism (Wolosiuk and Buchanan, 1976), extensive biochemical and proteomic work has shown the central role of $f$-type Trxs in the reductive activation of most of the Calvin–Benson cycle enzymes. In this regard, the findings that overexpression of Trx $f$, but not Trx $m$, enhanced starch accumulation and increased the content of sugars in tobacco leaves (Sanz-Barrio et al., 2013) lend further support to the idea that, among the complex set of plastidial Trxs, those of type $f$ play a key role in the redox regulation of carbon metabolism. However, Arabidopsis knockout mutants for Trx $f_1$, which accounts for up to 95% of the total $f$-type Trxs in this plant, showed impairment of AGPase redox regulation and leaf diurnal starch turnover alterations, yet the visible phenotype of these mutants was indistinguishable from the wild type (Thormählen et al., 2013). Moreover, double mutants lacking both Trx $f_1$ and Trx $f_2$ were also indistinguishable from wild type plants (Yoshida et al., 2015). Thus, the lack of $f$-type Trxs seems not to affect plant growth despite the relevant function proposed for these Trxs in the redox regulation of chloroplast metabolism based on biochemical analyses. These results suggest that additional redox systems may compensate for the function of $f$-type Trxs being sufficient to support the redox regulation that allows plant growth.

To address this issue, in the present work we have generated a double mutant of Arabidopsis, here termed trxf1f2, lacking both Trx $f_1$ and Trx $f_2$. Both qRT-PCR and Western blot analyses (Fig. 1A, B) confirmed that the trxf1f2 double mutant is a null mutant devoid of $f$-type Trxs. Western blot analysis of leaf extracts from wild-type plants detected a double band, which might correspond to $f_1$ and $f_2$ Trxs. However, this possibility was ruled out because the double band was detected in the trxf2 mutant but not in the trxf1 mutant. Thus, the double band is most probably indicative of a post-translational modification, the nature of which is still unknown. Under long-day conditions the growth of the single mutants, trxf1 and trxf2, showed slightly retarded growth (Fig. 3B), in agreement with the behaviour of the Trx $f_1$-deficient mutant, which shows retarded growth under short-days, but not under the long-day photoperiod (Thormählen et al., 2015). Adult plants of the trxf1f2 double mutant showed retarded growth (Fig. 3).
which was observed only at later stages of development, indicating that the effect of the deficiency of \textit{f-type} Trxs reported here (lower efficiency of photosynthetic parameters, impaired reduction of Calvin–Benson enzymes, lower starch content) requires time to affect plant growth. Most likely, the delayed growth of the \textit{trxf}-deficient mutant under short-day conditions is not attributable to short-term kinetics of activation, but rather to the lower final level of reduction of enzymes, such as FBPase and Rubisco activase, which can be seen after growth of the \textit{trxf}-deficient mutant but also of the single mutants after 34 d of growth (Fig. 4B). Therefore, despite the central function attributed to \textit{f-type} Trxs in chloroplast redox regulation based on biochemical \textit{in vitro} analyses, the \textit{in vivo} approach reported here shows that these Trxs are dispensable for plant growth at least under the standard long-day conditions performed in this study. It should be noted, however, that conditions that may cause light limitation, such as growth under a short-day photoperiod, have a negative effect on the growth rate of \textit{trxf}-deficient mutants. Moreover, higher light intensity failed to stimulate the growth of mutant plants to the extent that it was stimulated in wild-type plants (Fig. 4), indicating the relevant function of \textit{type-f} Trxs is for plant adaptation to varying light conditions.

The genes encoding \textit{Trx f1} and \textit{Trx f2} isoforms are subject to different regulation in \textit{Arabidopsis}. The expression of the \textit{TRX f1} gene responds to light, whereas the \textit{TRX f2} gene is under circadian control (Barajas-López et al., 2011). The differential pattern of expression of these genes might indicate different functions for the respective \textit{Trx f} isoforms. However, all the photosynthetic parameters analysed in this study, such as photosynthetic electron transport and response to illumination of the rate of CO$_2$ fixation ($A_N$), were more affected in the \textit{trxf1} than in the \textit{trxf2} mutant, while the double mutant showed an additive effect. Most probably, these results reflect the higher content of \textit{Trx f1} in wild-type plants (Fig. 1B) and support a redundant function for both \textit{f-type} Trxs in \textit{Arabidopsis}.

A relevant question concerning chloroplast redox regulation is the level of redundancy or specificity among the
illumination is impaired in the trxf1f2 knockout mutant that reduction of both FBPase and Rubisco activase upon of the Calvin–Benson cycle enzymes, our studies in vivo show function of f-type Trxs in light-dependent redox regulation (Zhang and Portis, 1999). In line with the proposed central is unable to reduce this enzyme f, whereas spinach Trx m shown to be readily reduced and activated by spinach Trx f (Thormählen et al., 2015). In addition, purified Rubisco activase has been confirmed in vitro studies showing the incomplete reduction of FBPase in mutant plants devoid of Trx f (Thormählen et al., 2015) and the double mutant devoid of both Trx f1 and Trx f2 (Yoshida et al., 2015). In addition, purified Rubisco activase has been shown to be readily reduced and activated by spinach Trx f, whereas spinach Trx m is unable to reduce this enzyme (Zhang and Portis, 1999). In line with the proposed central function of f-type Trxs in light-dependent redox regulation of the Calvin–Benson cycle enzymes, our studies in vivo show that reduction of both FBPase and Rubisco activase upon illumination is impaired in the trxf1f2 knockout mutant (Figs 8, 9; Supplementary Fig. S9). Nevertheless, despite the complete absence of Trx f, both enzymes become partially reduced during illumination. These results suggest that the in vitro studies showing the predominant function of f-type Trxs in the redox regulation of the Calvin–Benson cycle enzymes cannot be extrapolated to the physiological situation and show that the double mutant relies on alternative system(s) capable of limited reductive activation of these enzymes in response to light. In this regard, it is worth mentioning that mutant plants devoid of NTRC, an alternative chloroplast redox system, show an incomplete level of FBPase reduction, this effect being even more dramatic in the ntrc-trxf1 double mutant (Thormählen et al., 2015). Therefore, light-dependent redox regulation of Calvin–Benson cycle enzymes, such as FBPase, seems to be the result of the action of different redox systems including type-f Trxs (Fig. 8, this work; Thormählen et al., 2015; Yoshida et al., 2015), type m Trxs (Okegawa and Motohashi, 2015), and NTRC (Thormählen et al., 2015). The finding that NTRC and Trx f act in concert (Thormählen et al., 2015) indicates the existence of cross-talk among these redox systems.

Fig. 8. In vivo redox status of FBPase in response to light in the wild type and Trx f mutant lines. The redox status of FBPase from the different lines under analysis, as indicated on the left, was determined in leaf extracts from plants grown under short-day conditions and harvested at the end of the dark period (time 0), and after 1, 5, and 10 min of illumination at the indicated light intensities. Total leaf proteins were extracted in the presence of 10% TCA and protein thiols were alkylated with 10 mM MM-PFGlu. Proteins were resolved in SDS-PAGE (9.5% polyacrylamide) under non-reducing conditions, transferred to nitrocellulose filters, and probed with an anti-FBPase antibody; red, reduced; ox, oxidized.
higher light intensity, 500 μE m⁻²s⁻¹, FBPase becomes fully reduced (Fig. 8) indicating that the redox-insensitive form, FBPase II, is present in minor amounts in chloroplasts, in agreement with previous results (Rojas-González et al., 2015).

An interesting observation regarding the redox state of FBPase and Rubisco activase is that these enzymes do not become fully reduced under the growth light intensity even in wild-type plants. This is in agreement with recent reports (Yoshida et al. 2014, 2015) showing that Arabidopsis plants display only partial reduction of the FBPase when illuminated at low light intensity. However, we observed the complete reduction of FBPase in wild-type plants after 10 min illumination at 500 μE m⁻²s⁻¹ light intensity, while the trx1f2 double mutant showed a partial reduction of the enzyme (Fig. 8). A remarkable implication of these results is that plants that have been adapted to low light conditions have a significant pool of inactive, not fully reduced, enzymes. A possible advantage of this seemingly wasteful synthesis of excess Calvin–Benson cycle enzymes is that, after a sudden increase in photon flux leading to higher rates of photosynthetic electron transport, the ATP and NADPH generated would immediately be utilized for carbon dioxide fixation and, thus, would not be a problem of light stress. Obviously, a prerequisite for this to occur is that there are sufficiently high amounts of f-type Trxs to catalyse the reductive activation of these enzymes. In addition, we have analysed the changes of redox status of FBPase in light–dark transitions. Re-oxidation of FBPase in response to darkness is again a very rapid process which is essentially completed in 5 min in wild-type plants (Fig. 10). The fact that re-oxidation is faster in the mutants could indicate that, until exhausted, a pool of reduced f-type Trxs continues to catalyse reduction in the dark.

Light-harvesting efficiency and photosynthetic electron transport are highly sensitive to changes in carbon assimilation. For example, sub-atmospheric levels of CO₂, which result in limited regeneration of ADP and NADP⁺, lead to elevated NPQ and decreased effective PSII quantum yield at low or moderate light intensities (Kramer et al., 2004). Similarly, inhibition of the Calvin–Benson cycle enzymes in vivo by iodoacetamide leads to higher NPQ and slower linear photosynthetic electron transport (Joliot and Alric, 2013). Notably, the qE component of NPQ ensures safe dissipation of the light energy absorbed and prevents excess excitation of PSII (Szabó et al., 2005; Baker, 2008; Ruban et al., 2012). Such a negative feedback control that arises from hampered CO₂ fixation is the most likely explanation for the results concerning the activities of PSII and PSI obtained from the trx1f2 mutant plants using chlorophyll fluorescence and P700 absorbance. Hence, despite normal maximum PSII quantum

**Fig. 9.** In vivo redox status of Rubisco activase in response to light in the wild type and Trx f mutant lines. The redox status of Rubisco activase from the different lines under analysis, as indicated on the left, was determined in leaf extracts from plants grown under short-day conditions and harvested at the end of the dark period (time 0), and after 1, 5, and 10 min of illumination. Total leaf proteins were extracted in the presence of 10% TCA and protein thiols were alkylated with 10 mM MM-PEG24. Samples were fractionated in SDS-PAGE (9.5% polyacrylamide) under non-reducing conditions, transferred to nitrocellulose filters, and probed with an anti-Rubisco activase antibody; red, reduced; ox, oxidized.

**Fig. 10.** Re-oxidation of FBPase in response to darkness. The redox status of FBPase from the different lines under analysis, as indicated on the left, was determined in leaf extracts from plants grown under short-day conditions and harvested at the end of the light period (time 0), and after 1 min and 5 min of darkness. Total leaf proteins were extracted in the presence of 10% TCA and protein thiols were alkylated with 10 mM MM(PEG)24. Proteins were resolved in SDS-PAGE (9.5% polyacrylamide) under non-reducing conditions, transferred to nitrocellulose filters, and probed with an anti-FBPase antibody; red, reduced; ox, oxidized. Band intensities were quantified and the percentage of reduced FBPase is indicated.
efficiency, $F_v/F_m$, the linear photosynthetic electron transport rate is significantly decreased in the double \textit{trxf1f2} knockout mutant compared with the wild type, particularly at lower light intensities. At 100–150 $\mu$E m$^{-2}$ s$^{-1}$, which corresponds to growth light intensity, the electron transport in the double mutant was only half that in wild-type plants (Fig. 6A) and the yield of NPQ was twice as high (Fig. 6B).

Although in plants devoid of \textit{f}-type Trxs the response of the rate of carbon assimilation ($A_N$) to light was delayed (Table 1) these plants still reached carbon assimilation rates similar to those of the wild type (Supplementary Fig. S8). However, the content of starch was diminished in the \textit{trxf1f2} mutant (Fig. 11) which is in line with the impaired activation of the AGPase of the wild-type plant (Fig. 11) which is in line with the impaired activation of the AGPase of the \textit{trxf1f2} mutant (Thornmählen et al., 2013). Nevertheless, the leaf starch accumulated during the day under a long-day light regime seems sufficient for the correspondingly short night and to support wild-type growth rates (Fig. 2).

**Conclusion**

In summary, the \textit{in vivo} approach undertaken in this work identifies the impact of type \textit{f} Trxs on photosynthetic performance in \textit{Arabidopsis}, such as the kinetics of activation of carbon assimilation, the redox status of Calvin–Benson cycle enzymes upon a dark–light transition, and the control of photosynthetic electron transport. The fact that these parameters were impaired in plants devoid of type \textit{f} Trxs indicates that the functions of these Trxs are specific and are not compensated for by other Trxs or chloroplast redox systems. On the other hand, FBPane and Rubisco activase showed a significant level of reduction upon dark–light transition in the \textit{trxf1f2} double mutant indicating that additional chloroplast redox systems participate in light-dependent reduction of these Calvin–Benson cycle enzymes. This stands out against the well-established notion, based on biochemical \textit{in vitro} analyses, of the almost exclusive role attributed to type \textit{f} Trxs in the redox regulation of these enzymes. Surprisingly, the growth of plants devoid of type \textit{f} Trxs is indistinguishable from wild-type plants when grown under standard conditions with a long-day photoperiod, indicating that the function of type \textit{f} Trxs are dispensable for growth.

**Supplementary data**

Supplementary data can be found at JXB online.

**Table S1.** Sequence of oligonucleotides used for genotype analyses.

**Table S2.** Sequence of oligonucleotides used for gene expression analyses.

**Figure S1.** Genotype of \textit{Trx} \textit{f}-deficient mutants.

**Figure S2.** Cross-reaction of the anti Trx \textit{f1} antibody with Trx \textit{f1} and Trx \textit{f2}.

**Figure S3.** Level of \textit{NTRC}, \textit{m}- and \textit{x}-type \textit{TRX} gene transcripts in \textit{Trx} \textit{f}-deficient mutants.

**Figure S4.** Chlorophyll \textit{a} fluorescence of PSI.

**Figure S5.** Photosystem II activity in wild type and \textit{Trx} \textit{f} deficient mutants grown under long-day conditions.

**Figure S6.** Light-dependent linear photosynthetic electron transport and NPQ in wild type and \textit{Trx} \textit{f} deficient mutants grown under long-day conditions.

**Figure S7.** Absorbance of the oxidized form of PSI.

**Figure S8.** Net CO$_2$ assimilation rate ($A_N$) in the wild type and \textit{Trx} \textit{f} deficient mutants.

**Figure S9.** Light-dependent reduction FBPane and Rubisco activase.

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