Journal of Experimental Botany, Vol. 63, No. 13, pp. 4887–4900, 2012
doi:10.1093/jxb/ers163 Advance Access publication 12 July, 2012
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

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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Received 17 April 2012; Revised 7 May 2012; Accepted 8 May, 2012

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 4 h 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 4 h. The increase in TRX protein levels occurred after 1 h DTT feeding, implying a rapid effect of the thiol status on TRX *f* and *m1* protein turnover rates, while transcriptional regulation took 3 h 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

Abbreviations: DOF, DNA binding with one finger; DTT, dithiotreitol; FBPase, fructose-1,6-bisphosphatase; MDH, malate dehydrogenase; Rbcs, small subunit of ribulose-1,5-bisphosphate-carboxylase; TRX, thioredoxin

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* (Martí *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 CO_2 assimilation rate during starch synthesis and the oxalate/malate 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*., 2006*b*; 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*., 2006*a*; 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 *m1* from pea have been found to be more abundant in 2- 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* promoter 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 Cys_2–Cys_2 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*, *PsTRXm1*, and PsDOF transcription factor was analysed 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 *in vitro* binding to its specific transcription factor PsDOF7, while transient expression studies in agroinfiltrated pea leaves were used to confirm *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 (*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 ºC, 12 h light period with 100 µmol m^{-2} s^{-2} 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 ºC until analysis of metabolites and transcripts.

Determination of sugars

Carbohydrates were extracted twice from frozen leaf rosettes with 80% ethanol (v/v) at 80 ºC for 30 min, followed by further washing with 50% ethanol at 80 ºC for 15 min (Stitt *et al*., 1978). After centrifugation, sucrose, glucose, and fructose were measured enzymatically in the soluble solution 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 retrotranscriptase RNase Super-Script III (Invitrogen) and oligo(dT)12–15 primer, at 50 ºC for 1 h. The PCR conditions were 2 min at 94 ºC, 28 cycles at 92 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 30 s, and 72 ºC for 5 min. When real-time PCR was used, the conditions were: 2 min at 50 °C; 10 min at 95 °C; 40 cycles of 15 s at 95°C, 1 min at 60°C. The Ct value was determined using the instrument's software (iQcycler, Bio-Rad). The different primers used were rtTrxmF, 5'-GTTTCACTTCGCTGGTGTTGC-3' and rtTrxmR, 5'-CTTTCTCAGACAGAGTAGCC-3' for *PsTRX m1*; rtTrxfF, 5'-TGATAAAACCGTCGTCCTCGAT-3' and rtTrxfR, 5'-ATTTCCTCATCTTCCCCTCAGC-3' for *PsTRXf*; and rtDOF7F, 5'-GTGGGAGGTGGAACAAGAAA-3' and rtDOF7R, 5'-CCTGCAAAGGAAACCC-3' for *PsDOF7*. The experimental results were normalized against the actin-2 gene, which was set as the endogenous control gene, and the primer sequences were PsAct-F, 5'-AATGGTGAAGGCTGGATTTG-3' and PsAct-R, 5'-AGCAAGATCCAAACG AAGGA-3'. Quantity One software (Bio Rad) was used to quantify band intensities of relative levels of expression.

Protein extraction and western blot analysis

Leaf samples were snap-frozen with liquid N_2 and tissue was solubilized adding extraction buffer [25 mM TRIS-HCl, pH 7.5, 75 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.1 % (v/v) NP-40]. After SDS–PAGE, western blot was performed as described in Barajas-López *et al*. (2007). Quantity One software (Bio Rad) was used to quantify band intensities of relative protein content.

Overexpression and purification of PsDOF7 transcription factor

The *PsDOF7* cDNA sequence was amplified from pea leaf cDNA, using DOF-1N/DOF-1C and DOF-EcoRI/DOF-NcoI primers in two sequential PCRs, and cloned into the pET-28b (Invitrogen) expression vector. *Escherichia coli* BL21 (DE3) cells were transformed with the recombinant plasmid pET28b-*PsDOF7* and used for protein overproduction. *PsDOF7* expression was induced at 28 ºC by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the *E. coli* culture medium at a final concentration of 0.4 mM. After 4 h, cells were collected and resuspended in buffer A (20 mM phosphate pH 7.0, 20 mM imidazole, 500 mM NaCl, and EDTA-protease inhibitor cocktail). Cells were lysed and the supernatant was treated with 100 µg of DNase, whereupon the solution was filtered through a HisTrap® Crude affinity column (GE-Healthcare). PsDOF7 was eluted with buffer C [20 mM phosphate pH 7.0, 500 mM imidazole, 500 mM NaCl, and 50% (v/v) glycerol]. The fractions containing PsDOF7 were analysed by SDS–PAGE (Laemmli, 1970). Primer sequences were DOF-1N, 5'-CTTTGATCATCATCAATCC-3'; DOF-1C, 5'-CATATAGATGACTTTTCCTTAA-3'; DOF-EcoRI, 5'-TTTTGAATTCGGATTACCATTATATCCTCC-3'; and DOF-NcoI, 5'-TTTTCCATGGACACAACTCAATGGCC-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 [α-^{32}P]dATP mixture (3000 Ci mM^{-1}) and Klenow enzyme, and gel purified. Reactions were performed in 20 µl of a mixture containing 4.87 mM HEPES–KOH (pH 7.9), 25 mM KCl, 2.5 mM DTT, 5% glycerol, 1–5 µg of poly(dI–dC), 20 000 cpm of radiolabelled probe, and 50 ng of the PsDOF7 protein. When required, 100–500 ng of competitor unlabelled double-stranded probe was added to the reaction. In unspecific competition experiments, 1–5 µg of poly(dI–dC) was also included in the mixes. After incubation for 1 h at 4 ºC, the DNA–protein complexes were resolved on a 6% polyacrylamide gel with 0.5× TBE at 100 V for 3.5 h at 4 °C. The gel was dried and subjected to autoradiography.

Transient expression analysis by agroinfiltration of pea leaves

In a previous study (Barajas *et al.*, 2007), *PsTRX f* and *m1* promoters were transcriptionally fused to the *uidA* [β-glucuronidase (GUS)] reporter gene via the pBI101 binary vector, and designated *PsTRXf1*::*GUS* (f1-GUS) and *PsTRXm::GUS* (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 *Cauliflower mosaic virus* (CaMV) 35S promoter and used as negative control and effector of f1-GUS and 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. *Agrobacterium tumefaciens* strains GV3101::pMP90 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 (*P. sativum*) plants, using a 1 ml Micro fine syringe. After agroinfiltration, pea

plants were maintained in a growth chamber at 22 ºC, 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.

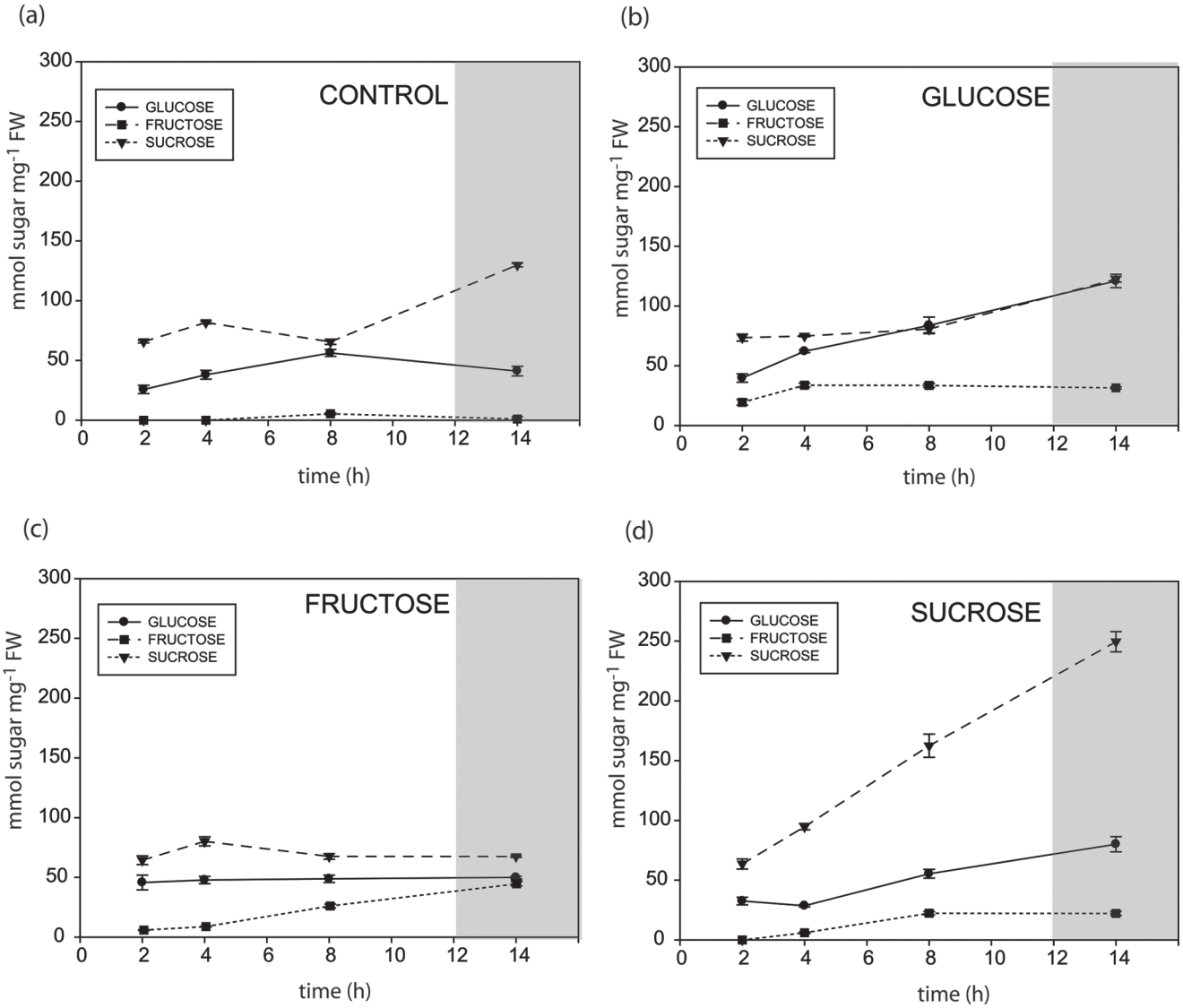

Fig. 1. Changes in the intracellular content of glucose, fructose, and sucrose after supplying buffer (2 mM MES) (a), glucose (b), fructose (c), or sucrose (d) via the roots of pea plants. The leaves were collected at 2, 4, 8, and 14 h (2 h dark) of the photoperiod. The results are the mean ±SE from three individual plant leaves of three different experiments.

Table 1 Mean hexose/sucrose ratios in pea leaves under different treatments at different times

	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

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).

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 8 h, 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 2 h and 8 h the mRNA level increased 63% in relation to control pea leaves. This effect disappeared after 2 h in the dark; however, the expression level of *PsTRX f* was still higher in relation to the value registered after 2 h of treatment. *PsTRX f* expression registered a maximum after 4 h 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 4 h and 8 h compared with the expression detected at 2 h (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 2 h. A very slight increase in TRX *m1* mRNA accumulation was detected at 2 h of light and 2 h (14 h) of dark, when plants were treated with sucrose, compared with control. Fructose supply induced a 2-fold increase of *PsTRX m1* expression only after 2 h 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 2 h 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.

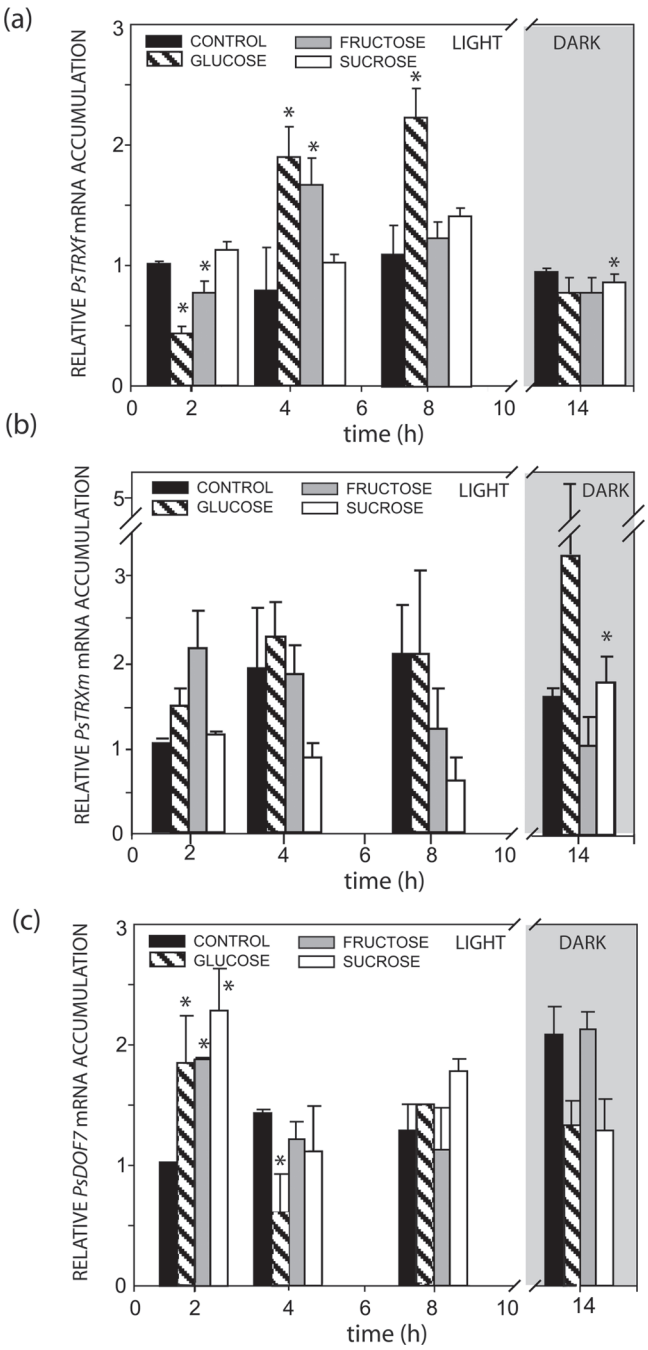

Fig. 2. Relative expression of (a) *PsTRX f*, (b) *PsTRX m1*, and (c) *PsDOF7* of pea plants supplied with 100 mM sorbitol, 100 mM glucose, 100 mM fructose, or 100 mM sucrose for 2, 4, 8, and 14 h (2 h dark). Levels of mRNA were analysed by RT-PCR with specific primers. The values were normalized to 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 Supplemenatry 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

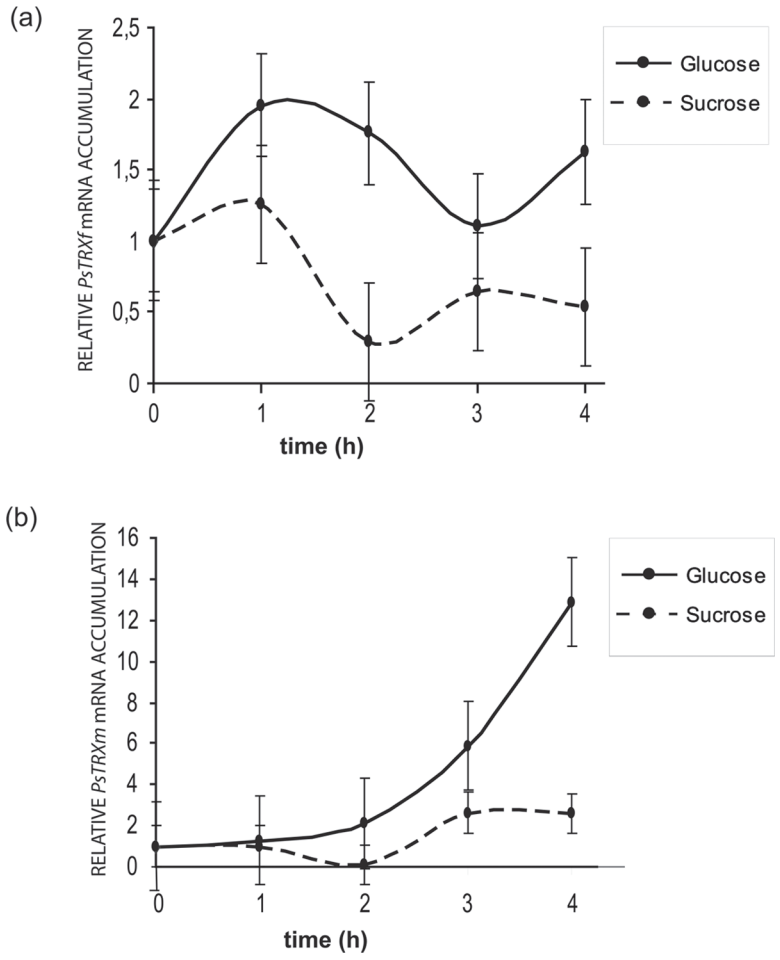

Fig. 3. Short-term feeding of sugars to leaf discs of pea plants at the end of the night leads to increased mRNA levels of TRX *f* and *m*. Leaf discs were incubated for 1, 2, 3, and 4 h in 100 mM sorbitol (control), 100 mM glucose or 100 mM sucrose, before samples were frozen to analyse TRX *f* (a) and TRX*m* (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.

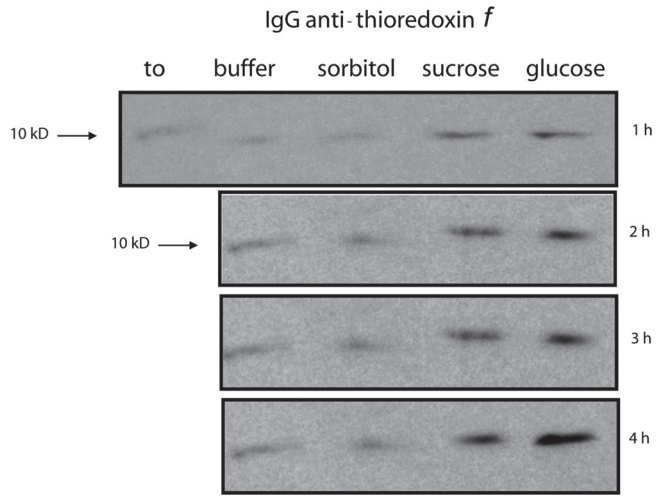

Fig. 4. Short-term feeding of sugars to leaf discs of pea plants at the end of the night leads to an increased TRX *f* protein level. Leaves of pea plants were harvested at the end of the night to prepare discs that were immediately incubated in the dark for 1, 2, 3, and 4 h in buffer (control), 100 mM sorbitol (control), 100 mM glucose, or 100 mM sucrose, before samples were frozen to analyse the Trx *f* protein level using western blots. t_0, leaf tissue before incubation. Western blot images are representative of three independent experiments.

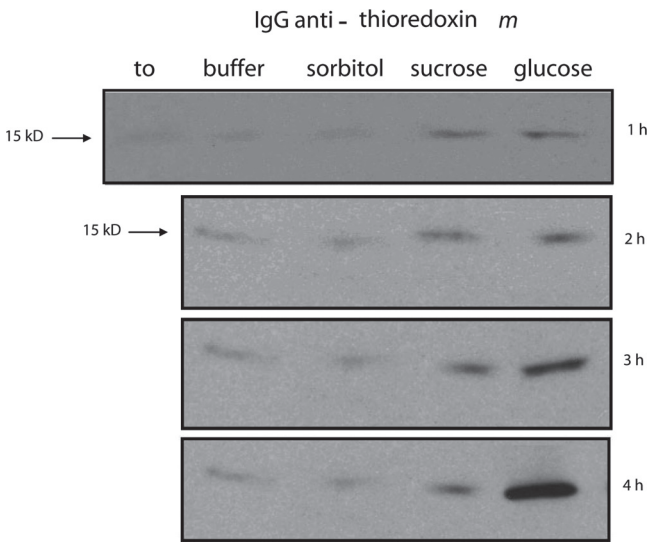

Fig. 5. Short-term feeding of sugars to leaf discs of pea plants at the end of the night leads to an increased TRX *m* protein level. Leaves of pea plants were harvested at the end of the night to prepare discs that were immediately incubated in the dark for 1, 2, 3, and 4 h in buffer, 100 mM sorbitol (control), 100 mM glucose, or 100 mM sucrose, before samples were frozen to analyse the Trx *m* protein level using western blots. t_0, leaf tissue before incubation. Western blot images are representative of three independent experiments.

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

Also TRX *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 *m1*, and shows that both TRX *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 *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 f *and* m1 *mRNA and protein levels*

To investigate the effect of the thiol status on *TRX f* and *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 *et al*., 2004; Serrato *et al*., 2009), NADP-MDH, and AGPase by thiol–disulphide modulation (Kolbe *et al*., 2006). As shown in Fig. 7a, DTT led to a strong increase in the *PsTRX f* and *m1* mRNA levels after 3–4 h. There were also strong increases in the TRX *f* (2.5-fold; Fig. 7b; Supplementary Fig. S3 at *JXB* online) and TRX *m1* (10-fold; Fig. 6c; Supplementary Fig. S3) protein levels, which developed gradually within the 4 h time course. Interestingly, TRX *f* and *m1* protein levels already increased markedly after 1 h incubation with DTT, while TRX *f* mRNA levels increased only slightly and TRX *m1* mRNA levels remained unchanged at this early time point. This indicates that the increase in TRX *f* and *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 *f* and *m1* protein content.

PsDOF7, a DOF transcription factor, binds in a sequence-specific manner to PsTRX f *and* PsTRX m1 *promoters*

PsTRX f and *PsTRX m1* regulatory regions exhibit four and nine DOF motifs (AAAG), respectively, surrounded by several light elements, as described previously (Barajas-López *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 *PsTRX* promoters, synthetic sequences carrying the DOF domain were incubated with the recombinant PsDOF7 transcription factor by using the EMSA technique. A database search revealed at least seven different *P. sativum* DOF transcription factor (Nakamura *et al*., 2003), and the alignment of all plant DOF protein sequences available showed that PsDOF7 was the transcription factor related to other DOF transcriptional factors involved in carbon metabolism regulation as was shown for DAG1 and AtDOF4.1

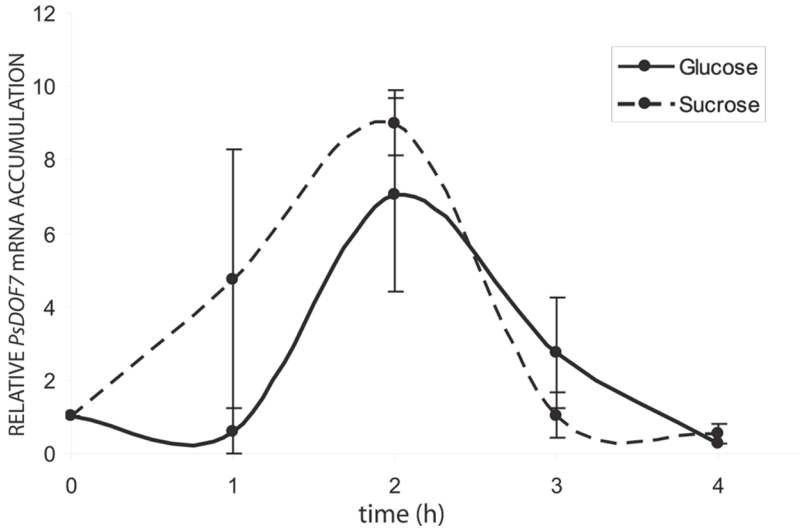

Fig. 6. Short-term feeding of sugars to leaf discs of pea plants at the end of the night leads to increased *PsDOF7* gene expression. Leaves of pea plants were harvested at the end of the night to prepare discs that were immediately incubated in the dark for 1, 2, 3, and 4 h in buffer, 100 mM sorbitol (control), 100 mM glucose, or 100 mM sucrose. mRNA levels were analysed by real-time PCR using specific oligonucleotides. The values were normalized to the sorbitol control. Each value is the mean ±SE 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.

(Yanagisawa and Sheen, 1998; Yanagisawa, 2000). 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

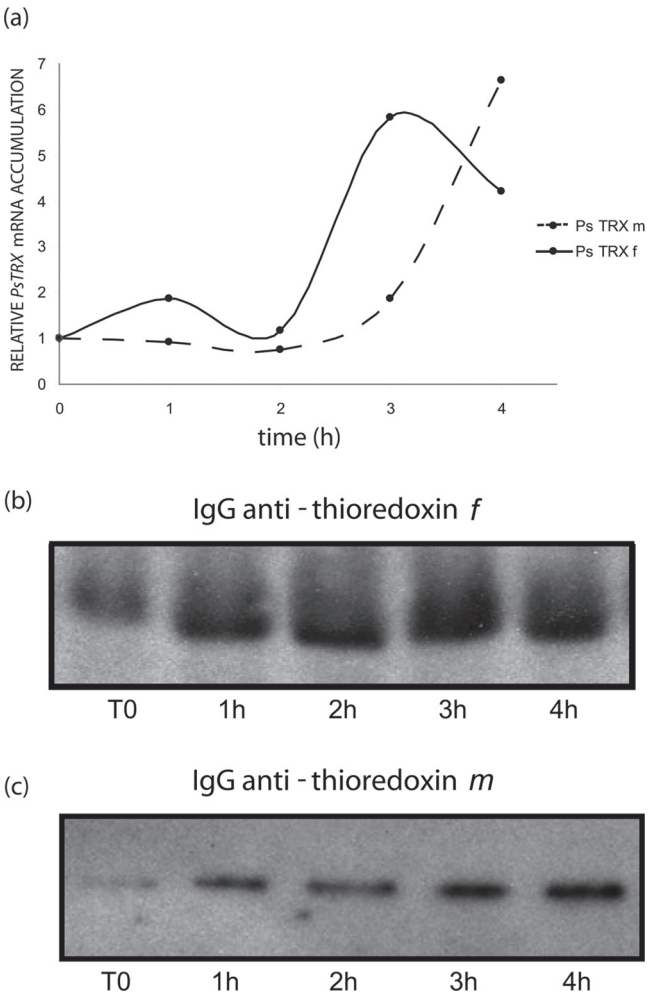

Fig. 7. Dithiotreitol (DTT) feeding leads to increased TRX *f* and *m1* expression in leaves. Leaves of pea plants were harvested

at the end of the night to prepare discs that were immediately incubated on zero and 5 mM DTT for 1–4 h in the dark. Discs were then quickly rinsed in buffer to remove external DTT before freezing in liquid nitrogen to analyse (a) TRX mRNA levels using real-time PCR or (b and c) TRX protein levels using western blots. The expression values were normalized to the buffer control. Each value of transcript 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.

PsTRXm1::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 *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.

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

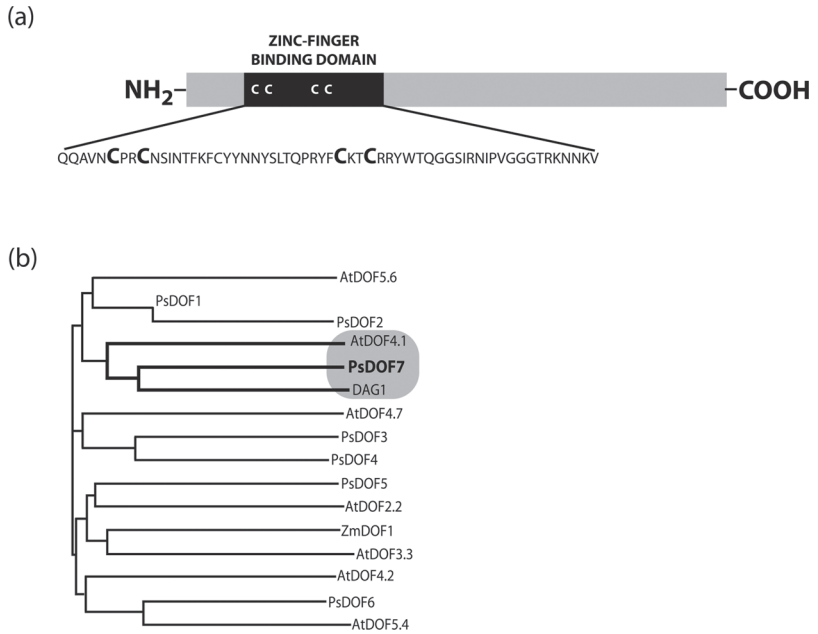

Fig. 8. (a) PsDOF7 protein sequence. The underlined sequence corresponds to the DOFmotif and Cs in bold show the cysteine residues involved in the zinc-finger domain binding to DNA. (b) Phylogenetic tree of plant DOF proteins. PsDOF7 is clustered with carbon-regulated metabolism transcription factors DAG1 and AtDOF4.1.

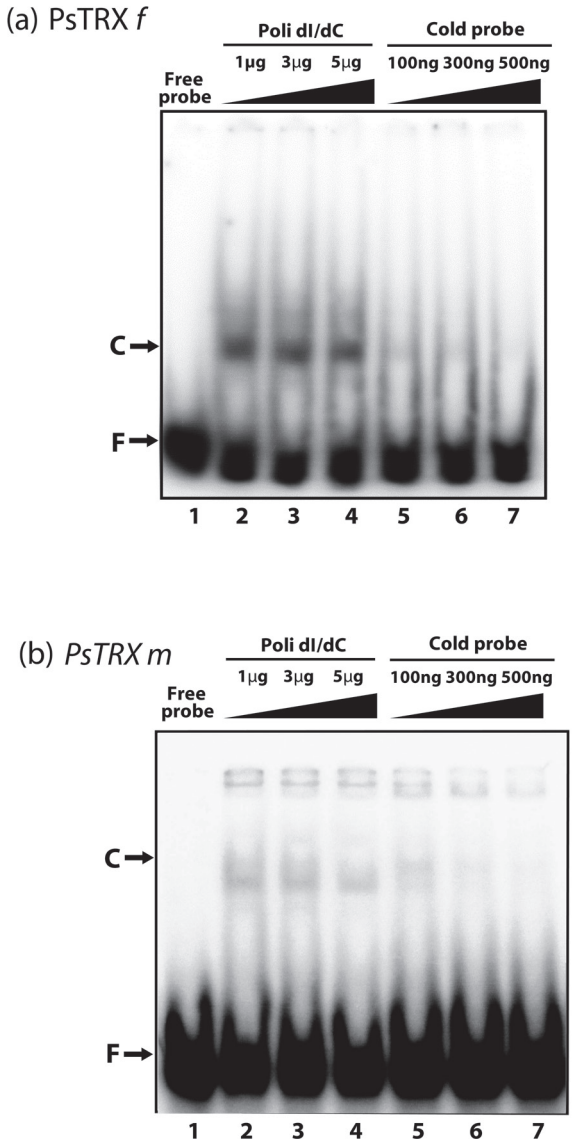

Fig. 9. Functional properties of the pea DOF motif present in *PsTRX f* and *PsTRX m1* promoters. EMSA of the recombinant PsDOF7 with oligonucleotide probes derived from (a) *PsTRX f* and (b) *PsTRX m* promoters. The probe without recombinant PsDOF7 is designated Free Probe (lane 1). Competition experiments were performed using increasing amounts of either the non-specific competitor [poly(dI–dC)] (lanes 2–4) or the unlabelled probe (lanes 5–7). Increasing molar amounts 100, 300, and 500 ng are indicated by triangles.
(a) A probe of 29 bp (LumF) derived from the *PsTRX f* promoters was ^{32}P labelled 5'-AGTGAAAAAAA**AAAG**AGATATTCGAAGGG-3'.
(b) A probe of 41 bp (LumM) derived from the *PsTRX m1* promoters was ^{32}P labelled 5'-TTGACATAAGTAGATATTG**AAAG**CA-AGATTGAAAAAAATGT-3'.

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) ($P \leq 0.05$). 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*., 2006*a*; 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

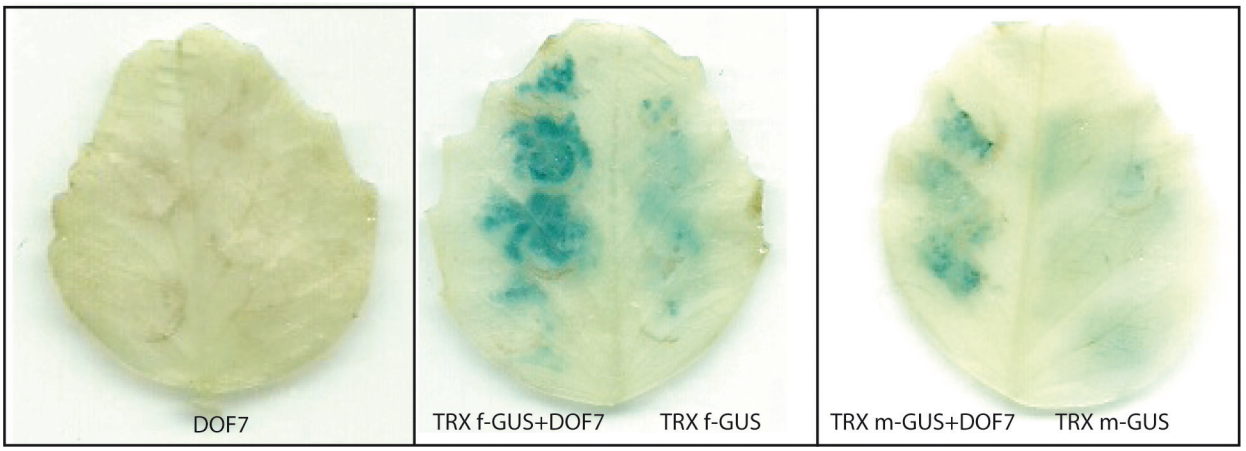

Fig. 10. Transient GUS expression in pea leaves driven by *PsTRX f* and *PsTRX m1* promoters. *Agrobacterium tumefaciens*-mediated transient transformation was conducted on near fully expanded leaves still attached to 2-week-old intact pea plants. Bacterial suspensions were infiltrated into mesophyll (Yang *et al*., 2000). *PsDOF* was used as the control (A). TRX f-GUS (B) and TRX m-GUS (C) were infiltrated into the right-hand side of the leaf, while the co-infiltration of TRX f-GUS (B) and TRX m-GUS (C) with DOF7 was into the left-hand side of the leaf. Histochemical assays were performed 3 d after infiltration. After GUS staining, plant tissues were incubated in 70% (v/v) ethanol at 37 ºC. The leaves in the figure are representative images of three different leaves of three independent experiments.

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 of *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 transcription *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.

Acknowledgements

The authors thank Trinidad Moreno and Ángel García-Díaz for their technical support during this work, and David Nesbitt for helpful editorial feedback on the manuscript. This work was supported by BIO2005-00157 and BIO2006-2816 from 'Ministerio de Educación y Ciencia', BIO2009-7297 from 'Ministerio de Ciencia e Innovación' and 'Fondo Europeo de Desarrollo Regional', and grants from Junta de Andalucía (Grupo BIO 154), Spain. JDBL was supported by a pre-doctoral fellowship from Junta de Andalucía, and AJS was supported by a post-doctoral fellowship from Junta de Andalucía (Spain). PG was supported by the Deutsche Forschungsgemeinschaft.

References

Arsova B, Hoja U, Wimmelbacher M, Greiner E, Üstün S, Melzer M, Petersen K, Lein W, Börnke F. 2010. Plastidial thioredoxin *z*interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in Arabidopsis and *Nicotiana benthamiana*. *The Plant Cell* **22,** 1498–1515.

Balmer Y, Vensel WH, Cai N, Manieri W, Schürmann P, Hurkman WJ, Buchanan BB. 2006a. A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. *Proceedings of the National Academy of Sciences, USA* **103,** 2988–2993.

Balmer Y, Vensel WH, DuPont FM, Buchanan BB, Hurkman WJ. 2006b. Proteome of amyloplasts isolated from developing wheat endosperm presents evidence of broad metabolic capability. *Journal of Experimental Botany* **57,** 1591–1602.

Barajas-López JD, Serrato AJ, Cazalis R, Reichheld JP, Meyer Y, Chueca A, Sahrawy M. 2011. Circadian regulation of the pea chloroplastic *f*and *m1*thioredoxins through control interaction of the CCA1 transcription factor. *Journal of Experimental Botany* **62,** 2039–2051.

Barajas-López JD, Serrato AJ, Olmedilla A, Chueca A, Sahrawy M. 2007. Localization in roots and flowers of pea chloroplast thioredoxin f and m proteins reveals new roles in non-photosynthetic organs. *Plant Physiology* **145,** 1–15.

Bläsing OE, Gibon Y, Gunther M, Hohne M, Morcuende R, Osuna D, Thimm O, Usadel B, Scheible WR, Stitt M. 2005. Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in Arabidopsis. *The Plant Cell* **17,** 3257–3281.

Bolouri-Moghaddam MR, Le Roy K, Xiang L, Rolland F, Van den Ende W. 2010. Sugar signalling and antioxidant network connections in plant cells. *FEBS Journal* **277,** 2022–37.

Bräutigam K, Dietzel L, Kleine T, *et al.*. 2009. Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation. *The Plant Cell* **21,** 2715–2732.

Buchanan BB, Balmer Y. 2005. Redox regulation: a broadening horizon. *Annual Review of Plant Biology* **56,** 187–220.

Buchanan BB, Crawford NA, Wolosiuk RA. 1978. Ferredoxin–thioredoxin system functions with effectors in activation of NADPH glyceraldehyde 3-phosphate dehydrogenase of barley seedlings. *Plant Science Letters* **12,** 257–264.

Carrasco JL, Chueca A, Sahrawy M, Hermoso R, Lázaro JJ, López Gorgé J. 1992. Role of light in the *in vivo*and *in vitro*synthesis of spinach thioredoxin *f*. *Physiologia Plantarum* **84,** 236-242.

Cazalis R, Chueca A, Sahrawy M, Lopez-Gorge J. 2004. Construction of chimeric cytosolic fructose-1,6-bisphosphatases by insertion of a chloroplastic redox regulatory cluster. *Journal of Physiology and Biochemistry* **60,** 7–21.

Cho Y-H, Yoo S-D. 2011. Signaling role of fructose mediated by FINS1/FBP in *Arabidopsis thaliana*. *PLoS Genetics* **7,** e1001263.

Couée I, Sulmon C, Gouesbet G, El Amrani A. 2006. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany* **57,** 449–459.

Fernández-Trijueque J, Barajas-López JD, Chueca A, Cazalis R, Sahrawy M, Serrato A. 2012. Plastid thioredoxins *f*and *m*are related to the developing and salinity response of post-germinating seeds of *Pisum sativum*. *Plant Science* **188–189,** 82–88.

Geigenberger P. 2011. Regulation of starch biosynthesis in response to a fluctuating environment. *Plant Physiology* **155,** 1566–1577.

Geigenberger P, Kolbe A, Tiessen A. 2005. Redox-regulation of carbon storage and partitioning in response to light and sugars. *Journal of Experimental Botany* **56,** 1469–1479.

Hall M, Mata-Cabana A, Akerlund HE, Florencio FJ, Schröder WP, Lindahl M, Kieselbach T. 2010. Thioredoxin targets of the plant chloroplast lumen and their implications for plastid function. *Proteomics* **10,** 987–1001.

Hendriks JHM, Kolbe A, Gibon Y, Stitt M, Geigenberger P. 2003. ADP-glucose pyrophosphorylase is activated by post-translational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. *Plant Physiology* **133,** 838–849.

Holmgren A. 1985. Thioredoxin. *Annual Review of Biochemistry* **54,** 237–271.

Jacquot JP, Vidal J, Gadal P, Schürmann P. 1978. Evidence for existence of several enzyme-specific thioredoxins in plants. *FEBS Letters* **96,** 243–246.

Jang JC, Leon P, Zhou L, Sheen J. 1997. Hexokinase as a sugar sensor in higher plants. *The Plant Cell* **9,** 5–19.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. Gus fusions: β-glucuronidase as a sensitive and versatile marker in higher plants. *EMBO Journal* **6,** 3901–3907.

Kolbe A, Oliver SN, Fernie AR, Stitt M, van Dongen JT, Geigenberger P. 2006. Combined transcript and metabolite profiling of Arabidopsis leaves reveals fundamental effects of the thiol–disulfide status on plant metabolism. *Plant Physiology* **141,** 412–422.

Kolbe A, Tiessen A, Schluepmann H, Paul M, Ulrich S, Geigenberger P. 2005. Trehalose-6-phosphate regulates starch synthesis via post-translational redox-activation of ADP-glucose pyrophosphorylase. *Proceedings of the National Academy of Sciences, USA* **102,** 11118–11123.

König J, Muthuramalingam M, Dietz K-J. 2012. Mechanisms and dynamics in the thiol/disulphide redox regulatory network: transmitters, sensors and targets. *Current Opinion in Plant Biology* **15** (in press).

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lijavetzky D, Carbonero P, Vicente-Carbajosa J. 2003. Genome-wide comparative phylogenetic analysis of the rice and Arabidopsis Dof gene families. *BMC Evolutionary Biology* **3**, 17.

López-Jaramillo J, Chueca A, Jacquot JP, Hermoso R, Lázaro JJ, Sahrawy M and López-Gorgé J. 1997. High-yield expression of pea thioredoxin *m*and assessment of its efficiency in chloroplast fructose-1,6-bisphosphatase activation. *Plant Physiology* **114,** 1169–1175.

Lunn JE, Feil R, Hendriks JH, Gibon Y, Morcuende R, Osuna D, Scheible WR, Carillo P, Hajirezaei MR, Stitt M. 2006. Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADP glucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochemical Journal***397,** 139–48.

Martí MC, Olmos E, Calvete JJ, Díaz I, Barranco-Medina S, Whelan J, Lázaro JJ, Sevilla F, Jiménez A. 2009. Mitochondrial and nuclear localization of a novel pea thioredoxin: identification of its mitochondrial target proteins. *Plant Physiology* **150,** 646–657.

Meyer Y, Siala W, Bashandy T, Riondet C, Vignols C, Reichheld JP. 2008. Glutaredoxins and thioredoxins in plants. *Biochimica et Biophysica Acta* **1783,** 589–600.

Meyer Y, Buchanan BB, Vignols F, Reichheld JP. 2009. Thioredoxins and glutaredoxins: unifying elements in redox biology. *Annual Review of Genetics* **43,** 335–367.

Michalska J, Zauber H, Buchanan BB, Cejudo FJ and Geigenberger P. 2009. NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts. *Proceedings of the National Academy of Sciences, USA* **106,** 9908–9913.

Montrichard F, Renard M, Alkhalfioui F, Duval FD, Macherel D. 2003. Identification and differential expression of two thioredoxin *h*isoforms in germinating seeds from pea. *Plant Physiology* **132,** 1707–1715.

Nakamura N, Marutani M, Sanematsu S, Toyoda K, Inagaki Y, Shiraishi T, Ichinose Y. 2003. Phylogenetic classification of Dof-type transcription factors in pea (*Pisum sativum*). *Plant Biotechnology* **20,** 247–252.

Pagano EA, Chueca A, López-Gorge J. 2000. Expression of thioredoxins *f*and *m*and of their targets fructose-1,6-bisphosphatase and NADP-malate dehydrogenase, in pea plants grown under normal and light/temperature stress conditions. *Journal of Experimental Botany* **51,** 1299–1307.

Pérez-Ruiz JM, Spínola MC, Kirchsteiger K, Moreno J, Sahrawy M, Cejudo FJ. 2006. Rice NTRC is a high-efficiency redox system for chloroplast protection against oxidative damage. *The Plant Cell* **18,** 2356–2368.

Rolland F, Baena-Gonzalez E, Sheen J. 2006 Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review of Plant Biology* **57,** 675–709.

Rosa M, Prado C, Podazza G, Interdonato R, González JA, Hilal M, Prado FE. 2009. Soluble sugars—metabolism, sensing and abiotic stress: a complex network in the life of plants. *Plant Signaling and Behaviour* **4,** 388–93.

Rueda-López M, Crespillo R, Cánovas FM, Ávila C. 2008. Differential regulation of two glutamine synthetase genes by a single Dof transcription factor. *The Plant Journal* **56,** 73–85.

Santos-Rosa M, Poutaraud A, Merdinoglu D, Mestre P. 2008. Development of a transient expression system in grapevine via agro-infiltration. *Plant Cell Reports* **27,** 1053–1063.

Schürmann P, Buchanan BB. 2008. The ferredoxin/thioredoxin system of oxygenic photosynthesis. *Antioxidants and Redox Signaling* **10,** 1235–1274.

Schürmann P, Wolosiuk RA, Breazeale VD, Buchanan BB. 1976. 2 Proteins function in regulation of photosynthetic CO2 assimilation in chloroplasts. *Nature* **263,** 257–258.

Sekin S. 1978. Enzymatic determination of glucose, fructose and sucrose in tobacco. *Tobacco Science* **23,** 75–77.

Serrato A, Barajas JD, Chueca A, Sahrawy M. 2009. Changing sugar partitioning in FBPase-manipulated plants. *Journal of Experimental Botany* **60,** 2923–2931.

Serrato AJ, Pérez-Ruiz JM, Cejudo FJ. 2002. Cloning of thioredoxin h reductase and characterization of the thioredoxin reductase–thioredoxin h system from wheat. *Biochemical Journal* **217,** 392–399.

Serrato A, Pérez-Ruiz JM, Spínola MC, Cejudo FJ. 2004. A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **279,** 43821–43827.

Sheludko YV, Sindarovska YR, Gerasymenko IM, Bannikova MA, Kuchuk NV. 2007. Comparison of several Nicotiana species as hosts for high-scale Agrobacterium-mediated transient expression. *Biotechnology and Bioengineering* **96,** 608–614.

Sivitz AB, Reinders A, Ward JM. 2008. Arabidopsis sucrose transporter AtSUC1 is important for pollen germination and sucrose-induced anthocyanin accumulation. *Plant Physiology* **147,** 92–100.

Stitt M, Bulpin PV, Rees TA. 1978. Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. *Biochimica et Biophysica Acta* **544,** 200–214.

Tanaka M, Takahata Y, Nakayama H, Nakatani M, Tahara M. 2009. Altered carbohydrate metabolism in the storage roots of sweetpotato plants overexpressing the SRF1 gene, which encodes a Dof zinc finger transcription factor. *Planta* **230,** 737–746.

Tiessen A, Hendriks JHM, Stitt M, Branscheid A, Gibon Y, Farre EM, Geigenberger P. 2002. Starch synthesis in potato tubers is regulated by post-translational redox-modification of ADP-glucose pyrophosphorylase: a novel regulatory mechanism linking starch synthesis to the sucrose supply. *The Plant Cell* **14,** 2191–2213.

Traverso JA, Vignols F, Cazalis R, Pulido A, Sahrawy M, Cejudo FJ, Meyer Y, Chueca A. 2007. PsTRX*h*1 and PsTRX*h*2 are both pea *h*-type thioredoxins with antagonistic behavior in redox imbalances. *Plant Physiology* **143,** 300–311.

Traverso JA, Vignols F, Cazalis R, Serrato AJ, Pulido P, Sahrawy M, Meyer Y, Cejudo FJ, Chueca A. 2008. Immunocytochemical localization of *Pisum sativum*TRXs *f*and *m*in non-photosynthetic tissues. *Journal of Experimental Botany* **59,** 1267–1277.

Umemura Y, Ishiduka T, Yamamoto R, Esaka M. 2004. The Dof domain, a zinc finger DNA-binding domain conserved only in higher plants, truly functions as a Cys2/Cys2 Zn finger domain. *The Plant Journal* **37,** 741–749.

van Bel AJ, Hess PH. 2008. Hexoses as phloem transport sugars: the end of a dogma? *Journal of Experimental Botany* **59,** 261–272.

VanderGheynst JS, Gou H-Y, Simmons CW. 2008. Response surface studies that elucidate the role of infiltration conditions on *Agrobacterium tumefaciens*-mediated transient transgene expression in harvested switchgrass (*Panicum virgatum*). *Biomass and Bioenergy* **32,** 372–379.

Van der Hoorn JAL, Laurent F, Roth R, De Wit PJGM. 2000. Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/cf-9-induced and Avr4/Cf-4-induced necrosis. *Molecular Plant-Microbe Interactions* **13,** 439–446.

VanEngelen FA, Molthoff JW, Conner AJ, Nap J-P, Pereira A, Stiekema WJ. 1995. pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Research* **4,** 288–290.

Wan CY, Wilkins TA. 1994. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum L*.). *Analytical Biochemistry* **223,** 7–12.

Wroblewski T, Tomczak A, Michelmore R. 2005. Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. *Plant Biotechnology Journal* **3,** 259–273.

Xiao W, Sheen J, Jang JC. 2000. The role of hexokinase in plant sugar signal transduction and growth and development. *Plant Molecular Biology* **44,** 451–461.

Yanagisawa S. 2000. Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize. *The Plant Journal* **21,** 281–288.

Yanagisawa S. 2002. The Dof family of plant transcription factors. *Trends in Plant Sciences* **7,** 555–560.

Yanagisawa S, Schmidt RJ. 1999. Diversity and similarity among recognition sequences of Dof transcription factors. *The Plant Journal* **17,** 209–214.

Yanagisawa S, Sheen J. 1998. Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. *The Plant Cell* **10,** 75–89.

Yang Y, Li R, Qi M. 2000. *In vivo*analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *The Plant Journal* **22,** 543–551.