RESEARCH PAPER

Expression of the chloroplast thioredoxins $f$ and $m$ is linked to short-term changes in the sugar and thiol status in leaves of Pisum sativum

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Abstract

Thioredoxins (TRXs) $f$ and $m$ are key components in the light regulation of photosynthetic metabolism via thiol–dithiol modulation in chloroplasts of leaves; however, little is known about the factors modulating the expression of these proteins. To investigate the effect of sugars as photosynthetic products on the expression of $PsTRX f$ and $m1$ genes, sucrose and glucose were externally supplied to pea plants during the day. There was an increase in the mRNA levels of $PsTRX f$ and $m1$ genes in response mainly to glucose. When leaf discs were incubated for up to 4h in the dark, glucose also led to an increase in both mRNA and protein levels of TRXs $f$ and $m$, while sucrose had no substantial effect. Expression of $PsDOF7$, a carbon metabolism-related transcription factor gene, was also induced by glucose. Protein–DNA interaction showed that PsDOF7 binds specifically to the DOF core located in $PsTRX f$ and $m1$ gene promoters. Transient expression in agroinfiltrated pea leaves demonstrated that PsDOF7 activated transcription of both promoters. The incubation of leaf discs in dithiotreitol (DTT) to increase the redox status led to a marked increase in the mRNA and protein levels of both TRXs within 4h. The increase in TRX protein levels occurred after 1h DTT feeding, implying a rapid effect of the thiol status on TRX $f$ and $m1$ protein turnover rates, while transcriptional regulation took 3h to proceed. These results show that the protein levels of both TRXs are under short-term control of the sugar and thiol status in plants.

Key words: Chloroplast, DOF, metabolic regulation, sugar signalling, thiol signalling, thioredoxins, transcription factors.

Introduction

Thioredoxins (TRXs) are small proteins with similar tertiary structures and are present in all living cells (Buchanan and Balmer, 2005). WCGPC is the conserved active site of these proteins that participates in disulphide interchange reactions with other protein targets (Holmgren, 1985). In the chloroplast, ferredoxin reduced during photosynthetic electron transport
reduces the bridge formed between the two cysteines of the TRX through ferredoxin–thioredoxin reductase (FTR) (Schürmann and Buchanan, 2008; Meyer et al., 2009). Reduced TRX then activates enzymes involved in photosynthesis in response to light. In the cytosol and other organelles, TRX h is reduced by NADPH via NADP thioredoxin reductases a and b (Serrato et al., 2002). In addition, an NADP thioredoxin reductase c containing a thioredoxin domain on the same polypeptide has been identified in the chloroplast (Serrato et al., 2004; Pérez-Ruiz et al., 2006) and shown to be involved in the post-translational redox regulation of ADP-glucose pyrophosphorylase (AGPase) and starch synthesis in leaves (Michalska et al., 2009).

The organization of the TRX superfamily in plants is based on the subcellular compartment in which they are located. Also, several TRXs can be found in the same organelle but with different functions. The genomic sequencing of Arabidopsis thaliana revealed a group of chloroplastic TRXs that included four TRX m genes (AtTRX m1, m2, m3, and m4), two TRX f genes (AtTRX f1 and f2), TRX x gene, two TRX y genes (AtTRX y1 and y2), and one TRX z gene (Arsova et al., 2010). In addition, the A. thaliana genome contains nine TRX h-type proteins presumably located in the cytosol, and two mitochondrial TRX o (Meyer et al., 2008). To date, in the pea genome, one TRX f (PsTRX f) and two TRX m (PsTRX m1 and m2) located in the chloroplast (López-Jaramillo et al., 1997; Pagano et al., 2000), one mitochondrial TRX o1 (Marti et al., 2009), and four cytosolic TRX h (Montrichard et al., 2003; Traverso et al., 2007, 2008) have been isolated. Initially, the main role of chloroplastic isoforms TRX f and TRX m were related to the exclusive redox activation of the chloroplastic fructose-1,6-biphosphatase (FBPase) and NADP-malate dehydrogenase (NADP-MDH), both enzymes being involved in the main steps of the Calvin cycle to improve the CO₂ assimilation rate during starch synthesis and the oxaloacetate redox pump, respectively (Schürmann et al., 1976; Buchanan et al., 1978; Jacquot et al., 1978). In the meantime, >100 TRX targets have been identified in plants (Balmer et al., 2006b; Hall et al., 2010), including AGPase, the key enzyme of starch biosynthesis (Hendriks et al., 2003; Geigenberger et al., 2005).

Moreover, both chloroplastic TRX f and m are also expressed in non-photosynthetic tissues such as seeds, roots, and flowers, suggesting that TRX f and m regulation is not limited to carbon fixation and photosynthesis (Balmer et al., 2006a; Barajas-López et al., 2007; Traverso et al., 2008). Indeed, TRXs have also been implicated in regulating starch synthesis in heterotrophic tissues in response to sugars (Tiessen et al., 2002; Michalska et al., 2009; Geigenberger, 2011). Recently, transcripts of TRXs f and m from pea have been found to be more abundant in 5- to 5-day-old cotyledons than in other pea organs, suggesting a role in reserve mobilization, such as amino acids or sugars stored in amyloplasts (Fernández-Trijueque et al., 2012).

While redox transfer pathways, mechanisms, and targets of TRX have been investigated in great detail in the past, relatively little is known about the factors regulating the expression of these TRXs in plants. Since the concentrations of TRXs have been found to be several magnitudes lower than the concentrations of the corresponding target proteins (König et al., 2012), modulation of the expression levels of TRX will be important to allow an efficient redox transfer to specific metabolic targets in specific tissues and conditions. Most research has focused on the regulation of TRX expression in response to light. It has been found that illumination of leaves leads to an increase in TRX f and m mRNA and protein levels (Carrasco et al., 1992). Furthermore, recently it has been shown that both PsTRX f and m1 transcripts and protein are under the regulation of the circadian clock (Barajas-Lopez et al., 2011). Analysis of PsTRX f and m1 promoters regions identified GATA, CAAT-box like, and GT1 cis-elements as responsible for light activation of gene expression (Barajas-Lopez et al., 2007). However, the underlying signalling components have not been clarified yet. Interestingly, deletion of light-regulated cis-element factors in the PsTRX f and m1 promoters did not induce the complete loss of PsTRX::GUS expression during darkness, suggesting that chloroplastic TRX genes are regulated not only by light but also by other metabolic factors including sugars (Barajas-López et al., 2007).

Carbohydrates may also possibly modulate the redox status of the cells by down- or up-regulating expression of many genes that code for some of the proteins displaying disulphide oxidoreductase activity, and TRXs could be a worthwhile example to analyse (Kolbe et al., 2005; Lunn et al., 2006). Transcriptome analyses suggest that sugar signalling and sugar-modulated gene expression are related to the control of oxidative stress, in which the TRXs might be involved (Couée et al., 2006; Rosa et al., 2009; Bolouri-Moghaddam et al., 2010). In addition, sugar regulation is closely related to diurnal changes in expression of genes assigned to starch and sucrose metabolism, trehalose metabolism, nutrient uptake, and assimilation and redox regulation (Bläsing et al., 2005). In addition to this, external glucose feeding has been found to increase the reduction status of the NADP system and the redox activation status of AGPase in Arabidopsis leaves (Kolbe et al., 2005), providing evidence for a link between glucose, the chloroplast redox status, and TRX targets.

Although sucrose is the major photosynthetic product and transport sugar in plants, many sugar signalling effects on growth and metabolism can be attributed to the action of its hydrolytic hexose products, glucose and fructose (or their downstream metabolic intermediates). Plants have developed different mechanisms for sensing and signalling sugars, where hexokinase (HXK) plays a central role (Jang et al., 1997; Rolland et al., 2006). However, the situation is far more complex, in that diverse sugar signals can activate different HXK-dependent and HXK-independent pathways.

Sugar regulation uses different molecular mechanisms to control transcription, translation, protein stability, and enzymatic activity. Within the sugar-mediated transcriptional mechanism, the plant-specific DOF (DNA binding with one finger) transcription factors are known to regulate the expression of genes that code for proteins involved in carbon metabolism (Yanagisawa, 2000; Lijavetzky et al., 2003). DOF protein contains a conserved DNA-binding domain that is generally located in the N-terminal regions of the proteins and includes a single Cys2-Cys2 zinc finger (Umemura et al., 2004). DOF transcriptional factors play critical roles as transcriptional regulators in plant growth and development that are closely related to carbon metabolism (Yanagisawa, 2002; Tanaka et al., 2009).
To gain better insight into the factors regulating the expression of chloroplastic TRXs, the expression pattern of \( PsTRXf, PsTRXm \), and \( PsDOF7 \) transcription factor was analyzed when pea plants were externally fed with glucose and sucrose to alter the carbon status, and with the reducing agent dithiothreitol (DTT) to alter the redox status of the leaves. As a means of evaluating the effect of light and sugar individually, the feeding experiments were performed with plants subjected to a normal photoperiod or with leaf discs in the dark. Finally, \( PsTRXf \) and \( m1 \) regulatory regions that carry the DOF cis-element were used to verify the \textit{in vitro} binding to its specific transcription factor \( PsDOF7 \), while transient expression studies in agroinfiltrated pea leaves were used to confirm \textit{in vivo} transregulation of both TRX promoters. The results show that glucose, but not sucrose, leads to a rapid increase in the expression of TRX \( f \) and \( m1 \) proteins probably mediated by \( PsDOF7 \), and that a similar effect is observed in response to DTT feeding.

**Materials and methods**

**Plant material and growth conditions**

Pea (\textit{Pisum sativum} var. Lincoln) plants were germinated on vermiculite for 2 d at 4 °C in the dark, and then transferred to a green cabinet (22 °C, 12 h light period with 100 µmol m \(^{-2}\) s \(^{-1}\) photosynthetically active radiation). Three weeks after germination, roots of entire plants were immersed in 2 mM MES (2-[\( N \)-morpholino]ethane-sulphonic acid) buffer pH 6.5, and different sugar solutions were prepared in 2 mM MES. Sucrose, fructose, and glucose were at 100 mM concentration. Leaves were harvested at 2, 4, 8, and 14 h (2 h dark). An additional experiment was performed at the end of the night period; leaf discs were immersed in glucose and sucrose solution at 100 mM, or DTT at 5 mM for up to 4 h in the dark. The plant material was instantly frozen in liquid nitrogen and stored at −80 °C until analysis of metabolites and transcripts.

**Determination of sugars**

Carbohydrates were extracted twice from frozen leaf rosettes with 80% ethanol (v/v) at 80 °C for 30 min, followed by further washing with 50% ethanol at 80 °C for 15 min (Stitt et al., 1978). After centrifugation, sucrose, glucose, and fructose were measured enzymatically in the soluble part by determining the reduction of NADP at 340 nm according to Sekin (1978).

**Reverse transcription, semi-quantitative, and real-time PCR analysis**

RNA from leaves was extracted from at least three different plants by a modified version of the hot borate method (Wan and Wilkins, 1994). First-strand cDNA was synthesized from 2.5 µg of total RNA using a modified version of the hot borate method (Wan and Wilkins, 1994). Primer sequences were \( \text{DOF-1N} \), 5'-CTTTGATCATCATCAATCC-3'; \( \text{DOF-1C} \), 5'-CATATAGATGACTTTTCCTTAA-3'; \( \text{DOF-EcoRI} \), 5'-TTTTGAATTCGGATTACCATTATATCCTCC-3'; and \( \text{DOF-NcoI} \), 5'-TTTTCGAATTCGATTACCATTATATCCTCC-3'; and \( \text{DOF-Neol} \), 5'-TTTTCGACAGAACCACATCGTGGCC-3'.

**Electrophoretic mobility shift assay (EMSA)**

The double-stranded oligonucleotides LumF and LumM, containing the DOF element described in Fig. 8, were synthesized by annealing synthetic primers LumFs and LumFa, LumMs and LumMa, and were end-labelled by a fill-in reaction with an \([\alpha-\text{\textit{32P}}]d\text{ATP} \) mixture (3000 Ci mm \(^{-1} \) in M). Primers were 5'-TTTTCCATGGACACAACTCAATGGCC-3'. Primer sequences were \( \text{DOF-1N} \), 5'-CTTTGATCATCATCAATCC-3'; \( \text{DOF-1C} \), 5'-CATATAGATGACTTTTCCTTAA-3'; \( \text{DOF-EcoRI} \), 5'-TTTTGAATTCGGATTACCATTATATCCTCC-3'; and \( \text{DOF-NcoI} \), 5'-TTTTCGACAGAACCACATCGTGGCC-3'.

**Transient expression analysis by agroinfiltration of pea leaves**

In a previous study (Barajas et al., 2007), \( PsTRXf \) and \( m1 \) promoters were transcriptionally fused to the \textit{uidA} (\[\beta\]-glucuronidase (GUS)) reporter gene via the pBI101 binary vector, and designated \( PsTRXf\text{-GUS} \) and \( PsTRXm\text{-GUS} \) (\textit{m1}-GUS). Both were used as reporter constructs for pea transformation. \( PsDOF7 \) was introduced into the pBINPlus35S binary vector (VanEngelen et al., 1995) without the GUS reporter gene but under the control of the \textit{Cauliflower mosaic virus} (CaMV) 35S promoter and used as negative control and effectors of \textit{fl-GUS} and \textit{m1-GUS}. Co-transfection experiments were performed according to the method of Yang et al. (2000) and Rueda-López et al. (2008). For transient co-expression experiments, bacterial cultures were mixed in a 1:1 ratio. \textit{Agrobacterium tumefaciens} strains GV3101::pMMP90 containing either a binary effector plasmid or a reporter construct were co-infiltrated into nearly fully expanded leaves of 10- to 15-day-old pea (\textit{P. sativum}) plants, using a 1 ml Micro fine syringe. After agroinfiltration, pea
Barajas-López et al. plants were maintained in a growth chamber at 22 °C, 16 h light for 3 d. Histochemical localization of GUS activity was performed as described by Jefferson (1987) using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Clontech).

**Results**

External feeding of sugars via the roots leads to changes in the levels of carbohydrates in leaves

Figure 1 shows the content of glucose, fructose, and sucrose in the upper leaves of pea plants fed through the roots with buffer (control) (Fig. 1a), glucose (Fig. 1b), fructose (Fig. 1c), and sucrose (Fig. 1d). The glucose content in control plants slightly increased after 8 h of exposure to light, and decreased in the dark (Fig. 1a). In the same control solution, after 14 h, sucrose content doubled compared with 2 h of light (Fig. 1a). During the same period, the fructose values did not change and they were much lower than for glucose or sucrose. Plants treated for 14 h with glucose underwent an increase in the glucose content, representing 300% of the content reached after 2 h (Fig. 1b). A progressive increase in the sucrose content was detected when pea plants were treated for 14 h with glucose (Fig. 1b). Fructose values increased after 2 h of glucose supply and remained constant at concentrations 20 times higher than in control plants. Whereas plants supplied with fructose did not clearly undergo changes in glucose with respect to control, sucrose augmentation at the end of the photoperiod was abolished (Fig. 1c). Interestingly, fructose rose significantly and it reached glucose values at the end of the treatment (Fig. 1c). Figure 1d shows a constant increase in the sucrose content of pea plants treated with the same sugar, rising after a 14 h photoperiod to almost 5-fold the value registered at 2 h. These plants also accumulated more glucose and fructose than controls, especially glucose.

Fig. 1. Changes in the intracellular content of glucose, fructose, and sucrose after supplying buffer (2 mM MES) (a), glucose (b), fructose (c), or sucrose (d) via the roots of pea plants. The leaves were collected at 2, 4, 8, and 14 h (2 h dark) of the photoperiod. The results are the mean ±SE from three individual plant leaves of three different experiments.
Table 1 shows the hexose:sucrose ratio in the plants treated with glucose, fructose, and sucrose in relation to control plants and throughout the photoperiod. The sucrose supply induced a lower hexose:sucrose ratio in relation to control plants, in contrast to the glucose or fructose treatment, in which the ratio was higher throughout the photoperiod, indicating a direct effect on this ratio by the type of carbohydrate supplied to the plants. All these results indicate that feeding with different sugars modifies the intracellular content of these sugars, and that each sugar treatment induced an important increase in the same sugar in pea leaves. These data suggest a specific uptake possibly through the phloem (van Bel and Hess, 2008) or the xylem, the accumulation of each sugar, and a direct effect on metabolism. Both sucrose and monosaccharide transporters have been described as the main sugar transport between source and sink organs in plants (Sivitz et al., 2008).

| Time (h) | Control | Glucose | Fructose | Sucrose |
|---------|---------|---------|----------|---------|
| 2 h     | 0.39    | 0.57    | 1.01     | 0.51    |
| 4 h     | 0.46    | 0.91    | 1.02     | 0.36    |
| 8 h     | 0.94    | 1.36    | 1.22     | 0.48    |
| 14 h    | 0.32    | 1.37    | 1.21     | 0.41    |

PsTRX f, PsTRX m1, and PsDOF transcript levels in leaves are differently modified in response to external feeding of glucose and sucrose to intact plants via the roots

PsTRX f transcripts rose slightly when the plants were kept in buffer, reaching maximum expression at 8h, and then declined in the dark period (Fig. 2a). Plants administered sucrose behaved similarly, with a decrease after 14 h supply. Nevertheless, in contrast to sucrose, glucose uptake induced a significant increase of PsTRX f transcript ($P \leq 0.05$), and at between 2h and 8h the mRNA level increased 63% in relation to control pea leaves. This effect disappeared after 2h in the dark; however, the expression level of PsTRX f was still higher in relation to the value registered after 2h of treatment. PsTRX f expression registered a maximum after 4h of treatment with fructose, but this declined rapidly. A comparable trend was noted for PsTRX m1 transcripts when plants were provided with buffer, increasing by close to 2-fold at 4h and 8h compared with the expression detected at 2h (Fig. 2b). When plants were given glucose in the light, the PsTRX m1 mRNA level slightly increased, ~30% compared with control, but statistically the difference is not significant. In the dark period, the transcript content in glucose-fed plants was increased by 2-fold the level detected at 2h. A very slight increase in TRX m1 mRNA accumulation was detected at 2h of light and 2h (14h) of dark, when plants were treated with sucrose, compared with control. Fructose supply induced a 2-fold increase of PsTRX m1 expression only after 2h light (Fig 2b) which decreased rapidly at the later time points.

In Fig. 2c it can be noted that glucose, fructose, and sucrose induced a significant increase in PsDOF7 expression after 2h in the light ($P \leq 0.05$). These data suggest that sugars may exert short-term control of the expression of TRXs and of DOF transcription factors.

Fig. 2. Relative expression of (a) PsTRX f, (b) PsTRX m1, and (c) PsDOF7 of pea plants supplied with 100 mM sorbitol, 100 mM glucose, 100 mM fructose, or 100 mM sucrose for 2, 4, 8, and 14 h (2h dark). Levels of mRNA were analysed by RT-PCR with specific primers. The values were normalized to the sorbitol control. Each value is the mean ± SE from three determinations of three cDNA preparations. Asterisks mark significant differences ($P \leq 0.05$) as determined by Student’s t-test comparing the treatment in relation to control.
External supply of glucose to leaf discs in the dark leads to a rapid increase in TRX f and m1 mRNA and protein levels, while sucrose has no substantial effect.

To investigate whether a similar effect of sugars also occurs in leaves in the dark, mRNA (Fig. 3) and protein contents (Figs 4, 5) of PsTRX f and PsTRX m1 were analysed in pea leaf discs prepared at the end of the night period and subsequently incubated with 100 mM glucose or 100 mM sucrose in the dark for 1, 2, 3, and 4 h. Previous experiments have shown that this treatment leads to an increase in the respective internal sugar levels in the leaf disc tissue (Kolbe et al., 2005).

Within 1–2 h, there was a significant increase of PsTRX f transcripts of ~2-fold when glucose was supplied ($P \leq 0.05$), while no change was observed in response to sucrose, compared with 100 mM sorbitol control (Fig. 3a). With increasing incubation time up to 4 h, the glucose effect on the TRX f mRNA level was diminished, while sucrose even led to a slight decrease in TRX f mRNA levels below the sorbitol control. Glucose feeding also led to an increase in PsTRX m1 transcript levels within 4 h, although the time course was different compared with PsTRX f.

As shown in Fig. 3b, PsTRX m1 increased more gradually, showing a significant increase at 3 h and reaching the maximum after 4 h ($P \leq 0.05$). Similar to the results with PsTRX f, sucrose feeding had no substantial effect on the PsTRX m1 transcript level. This shows that glucose, but not sucrose, feeding to leaves leads to an increase in the mRNA levels of both TRXs in the dark. However, it is worth mentioning that the glucose induction of the expression of both TRX genes displays a different pattern of regulation.

To investigate whether the changes in TRX f and m1 transcript levels in response to sugars are translated into changes in the respective protein levels, western blot analyses were performed using pea TRX f and m1 antibodies (Barajas et al., 2007). Inspection of the blot in Fig. 4 and the quantification of the intensity of the bands in Supplementary Fig. S1 available at JXB online shows a 2- to 3-fold increase in TRX f protein levels after 4 h feeding of glucose, while sucrose feeding induces an increase of the protein content between 3 h and 4 h of treatment. This confirms the results of the TRX f mRNA analysis (see Fig. 3a) and shows that the increase in TRX f mRNA observed

![Graph](image)

**Fig. 3.** Short-term feeding of sugars to leaf discs of pea plants at the end of the night leads to increased mRNA levels of TRX f and m. Leaf discs were incubated for 1, 2, 3, and 4 h in 100 mM sorbitol (control), 100 mM glucose or 100 mM sucrose, before samples were frozen to analyse TRX f (a) and TRXm (b) mRNA levels by real-time PCR using specific oligonucleotides. The values were normalized to the sorbitol control. Each value is the mean ± SE from three determinations of three cDNA preparations. The transcript amounts are represented as arbitrary units in relation to the control level, time 0 h set to 1.0.
Sugar and thiol status affects TRX \textit{f} and \textit{m} expression

After 1 h and 2 h is accompanied by an increase in TRX \textit{f} protein after 4 h of glucose feeding. It also confirms that the effect of sucrose feeding on TRX \textit{f} expression is only minor, at the mRNA as well as the protein level.

Also TRX \textit{m1} protein levels gradually increased up to 3-fold when glucose was supplied for 4 h to leaf discs in the dark, while sucrose feeding showed no major effect, compared with sorbitol or buffer control (Fig. 5; Supplementary Fig. S2 at JXB online). This again confirms the results of the mRNA analysis of TRX \textit{m1}, and shows that both TRX \textit{m1} mRNA and protein levels were strongly increased in response to glucose, but the sucrose feeding showed a slight effect.

Figure 6 shows that glucose and sucrose external supply induced a strong increase in the \textit{PsDOF} transcripts after 2 h in the dark that decay rapidly at 4 h, indicating its participation in a general sugar transcriptional regulation of genes.

External supply of DTT to alter the thiol status of leaf discs in the dark leads to a rapid increase in TRX \textit{f} and \textit{m1} mRNA and protein levels

To investigate the effect of the thiol status on TRX \textit{f} and \textit{m1} expression independently of sugars, 5 mM DTT was supplied to the leaf discs for up to 4 h. Previous studies have shown that this treatment leads to an increase in the thiol status of the tissue, leading to post-translational redox activation of known TRX targets such as FBPase (Cazalis \textit{et al.}, 2004; Serrato \textit{et al.}, 2009), NADP-MDH, and AGPase by thiol–disulphide modulation (Kolbe \textit{et al.}, 2006). As shown in Fig. 7a, DTT led to a strong increase in the \textit{PsTRX f} and \textit{m1} mRNA levels after 3–4 h. There were also strong increases in the TRX \textit{f} (2.5-fold; Fig. 7b; Supplementary Fig. S3 at JXB online) and TRX \textit{m1} (10-fold; Fig. 6c; Supplementary Fig. S3) protein levels, which developed gradually within the 4 h time course. Interestingly, TRX \textit{f} and \textit{m1} protein levels already increased markedly after 1 h incubation with DTT, while TRX \textit{f} mRNA levels increased only slightly and TRX \textit{m1} mRNA levels remained unchanged at this early time point. This indicates that the increase in TRX \textit{f} and \textit{m1} protein levels in response to DTT can only partly be explained by the increase in the respective transcript levels and implies possible additional thiol-dependent effects that enhance TRX \textit{f} and \textit{m1} protein content.

\textit{PsDOF7}, a DOF transcription factor, binds in a sequence-specific manner to \textit{PsTRX f} and \textit{PsTRX m1} promoters

\textit{PsTRX f} and \textit{PsTRX m1} regulatory regions exhibit four and nine DOF motifs (AAAG), respectively, surrounded by several light elements, as described previously (Barajas-López \textit{et al.}, 2007). These elements serve as binding sites for DOF transcription factors and may contribute to the sugar-dependent regulation of TRXs. In an effort to detect the potential interaction with the DOF regulatory motifs of both chloroplastic \textit{PsTRX} promoters, synthetic sequences carrying the DOF domain were incubated with the recombinant \textit{PsDOF7} transcription factor by using the EMSA technique. A database search revealed at least seven different \textit{P. sativum} DOF transcription factor (Nakamura \textit{et al.}, 2003), and the alignment of all plant DOF protein sequences available showed that \textit{PsDOF7} was the transcription factor related to other DOF transcriptional factors involved in carbon metabolism regulation as was shown for DAG1 and AtDOF4.1.
The PsDOF7 coding sequence was isolated by PCR, subcloned, and overexpressed as a His-tag fusion protein in *E. coli*. The phylogenetic tree of plant DOF proteins and predicted amino acid sequence of PsDOF7 is shown in Fig. 8a and 8b, together with the conserved 52 amino acids of the DOF domain.

The specific interaction of the PsDOF7 transcription factor in a gel-shift assay was carried out with four different radioactively labelled oligonucleotide probes for LumF and LumM that contain the AAAG motif of the *PsTRX* f and *PsTRX* m1 promoter sequences, respectively (Barajas-López et al., 2007). As shown in Fig. 9, a retarded band was observed when PsDOF7 protein was incubated with LumF and LumM (Fig. 9a, 9b, lanes 2–4). Binding specificities were confirmed by competition titrations, up to 500 ng, with the corresponding unlabelled homologous probes LumF and LumM (Fig. 9a, 9b, lanes 5–7) and by using different amounts of poly(dI–dC) in binding reactions of PsDOF7–LumF/LumM. These results demonstrated that the PsDOF7 transcription factor can strongly interact in a sequence-specific manner with the regulatory AAAG element of both pea chloroplastic TRXs promoters.

**PsDOF7 regulates the expression of the TRX f and TRX m1 promoters in agroinfiltrated pea leaves**

To verify the *in vivo* transcriptional regulation of *TRX f* and *TRX m1* genes, transient expression experiments were performed in planta using infiltration of *A. tumefaciens* cells into pea leaves. Agroinfiltration has been demonstrated to be effective for transient expression in many plant species including tobacco (Sheludko et al., 2006), grapevine (Santos-Rosa et al., 2008), lettuce, tomato, *Arabidopsis* (Wroblewski et al., 2005), witchgrass (VanderGheynst et al., 2008), radish, pea, lupine, and flax (Van der Hoorn et al., 2000). *PsTRXf::GUS* (f1-GUS) and
Sugar and thiol status affects TRX \( f \) and \( m \) expression

PsTRX\( m1 \)::GUS (m1-GUS) were used as reporter constructs (Fig. 10b, 10c, leaf right-hand side). The whole open reading frame of PsDOF7 was used as the negative control (Fig. 10a) and as an effector under the control of the CaMV 35S promoter (Figs. 10b, c, leaf left-hand side). Co-infiltration experiments in \( P. \ sativum \) leaves were performed essentially as described by Yang et al. (2000).

As shown in Fig. 10b and 10c, infiltration of PsTRX\( f1 \)::GUS into the leaf right-hand side resulted in a visible GUS activity in the areas where \( \text{Agrobacterium} \) cells were applied. Co-expression of the reporter \( f1 \)-GUS and 35S::DOF7 effector construct significantly increased the GUS activity under the control of the TRX \( f1 \) promoter (Fig. 10b, leaf left-hand side). These data provide evidence that PsDOF7 functions as a positive regulator in the transient experiments. Co-expression experiments performed as described before for the m1-GUS construct (Fig. 10c, leaf left-hand side) and the effector construct PsDOF7, showed increased GUS activity under the control of the TRX \( m1 \) promoter, suggesting that PsDOF7 may function as well as a positive regulator of the gene, but with lower intensity.

![Fig. 8.](image)

**Discussion**

TRXs \( f \) and \( m \) are key components in the light regulation of photosynthesis and chloroplast metabolism (Buchanan and Balmer, 2005). However, the factors regulating the expression of these TRXs have not been fully clarified yet. In this report, evidence is provided that sugars and the redox status are involved in the transcriptional and post-translational regulation of PsTRX \( f \) and \( m1 \) expression in leaves. Sugars also lead to an increase in the expression of the carbon metabolism-related transcription factor PsDOF7, which binds to PsTRX \( f \) and \( m1 \) promoter regions as shown by DNA–protein interaction studies. The results provide evidence for a link between photosynthesis and TRX expression in leaves and may explain the expression of these TRXs in sink tissues importing sugars from the phloem.

In previous studies, light has been found to be an important factor regulating the expression levels of pea TRX \( f \) and TRX \( m1 \), that additionally are under the control of the circadian oscillation exerted at both transcriptional and protein levels (Carrasco et al., 1992; Barajas-López et al., 2011). In these studies Barajas and colleagues (2011) reported that Arabidopsis transgenic plants expressing PsTRX \( f \) and \( m1 \) gene promoters fused to the GUS reporter gene showed an oscillatory expression pattern, which persists during subjective night. The night and subjective night phases did not induce the complete loss of TRX \( f \) and \( m1 \) expression, suggesting that other factors besides light, such as sugars, could control the TRX \( f \) and \( m1 \) mRNA level. The present results confirm these previous studies, showing that TRX \( f \) and \( m1 \) transcript levels are increased during the day (Fig. 2a, 2b). In addition to this, it was found that two further factors are important in regulating TRX expression, sugars and redox. These two internal inputs are linked to light via photosynthesis, but can also act independently of...
light in the dark. Sugars affected the expression of *PsTRX f* and *m1*, at both the mRNA and protein level. The effect was mainly found with glucose, and the effect on *PsTRX m1* was stronger than on *PsTRX f* (Figs. 4, 5; Supplementary Figs S1, S2 at JXB online). There was a 3-fold increase in TRX *f* and a 5-fold increase in TRX *m1* protein levels (see Supplementary Figs S1 and S2). Glucose led to a significant increase mainly in the expression of *PsTRX f* in the light and of both PsTRXs in the dark (Figs 2, 3) (*P* ≤ 0.05). Despite the different pattern of expression, this indicates that light regulation of TRX expression may be mediated by an increase in glucose levels. Moreover, glucose could also be involved in inducing TRXs in heterotrophic tissues, since the glucose effect on TRX expression also occurred independently of light. This may explain the expression of *TRX f* and *m* isoforms in heterotrophic sink tissues importing sugars from the phloem (Balmer et al., 2006a; Barajas-López et al., 2007; Traverso et al., 2008). Plastid TRX expression has been observed in cotyledons of etiolated seedlings of *A. thaliana* lines carrying constructs corresponding to *PsTRX f* and *m1* promoters fused to the reporter gene GUS. These results indicated a role in reserve mobilization and an influence of other internal or environmental stimuli separate from the light (Fernández-Trijueque et al., 2012). Furthermore, TRXs have been implicated in regulating carbon metabolism in tubers and roots, as well as in leaves, in response to sugar supply by acting on AGPase as a target (Tiessen et al., 2002; Hendriks et al., 2003; Michalska et al., 2009; Geigenberger, 2011). The results of the present study therefore raise the possibility that glucose-induced redox activation of AGPase might be partly due to an increase in TRX protein level.

Both light and sugar factors could be bolstered or inhibited in their effects and could share several steps of their regulatory pathways. Additionally, the different effects noted with glucose and sucrose on *PsTRX f* and *m1* expression point to specific and differential signalling pathways used by each carbohydrate to control *PsTRX* transcripts (Jang et al., 1997). In fact, glucose incubation provokes the higher glucose augmentation in pea leaves (Fig. 1b), suggesting either a direct or an indirect role in the increase of *PsTRX f* mRNA accumulation (Fig. 2). In contrast to glucose and sucrose, fructose supply inhibits sucrose formation (Fig. 1c). This is in line with a recent report of Cho and Yoo (2011) describing fructose as a signalling molecule that arrests seedling development.

The increase in *PsTRX f* and *m1* protein level upon glucose feeding occurred rapidly within 4 h in the dark and was preceded by an increase in the respective mRNA levels, indicating transcriptional regulation. Sucrose feeding only had minor effects, indicating that transcriptional regulation is mainly linked to a glucose-specific sugar signalling pathway. This may involve signalling via HXK, which has been found to be involved in signalling pathways regulating transcriptional regulation of photosynthesis in response to sugars (Jang et al., 1997; Xiao et al., 2000; Rolland et al., 2006).

The results also implicate the PsDOF7 transcription factor as being involved in the sugar signalling pathway regulating TRX expression (Yanagisawa and Schmidt, 1999). First, the *PsDOF7* expression level is induced by sugars such as glucose, which also lead to induction of *PsTRX f* and *m1* expression under the same experimental conditions. Secondly, protein–DNA interaction assays show that the selected regions of both *PsTRX* gene promoters carrying DOF core elements interact specifically with the transcription factor DOF7. These results suggest that sugars could
regulate TRX expression at the transcriptional level through the binding of PsDOF7 transcription factor to chloroplastic TRX promoters, although additional nucleotides flanking the DOF motif might be necessary for the interaction in PsTRX \( f \) and \( m1 \) promoters. The different elements surrounding the canonical DOF-binding site of PsTRX \( f \) and \( m1 \) promoters might be responsible for their gene-differential transcriptional regulation in the sugar-treated plants. The results of in vivo co-transformation support the ability of PsDOF7 to activate transcription of TRX \( f \) and TRX \( m1 \) in pea leaves. PsDOF7 is able to bind a region of the TRX \( f \) and TRX \( m1 \) in vitro (Figs 8, 9) and to regulate gene expression in vivo in pea leaves (Fig. 10). While the present results provide in vitro and in vivo evidence that the DOF7 transcription factor is a likely candidate for linking TRX expression to sugars, they do not rule out the possibility that other transcription factors including PsDOF could bind in vivo to the promoters analysed.

The results show that redox status provides a further input in regulating the expression of TRX \( f \) and \( m1 \) in leaves. Using a similar approach as in Kolbe et al. (2006), the effect of an increased redox status on TRX \( f \) and \( m1 \) expression levels was investigated by feeding diluted DTT to leaf tissue in the dark. The protein levels of both TRXs increased markedly within 1 h and showed a further increase up to 4 h incubation with DTT (Fig. 7b, 7c; Supplementary Fig. S3 at JXB online). As shown in Fig. 7a, this can only partly be explained by transcriptional mechanisms, since the main increase in the respective mRNA levels occurred in the second half of the time course after 3 h. The very rapid increase in TRX protein levels after 1 h is most likely to be due to post-translational rather than transcriptional mechanisms. Obviously, there is an immediate link between the redox status of the tissue and TRX protein turnover. This may indicate that reduced TRX is more stable than oxidized TRX, or the possible involvement of redox in regulating TRX protein turnover pathways. Transcriptional regulation mediates the longer term effects of redox on TRX expression. Photosynthetic redox signals have been shown to regulate gene expression in photosynthetic acclimation in leaves in response to changes in the light conditions (Bräutigam et al., 2009). Similar redox-based mechanisms may also be involved in the transcriptional regulation of TRX expression in response to light signals. Also, the glucose-induced transcriptional activation of TRX expression may involve a redox component, since glucose feeding to leaf discs in the dark leads to an increase in the NADPH/NADP redox status and increased redox activation of AGPase (Kolbe et al., 2006).

In conclusion, the present results show that beside light, expression of PsTRX \( f \) and \( m1 \) is regulated by sugar, mainly glucose, and thiol signals at the transcriptional and post-translational level. While the sugar signal probably involves a DOF7 transcription factor, the nature of the redox signalling pathways leading to changes in gene expression remains to be resolved. Revealing new factors involved in the expression of plastidial TRXs shows a complex but fine regulation network used by the plants to preserve the steady state of its metabolism. Furthermore, these findings open up a wide field of research needed to investigate the interplay between light, redox, and sugars in regulating the expression levels of TRXs and the possible implications for TRX targets in plants.

**Supplementary data**

Supplementary data are available at JXB online.

Figures S1. Effect of short-term sugar feeding on TRX \( f \) protein levels in pea leaf discs in the dark.

Figure S2. Effect of short-term sugar feeding on TRX \( m \) protein levels in pea leaf discs in the dark.

Figure S3. Effect of short-term DTT feeding on TRX \( f \) and \( m \) protein levels in pea leaf discs in the dark.
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