

RESEARCH PAPER

Circadian regulation of chloroplastic *f* and *m* thioredoxins through control of the CCA1 transcription factor

Juan de Dios Barajas-López^{1,*}, Antonio Jesus Serrato¹, Roland Cazalis², Yves Meyer³, Ana Chueca¹, Jean Philippe Reichheld³ and Mariam Sahrawy^{1,†}

¹ Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain

² Université de Namur, UR BV, 61 rue de Bruxelles, 5000 Namur, Belgium

³ Laboratoire Génome et Développement des Plantes, Université de Perpignan, UMR 5096 CNRS-UP-IRD, F-66860 Perpignan, France

* Present address: Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, SE-901 87 Umeå, Sweden.

† To whom correspondence should be addressed. E-mail: mariam.sahrawy@eez.csic.es

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Abstract

Chloroplastic thioredoxins *f* and *m* (TRX *f* and TRX *m*) mediate light regulation of carbon metabolism through the activation of Calvin cycle enzymes. The role of TRX *f* and *m* in the activation of Calvin cycle enzymes is best known among the TRX family. However, the discoveries of new potential targets extend the functions of chloroplastic TRXs to other processes in non-photosynthetic tissues. As occurs with numerous chloroplast proteins, their expression comes under light regulation. Here, the focus is on the light regulation of TRX *f* and TRX *m* in pea and *Arabidopsis* during the day/night cycle that is maintained during the subjective night. In pea (*Pisum sativum*), TRX *f* and TRX *m1* expression is shown to be governed by a circadian oscillation exerted at both the transcriptional and protein levels. Binding shift assays indicate that this control probably involves the interaction of the CCA1 transcription factor and an evening element (EE) located in the *PsTRX f* and *PsTRX m1* promoters. In *Arabidopsis*, among the multigene family of TRX *f* and TRX *m*, *AtTRX f2* and *AtTRX m2* mRNA showed similar circadian oscillatory regulation, suggesting that such regulation is conserved in plants. However, this oscillation was disrupted in plants overexpressing CCA1 (*cca1-ox*) or repressing CCA1 and LHY (*cca1-lhy*). The physiological role of the oscillatory regulation of chloroplastic TRX *f* and TRX *m* in plants during the day/night cycle is discussed.

Key words: *Arabidopsis* mutants, CCA1, chloroplastic thioredoxins, circadian regulation, EE, *Pisum sativum*.

Introduction

The ferredoxin/thioredoxin system constitutes a notable example of modulation of light-regulated Calvin cycle enzymes by chloroplastic thioredoxins (TRXs), a process that involves thiol–disulphide exchange. During photosynthesis, the reduced ferredoxin transfers its electron via a reductase to the TRX proteins, which in turn can reduce enzymes involved in carbon metabolism. Found in all organisms, TRXs are small proteins (14 kDa) that have a conserved redox active centre (WCXPC) involved in the reduction of a wide variety of proteins (Holmgren, 1985, 1989; Balmer *et al.*, 2003, 2006a; Meyer *et al.*, 2009). The genome of *Arabidopsis thaliana* contains four TRX *m* genes (*AtTRX m1*, *m2*, *m3*, and *m4*),

two TRX *f* genes (*AtTRX f1* and *f2*), one 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* genes (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. In the chloroplast, ferredoxin reduces the bridge formed between the two

cysteines of TRX through ferredoxin-thioredoxin reductase (FTR) (Schürmann and Jacquot, 2000). In the cytosol and organelles, TRX *h* proteins are reduced by NADPH via NADP thioredoxin reductase (NTR).

Activation of Calvin cycle enzymes by TRX *f* and *m* is one of the best characterized processes, and the function of the proteins involved in carbon metabolism of the chloroplast has been well defined (Bassham and Krause, 1969; Jacquot *et al.*, 1978; Crawford *et al.*, 1979; López-Jaramillo *et al.*, 1997; Collin *et al.*, 2003, 2004). Among the Calvin cycle enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SBPase), phosphoribulokinase (PRK), and ADP-glucose pyrophosphorylase (AGPase) are activated by TRXs (Buchanan *et al.*, 2002; Geigenberger *et al.*, 2005). A chloroplast detoxifying protein, a subunit of ATP synthase, and a chlorophyll biosynthetic enzyme that belongs to the photosynthetic electron chain are also protein targets of the chloroplastic TRXs (Lemaire *et al.*, 1999; Rey *et al.*, 2005; Pérez-Ruiz *et al.*, 2006; Ikegami *et al.*, 2007; Kirchsteiger *et al.*, 2009). Recently an additional mechanism of control has been proposed in which a Calvin cycle ternary complex including PRK, GAPDH, and a small protein, CPI2, may represent a reservoir of inhibited enzymes that are reduced and dissociated by TRX *f* during the dark to light transition (Wedel *et al.*, 1997; Howard *et al.*, 2008; Marri *et al.*, 2009). The activation of these enzymes at specific times of the day to avoid futile steps is crucial for metabolic processes, and this activation requirement suggests that TRX activation adapts the activities of the chloroplast to the availability of light energy and carbon status.

Light is the main environmental factor involved in the biosynthesis and regulation of chloroplast thioredoxin expression, and can affect many processes in which they are involved (Carrasco *et al.*, 1992; Meyer *et al.*, 2008). In addition, plants have endogenous biological clocks that enable them to organize their physiological, metabolic, and developmental processes to occur at optimal times (Dodd *et al.*, 2005). The assimilation of carbon in the Calvin cycle is under the control of the light/dark cycle that regulates the activity of the enzymes to prevent futile steps and control the balance between sucrose and starch synthesis. It is therefore not surprising that the expression and function of the genes encoding proteins involved in sugar synthesis are under tight regulation by light and the biological clock. To date, transcripts of the genes of *Neurospora crassa* GAPDH, *Chlamydomonas* PRK, and Rubisco activase in several plant species have been shown to be under the control of the circadian clock (Martino-Catt and Ort, 1992; Pilgrim and McClung, 1993; Shinohara *et al.*, 1998; Lemaire *et al.* 1999).

Work on circadian clock control in plant TRXs is limited to observations by Harmer *et al.* (2000) regarding the abundance of *AtTRX f2* and *AtTRX like5* mRNA (WCRKC THIOREDOXIN 1), which responds to oscillatory regulation. None of the well-known genes that code for enzymes activated by chloroplast TRXs has been found among the genes involved in carbon metabolism that function under

the circadian clock. However, researchers have detected a cluster of genes that encode enzymes involved in starch mobilization that are under clock control, suggesting the presence of a circadian component in the regulation of carbohydrate metabolism (Jeannette and Prioul, 1994; Mérida *et al.*, 1999). Interestingly, *Chlamydomonas reinhardtii* TRX *m*, TRX *h*, ferredoxin, PRK, and ferredoxin-NADP reductase (FNR) genes have been shown to be controlled by the circadian clock under continuous light conditions (Lemaire *et al.*, 1999, 2002).

In this context, it is reasonable to conjecture that in addition to the light-dependent redox regulation exerted by chloroplastic TRXs to activate the proteins involved in photosynthesis and CO₂ fixation, the expression of the TRX genes could be regulated by the circadian clock and anticipate dawn to develop their function. However, only a few studies examining the day/night cycle have focused on the transcriptional regulation of the TRX *f* and *m* genes under different light conditions, and no transcription factor controlling expression of these genes has yet been described. In this work, the effect of light to dark transition on pea TRX *f* and *m1* gene expression as well as protein biosynthesis is characterized, and support for the hypothesis that these genes are regulated under the circadian clock is presented. The gel-shift assay findings document binding of the CCA1 recombinant transcription factor on a fragment that carries the evening element (EE) of the promoter regions of both chloroplastic PsTRX *f* and *m1*. In addition, the expression of *Arabidopsis thaliana* TRX *f* and *m* genes (*AtTRX f1*, *AtTRX f2*, *AtTRX m1*, *AtTRX m2*, *AtTRX m3*, and *AtTRX m4*) is analysed in *Arabidopsis* plants as well as in plants overexpressing CCA1, and in plants repressing CCA1 and LHY.

Materials and methods

Plant material and growth conditions

Pea (*Pisum sativum* var. Lincoln) leaves were used in protein, DNA, and RNA extraction and promoter isolation, as well as for northern blot, reverse transcription-PCR (RT-PCR), and western blotting experiments. Plants were grown in a green cabinet for 15 d, first in vermiculite and then in compost, with a 12 h/12 h photoperiod and light intensity of 80 μmol photons m⁻² s⁻¹ PAR, 23 °C temperature, and daily watering.

Arabidopsis thaliana ecotype Columbia seeds overexpressing CCA1 (*cca1-ox*) or repressing CCA1 and LHY (*cca1-lhy*) and transgenic seeds carrying constructions f444-β-glucuronidase (GUS; 444 bp), f126-GUS (126 bp), m1874-GUS (1874 bp), m200-GUS (200 bp), and m100-GUS (100 bp), described in a previous work (Barajas *et al.*, 2007), were grown in MS medium supplemented with sucrose, agar, and kanamycin in a light cabinet under a 12 h light/12 h dark photoperiod, at 140 μmol photons m⁻² s⁻¹ PAR and 23 °C temperature.

For the circadian experiments with day/night cycles, the samples were collected every 4 h or 12 h over 48 h, and during the second night the light was kept on to simulate subjective night.

Extraction and gel analysis of RNA

Total RNA extracted from pea with Trizol (BD, Sparks, MD, USA) was used for northern blot analysis and reverse

transcription. For northern blots, total pea leaf RNA (15 µg) was subjected to electrophoresis through agarose/formaldehyde gels and transferred onto Hybond-N+ membranes (Amersham Pharmacia Biotech). Hybridization was performed at 42 °C in a MOPS buffer (10× MOPS, 50% formamide, 0.5% SDS, 6× SSC, 5× Denhardt's solution, 50 µl of calf thymus) to random primed 3'-specific probes of *PsCab*, *PsTRXf*, and *PsTRXm1* genes amplified by PCR (Kit of Readyprime, Amersham II Labelling System).

RT-PCR and semi-quantitative PCR analysis

For semi-quantitative RT-PCR, 2.5 µg or 5 µg of RNAs treated with DNase (DNase Turbo[®], Invitrogen) were used for reverse transcription with retrotranscriptase RNase SuperScript-III[®] (Invitrogen, Carlsbad, CA, USA) and oligo(dT)₁₂₋₁₅ primer, at 50 °C for 1 h. After selecting the dilutions, 1/50 of the reaction was taken for PCR using specific oligonucleotides (GUS I and II) for the GUS gene (Table 1). Amplified fragments of *Pisum* or *Arabidopsis* 18S were used as housekeeping genes to normalize the expression level.

Real-time quantitative RT-PCR analysis

For gene expression analysis by real-time PCR, samples of total RNA (500 ng) extracted from wild-type, *ccal-ox*, and *ccal-lhy* double-mutant rosettes using the Aurum[™] Total RNA Mini kit (BioRad) were reverse transcribed with Multiscribe Reverse Transcriptase and random hexamer primers (Applied Biosystems, Foster City, CA, USA). For quantification, real-time PCRs were performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and Taqman technology. The cDNA was amplified in a 2× TaqMan Master mix (Eurogentec S.A., University of Liège, Belgium) in a final volume of 25 µl. The PCR

conditions were: 2 min at 50 °C; 10 min at 95 °C; and 40 cycles of 15 s at 95 °C, 1 min at 60 °C. The Ct value was determined using the instrument's software. Specific primers and probes of each gene were designed using Primer Express (Applied Biosystems) software, the corresponding pair of primers and probe being Pcab up/down, AtCab2 for AtCAB, Pf1 up/down, ATf1 for AtTRX *f1*, Pf2 up/down, ATf2 for AtTRX *f2*; Pm1 up/down, ATm1 for AtTRX *m1*, Pm2 up/down, ATm2 for AtTRX *m2*, Pm3 up/down, ATm3 for AtTRX *m3*, Pm4 up/down, ATm4 for AtTRX *m4*, and 18S up/down, P18S for rRNA 18S (Table 1). Relative quantification of gene expression was monitored after normalization by 18S rRNA expression as internal control, as fold variation over a calibrator using the 2- $\Delta\Delta$ CT method (Livak and Schmittgen, 2001).

Electrophoretic mobility shift assays (EMSA)

The double-stranded oligonucleotides LumF and LumM containing the EE were obtained by annealing oligonucleotides LumFs (5'-AAGTGAAAAAAAAAAGAGATATTCGAAGGG-3') and LumFa (5'-GATTCCCTTCGAATATCTCTTTTTTTTTTTC-3'), and oligonucleotides LumMs (5'-TAAGTAGATATTGAAAGCAAGATTGAAAAAAAAATGTTG-3') and LumMa (5'-AATCAAACATTTTTTCAATCTTGCTTTCAATATCT-3'), respectively. A single mutation in the EE was introduced in the oligonucleotides LumFDs (5'-AAGTGAAAAAAAAAAGAGcTATTCGAAGGG-3') and LumFDa (5'-GATTCCCTTCGAATAgCTCTTTTTTTTTTTC-3'), and oligonucleotides LumMDs (5'-TAAGTAGcTATTGAAAGCAAGATTGAAAAAAAAATGTTG-3') and LumMDa (5'-AATCAAACATTTTTTCAATCTTGCTTTCAATAgCT-3'). The double-stranded mutated oligonucleotides were obtained by annealing. Fragments were labelled with [³²P]dATP by fill-in reaction with Klenow. The radioactive probes were purified from 8%

Table 1 Gene-specific oligonucleotides used for qPCR analysis

	Gene analysed	Oligosequence
Pcab up	AtCAB	5'-CTGCGGCATCAGAAGTCCTT-3'
Pcab down	id	5'-CCTTTGGCTTGGCAACAGTC-3'
Pf1 up	AtTRXf1	5'-AAACAGCGAGGTCTGCTGCT-3'
Pf1 down	id	5'-TAACACCAGATTTACATTACATACAAACA-3'
Pf2 up	AtTRXf2	5'-TCCGTTATTCTCCGATTACATCTACC-3'
Pf2 down	id	5'-GAATTCGGGATCCGGCA-3'
Pm1 up	AtTRXm1	5'-AATTCTAGGGTTTCCCATTACG-3'
Pm1 down	id	5'-GAGTCCCATTGTAATCGTTGA-3'
Pm2 up	AtTRXm2	5'-TCTCCGGCTTCGTTGACC-3'
Pm2 down	id	5'-GAGCTTCACAGACGACGGCT-3'
Pm3 up	AtTRXm3	5'-AGCGAAACCCCGGTGTAG-3'
Pm3 down	id	5'-TTATCCTGTGGACCATCCGAC-3'
Pm4 up	AtTRXm4	5'-AATCGCTCGCGGTGGAC-3'
Pm4 down	id	5'-GATTTGGTACTTCGACGGCG-3'
18S up	rRNA18s	5'-AGTAAGCGCGAGTCATCAGCT-3'
18S down	id	5'-CATTCAATCGGTAGGAGCGAC-3'
Act up	rRNAAct	5'-TGGTCGTACAACCGGTATTG-3'
Act down	id	5'-CAGTAAGGTACAGTCCAGCA-3'
GUS I	GUS	5'-AACGGGGAAACTCAGCAAGC-3'
GUS II	id	5'-TGTGAGCGTCGCAGAACATT-3'
Probes		
AtTRXf1 ATf1		5' FAM -CCGGATGAACCACTCTCTCATGTCTTTACC- Tamra3'
AtTRXf2 ATf2		5' FAM -CCGGAGGATTTCCCCCGTGAA- Tamra3'
AtTRXm1 ATm1		5' FAM -CGAGGCGTTATCTGTGAAGCTCAGGACAC- Tamra3'
AtTRXm2 ATm2		5' FAM -CGATTCATCAACCTAGGGTTTCTCGATTACGAA- Tamra3'
AtTRXm3 ATm3		5' FAM -AGAATTCTACACGAGTTGGTCCGGTCCA- Tamra3'
AtTRXm4 ATm4		5' FAM -AATCGCCTGTGAGGCTCAGGACACC- Tamra3'
AtCAB2 ATcab2		5' TET -AAGCGGCCGTGTGACAAATGAGGA- Tamra3'
rRNA18s P18S		5' FAM -CTGCCCTTTGTACACACCGCCCG- Tamra3'

polyacrylamide gels. CCA1 protein was purified with plasmid pXCA-24, kindly provided by Elaine Tobin (UCLA, USA), which contains the full-length CCA1 cDNA fused to glutathione *S*-transferase (GST) cloned in pGEX-3X (Amersham Pharmacia Biotech) (Wang et al., 1997). Binding was carried out in 20 µl of 30 mM HEPES, pH 7.9, 125 mM KCl, 0.25 M dithiothreitol (DTT), 12% glycerol (Diaz et al., 2002). Mixes were incubated for 30 min on ice. In unspecific competition experiments, 0–0.5 µg of poly(dI–dC) was also included in the mixes. In specific competition experiments, the unlabelled double-stranded oligonucleotides (0.1–0.5 µg) were included and assayed under standard conditions. EMSAs were performed after adding 1/10 of loading buffer to the mixes in a 6% polyacrylamide pre-electrophoresed gel.

Results

Oscillation of pea TRX *f* and *m1* mRNA in leaves during the day/night cycle

The levels of *TRX f* and *TRX m1* mRNAs were measured during growth of pea seedlings in a photoperiod of 12 h light and 12 h darkness. To study the circadian regulation, the second dark period was replaced by a light period called subjective night. Figure 1 shows that *PsTRX f* transcripts peak after 6 h of illumination and then rapidly decline. The level remains almost undetectable until 21 h (night period) and starts to rise by the end of the same period, reaching a maximum of mRNA expression at 27 h (during the light period). A low transcript level is detected during the

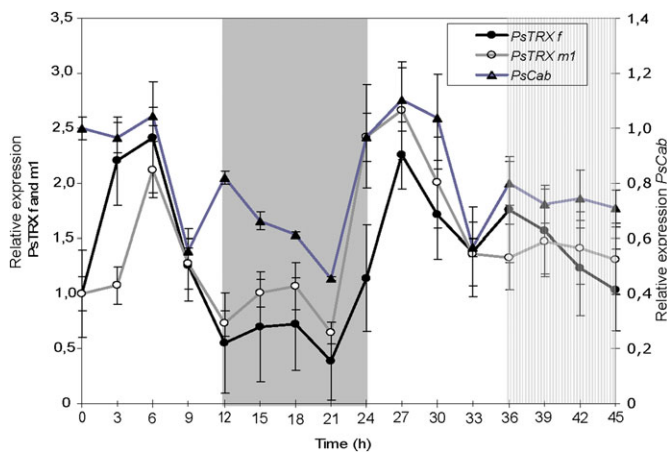


Fig. 1. Relative expression of *PsCab*, *PsTRX f*, and *PsTRX m1* genes in leaves of pea during the day/night cycle. Plants were grown under a 12 h light/12 h night photoperiod, and leaf samples were collected at 3 h intervals over a period of 45 h. Total RNA was extracted and analysed by northern blotting (15 µg). Filters were hybridized with 3'-specific probes of *PsCab*, *PsTRX f* and *PsTRX m1* genes, and loading differences were corrected by re-probing the filter with a cDNA fragment of the 18S rRNA. Clear areas in the graph indicate the day during the day/night periods, the grey area indicates the night, and the hatched area indicates the night period in which the light was kept on. The graph shows quantification of northern blot data normalized considering the lowest value in each case. Numbers on the ordinates indicate the real time in hours at which samples were collected for RNA isolation. (Fisher's exact test, $P=0.01$).

subjective night. *PsTRX m1* transcripts also peak after 6 h of light of the first day and at 27 h of the second light phase (Fig. 1). The transcript levels steadily declined throughout the dark period and subjective night. This suggests that the oscillation of the *PsTRX f* and *m1* mRNA level is likely to be under the regulation of the circadian clock. The decreases detected at 9 h and 33 h, a few hours before the end of the light period, anticipating the dark effect, reflect an oscillatory control and a circadian clock-related mechanism. The chlorophyll *alb*-binding (*CAB*) genes are known to be light regulated and under the control of the circadian clock (Buetow et al., 1988). The *P. sativum* *Cab-8* (*PsCab8*) gene is a member of the type-I *Lhcb* (chlorophyll *alb*-binding protein) gene family and exhibits the most important rhythmic expression among them (Alexander et al., 1991), and a cDNA fragment (500 bp) was used as an *Lhcb*-specific probe. The *PsCab8* gene shows an oscillatory expression pattern, which persists during subjective night (Fig. 1). During the daytime, the peaks appeared at 6 h and 27 h. The increase in mRNA content started before the end of the night phase, indicating anticipation of the expression before the light is switched on. According to previous reports (Millar and Kay, 1991; White et al., 1992), these results indicate that *PsCab* expression is under a circadian regulation, validating the experimental conditions.

Oscillation of pea TRX *f* and TRX *m1* proteins during the day/night cycle

Immunoblot analyses were performed with pea anti-TRX *f* and anti-TRX *m* antisera. Figure 2 shows that the amount of pea TRX *f* and *m1* protein detected in leaves reaches

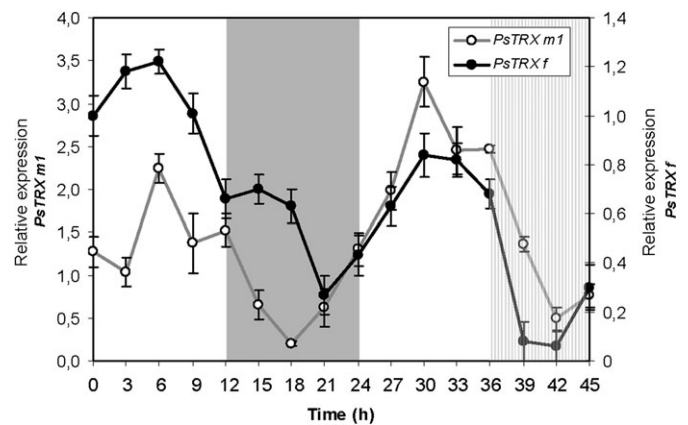


Fig. 2. Relative expression of PsTRX *f* (A) and PsTRX *m1* (B) proteins determined after immunodetection by western blotting using specific antibodies against *Pisum sativum* TRX *f* and TRX *m1* antibodies. Western blot analysis was performed in 50 µg of pea total protein extracts. Clear areas in the graph indicate the day during the day/night periods, the grey area indicates the night, and the hatched area indicates night period in which the light was kept on (hatched area). The graph is representative of two repetitions and shows relative quantification of bands from western blot that were normalized considering the lowest value in each case. Numbers on the ordinates indicate the real time in hour at which samples were collected for protein isolation.

maximum accumulation at 6 h and 30 h of the light periods, then drops to minimum levels during the night (21–18 h) and subjective night (42 h). The oscillation of pea TRX *f* and *m* proteins follows the same mRNA oscillation behaviour during the day/night cycle. Both TRX *f* and *m* mRNA and protein increases are seen to anticipate the light effect, indicating a circadian regulation.

Oscillation of GUS mRNA levels under the control of the pea TRX f promoter in transgenic plants during the day/night cycle

To ascertain whether the circadian oscillation of *PsTRX f* and *PsTRX m1* gene expression is governed by transcriptional control, a 444 bp fragment and a deleted fragment of the same region (126 bp) corresponding to the 5' upstream regions of *PsTRX f*, as well as a fragment of 1894 bp and two deleted fragments of 200 bp and 100 bp of *PsTRX m1* promoter were isolated (Fig. 3). All were transcriptionally fused to the *uidA* (GUS) reporter gene via the pBI101 vector, and designated as: f444 and f126; and m1874, m200, and m100, respectively (Barajas *et al.*, 2007). *Arabidopsis* plantlets carrying the different promoter constructs were used to determine the abundance of GUS transcripts. Figure 3a shows a peak of f444–GUS expression in the middle of light periods that decayed 6- to 7-fold during the night and the subjective night in comparison with the light periods. The f126–GUS, which lacks most of the light-dependent *cis*-elements, lost its GUS expression. These results suggest that the 444 bp 5' upstream region suffices to confer an oscillatory pattern of *PsTRX f* expression, and that the *cis*-elements necessary for the gene's transcription are located in the –444/–126 region.

The m1874–GUS construct displayed a peak of expression during the mid-light periods that decayed 2.5 times in the night phase and 1.2 times during the subjective night. The m200–GUS construct shows a weaker oscillatory pattern of expression, suggesting that additional elements control the circadian process. Removing another 100 bp was sufficient to lose GUS expression.

CCA1 transcription factor binds specifically to EEs located in PsTRX f and m1 promoters

Analysis of the sequence of the proximal promoter of *PsTRXs f* in the –444/–126 region revealed an AATATCT sequence (–271 to –277 in an antisense orientation) similar to the core of a putative EE (Fig. 3a). EEs have been reported to be over-represented in a cluster of genes regulated by the circadian clock (Lam and Chua, 1989; Gilmartin *et al.*, 1990; Harmer *et al.*, 2000; Barajas *et al.*, 2007). The EE sequence (AAA/TATATCT) is identical to the sequence CBS (CCA1-binding site) recognized in the TOC1 (Timing of Cab Expression 1) promoter (AAAATATCT) by the transcriptional repressors CCA1 (Circadian Clock Associated 1) and LHY (Late Elongated Hypocotyl) (Alabadi *et al.*, 2001; Yanovsky and Kay, 2001). The homologous Myb-like transcription factors CCA1 and LHY together with TOC1

constitute a transcriptional feedback loop that is essential for a functional circadian clock in *Arabidopsis*.

As shown in Fig. 3b, the *PsTRX m1* promoter showed two putative EE motifs in a sense orientation that differed by only one nucleotide from the core seven-nucleotide consensus sequence (EE1, –823 to –817; and EE2, –1131 to –1125; all numbered from the transcription start site).

EMSA were performed to determine whether a recombinant *Arabidopsis* CCA1 protein could bind the putative EE from the TRX *f* and TRX *m* upstream regions. Double-stranded probes of 30 bp (LumFs/LumFa) and 37 bp (LumMs/LumMa) (described in the Materials and methods) carrying the putative EE of the TRX *f* and TRX *m* regulatory regions, respectively, were constructed. As shown in Fig. 4a and b, the migration of both sequences was retarded in the presence of the recombinant *Arabidopsis* CCA1 protein. The shifted probes were stable with the addition of a large excess of the non-specific competitor poly(dI–dC) (Fig. 4a, lines 6–8; b, lines 2–4). However, when 300 ng of cold specific LumFs/LumFa or LumMs/LumMa competitors were added, the bandshift was fully displaced, suggesting a specific binding of CCA1 to the EE of *PsTRX f* and *m1* promoters (Fig. 4a, b, specific competitor).

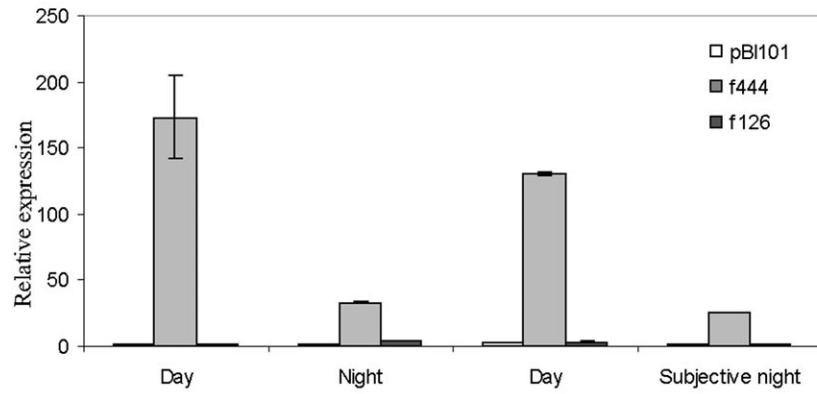
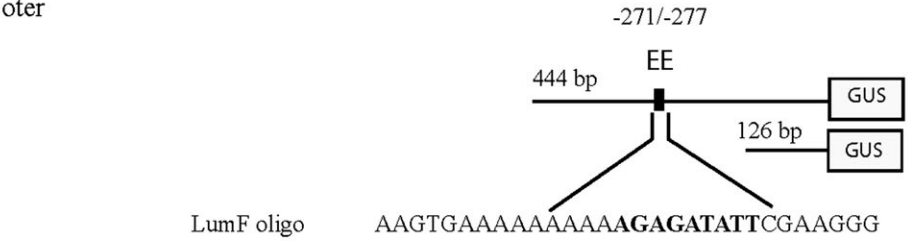
To identify the bases involved in the binding, EMSA was performed using new pairs of oligonucleotides where the ATATT core sequence was changed to cTATT. In Fig. 4a (lines 9 and 10) and b (lines 8 and 9), no binding was observed between the mutated variant and the CCA1 protein, suggesting that the first A base of the core sequence is essential to establish the complex. These results indicate that CCA1 binds in a specific manner to the EE sequence of the *PsTRXs f* and *m1* promoters.

Oscillation of A. thaliana TRX f2 and m2 mRNA in rosettes of wild-type plants during the day/night cycle and day/subjective night

The *in vitro* interaction of CCA1 with the putative EE of the *PsTRX f* and *PsTRX m1* upstream regions suggests CCA1 involvement in the circadian regulation of TRX *f* and TRX *m1* in pea. To test this hypothesis, the circadian regulation of TRX *f* and TRX *m* was studied in the *cca1* mutant background. Because such mutants are not yet available in pea, *Arabidopsis* and real-time PCR with specific oligonucleotides (Table 1) were used to establish the expression of the two TRX *f* [*AtTRX f1* (locus At3g02730) and *AtTRX f2* (locus At5g16400)], the four TRX *m* [*AtTRX m1* (locus At1g03680), *AtTRX m2* (locus At4g03520), *AtTRX m3* (locus At2g15570), and *AtTRX m4* (locus At3g15360)], and *AtCab2* (locus At1g29920) genes during the diurnal cycle in *Arabidopsis* plantlets grown under the same oscillatory conditions as pea plantlets.

Figure 5c shows a peak of the *AtTRX f2* transcripts after 4 h and 28 h illumination, corresponding to the light periods. Expression was almost undetectable during the first night and the subjective night, and a noteworthy oscillatory pattern was seen for *AtTRX m2* (Fig. 5e), displaying two peaks of expression, at 4 h and 28 h of the corresponding

(a) *PsTRX f* Promoter



(b) *PsTRX m* Promoter

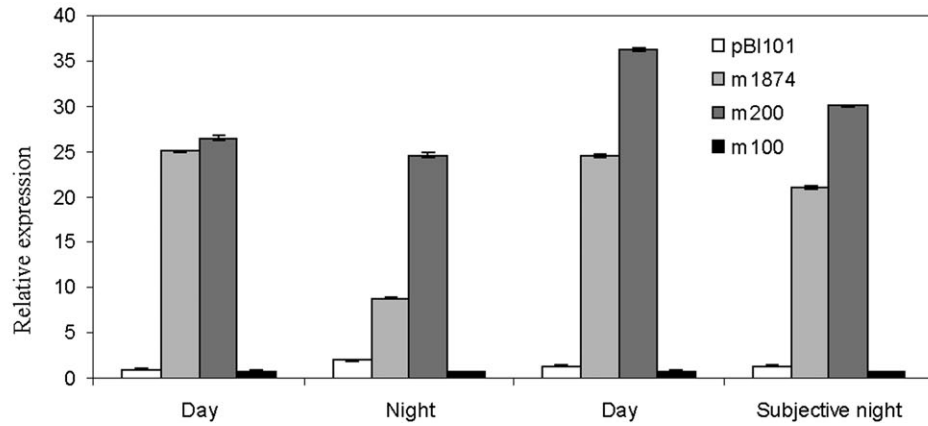
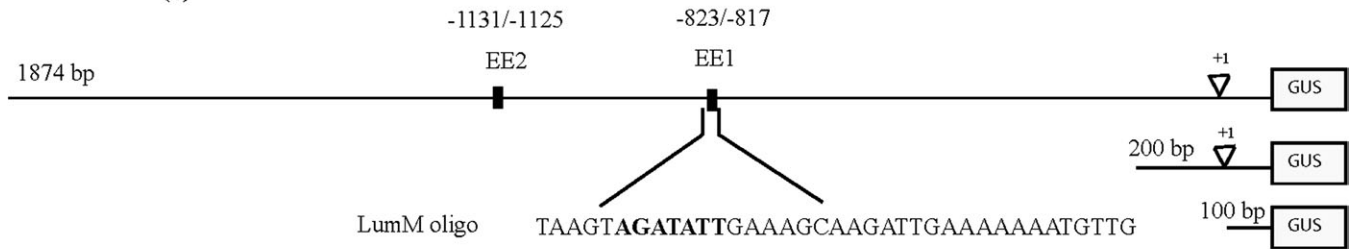


Fig. 3. Light/dark regulation of GUS mRNA expression under the *PsTRX f* and *PsTRX m1* promoter. Transgenic plants carrying complete promoter constructs (f444–GUS or m1894–GUS) or truncated promoter constructs (f126–GUS, m200–GUS, or m100–GUS) were subjected to a 12 h/12 h photoperiod, the second night the light was kept on, and rosettes were collected at 12 h intervals over a 48 h period; mRNA levels of the GUS-encoding gene were determined in leaves by semi-quantitative RT-PCR as described in the Materials and methods. (a) EE site localization, LUMFs oligo sequence, and GUS expression under the *PsTRX f* promoter; (b) EEs site localizations, LUMMs oligo sequence, and GUS expression under the *PsTRX m* promoter. Samples were harvested for 48 h at midday and midnight from 20-day-old plants. Values are the average of three determinations of two cDNA preparations.

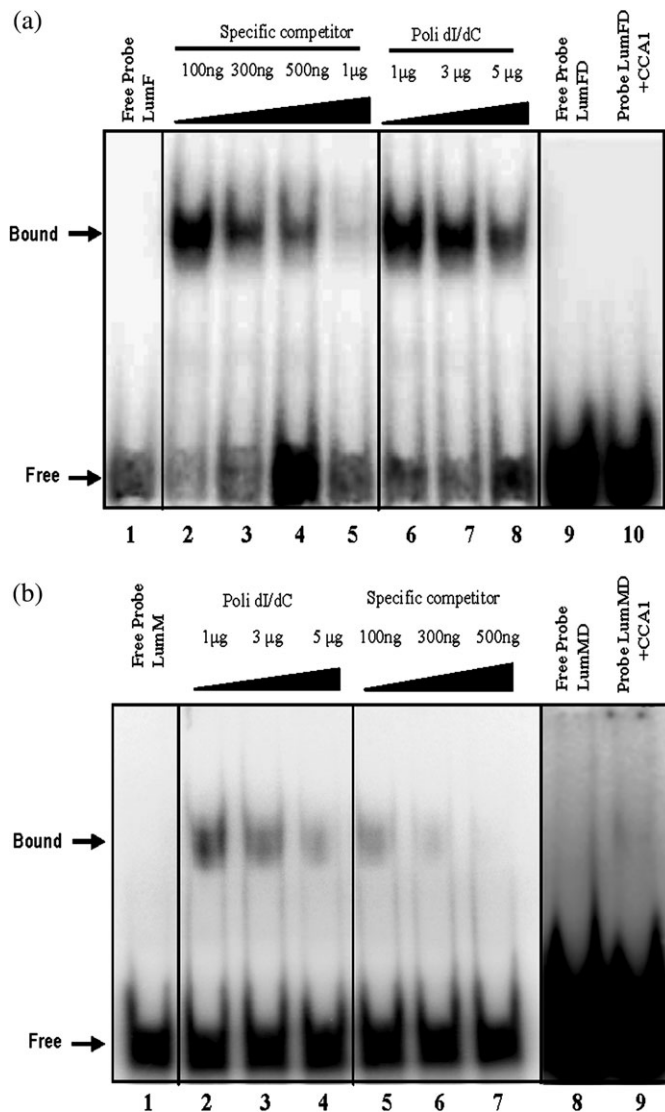


Fig. 4. Functional properties of the pea EE motif present in *PsTRX f* (a) and *PsTRX m1* (b) promoters. Electrophoretic mobility shift assays (EMSA) of the recombinant *Arabidopsis* CCA1 with oligonucleotide probes derived from the *PsTRX f* and *PsTRX m1* promoter. (a) The probe without recombinant CCA1 is designated Free Probe (lanes 1 and 9). Competition experiments were performed using increasing amounts either of the non-specific competitor [poly(dI-dC)] (lanes 6, 7, 8, and 10) or of the unlabelled probe (lanes 2, 3, 4, and 5). Increasing molar amounts of the unlabelled probe, 100, 300, and 500 ng, and 1 µg, are indicated by triangles. Lanes 1–8: a probe of 30 bp (LumF) derived from the *PsTRX f* promoters was ³²P labelled 5'-AAGTGAAAAAAAAAAGA-GATATTCTGAAGGG-3'. Lanes 9 and 10: a substitution of the ATATT EE core by CTATT was made in the LumFD oligonucleotide and was ³²P labelled 5'-AAGTGAAAAAAAAAAGAGcATATTC-GAAGGG-3'. (b) The probe without recombinant CCA1 is designated Free Probe (lanes 1 and 8). Competition experiments were performed using increasing amounts either of the non-specific competitor [poly(dI-dC)] (lanes 2, 3, 4, and 9) or of the unlabelled probe (lanes 5, 6, and 7). Increasing molar amounts, 100, 300, and 500 ng, are indicated by triangles. Lanes 1–7: a probe of 37 bp (LumM) derived from the *PsTRX m1* promoters was ³²P labelled

mid-light periods. During the dark phase and subjective night the level of transcripts diminished 8-fold with respect to the maximum found in the light phase. These results suggest circadian control of the *AtTRX f2* and *AtTRX m2* genes. Although *AtTRX f1*, *AtTRX m1*, and *AtTRX m4* are light induced, no oscillatory effect was observed over the photoperiod (Fig. 5b, d, g). These findings suggest that, among the TRX *f* and *m* genes of *Arabidopsis*, only *AtTRX f2* and *m2* are under circadian clock regulation.

Figure 5a shows an oscillatory pattern of expression of the wild-type *AtCab2* gene that is maintained throughout subjective night. Peaks of expression appeared at 4 h and 28 h of the light periods, similar to the case of *PsCab8* transcripts. Expression was undetectable during the night phase and subjective night.

Disruption of *Arabidopsis* TRX *f2* and *m2* oscillatory expression in *cca1-ox* and *cca1-lhy* double-mutant plants

To determine whether CCA1 and LHY transcription factors are involved in the circadian oscillatory regulation of *AtTRX f2* and *m2* in *Arabidopsis*, the relative mRNA content of both genes was determined in plants that overexpress the CCA1 transcription factor (*cca1-ox* plants) and in plants that are defective in CCA1 and LHY transcription factors (*cca1-lhy* double-mutant) (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Mizoguchi *et al.* 2002). Figure 6a indicates that the peak of *AtCab2* mRNA accumulation in *cca1-ox* plants was 12 times lower than that found in wild-type plants, and started to decrease rapidly after 4 h of illumination, the transcript level being very low during the rest of the photoperiod. Moreover, the expected peak of expression in the second light phase disappeared. In the *cca1-lhy* double-mutant line, the transcripts level was undetectable. Two small peaks of *AtTRX f2* transcripts were detected in *cca1-ox* mutant plants during the first day (Fig. 6b). One peak at 4 h of the light phase coincided with the same peak found in wild-type plants, but with mRNA content 4.5 times lower; the second peak was detected at 16 h of the night phase. From midnight of the first day onward, expression became undetectable.

In the *cca1-lhy* double-mutant line, *AtTRX f2* transcripts displayed a small peak at the end of the light phase and a broad accumulation of mRNA from 24 h to 32 h of the second light period, declining during subjective night. Plants overexpressing CCA1 showed half the mRNA accumulation of *AtTRX m2* detected in wild-type rosettes after 4 h of illumination, which decreased rapidly (Fig. 6c). In the *cca1-lhy* double-mutant line, *AtTRX m2* transcripts were at least 10 times lower, and in the pattern of expression a single

5'-TAAGTAGATATTGAAAGCAAGATTGAAAAAATGTTG-3', Lanes 8 and 9: a substitution of the ATATT EE core by CTATT was made in the LumMD oligonucleotide and was ³²P labelled 5'-TAAGTAGcTATTGAAAGCAAGATTGAAAAAATGTTG-3'.

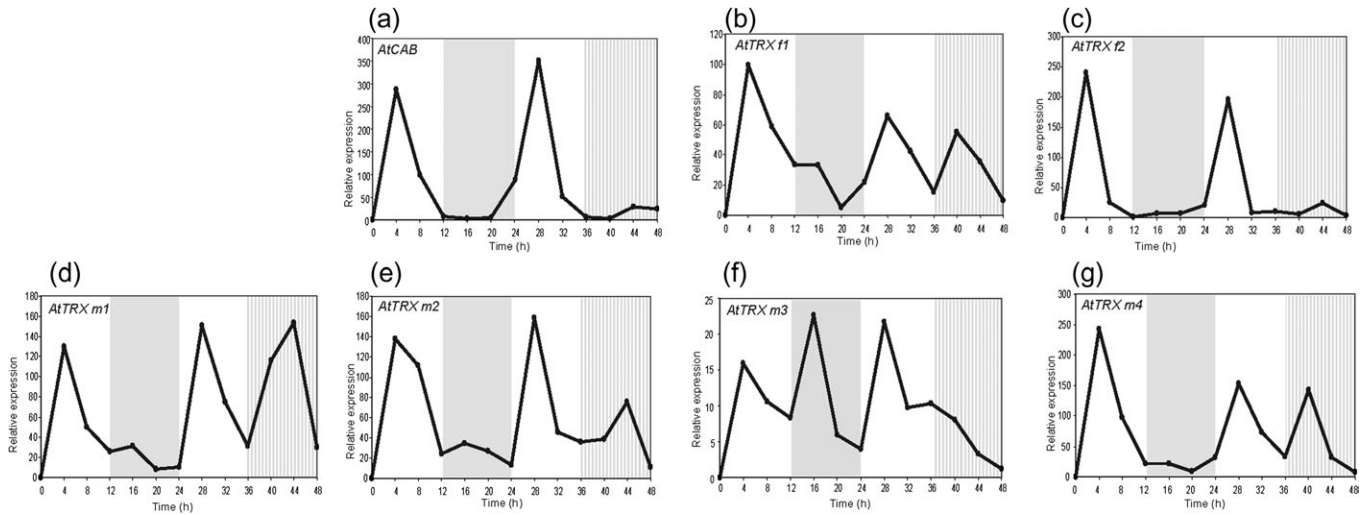


Fig. 5. Relative expression of *AtTRXCAB* (a), *AtTRX f1* (b), *AtTRX f2* (c), *AtTRX m1* (d), *AtTRX m2* (e), *AtTRX m3* (f), and *AtTRX m4* (g) genes in rosettes of *Arabidopsis* wild-type plants during the day/night cycle. Seedlings were grown under a 12 h light/12 h night photoperiod and rosette samples were collected at 4 h intervals over a period of 48 h; during the second night, the light was kept on. Total RNA was extracted and analysed by real-time PCR using specific oligonucleotides. Clear areas in the graph indicate the day during the day/night periods, the grey area indicates the night, and the hatched area indicates subjective night. The graph shows relative quantification of gene expression monitored after normalization by 18S rRNA expression as internal control, considering the lowest value in each case. Numbers on the ordinates indicate the real time in hours at which samples were collected for RNA isolation.

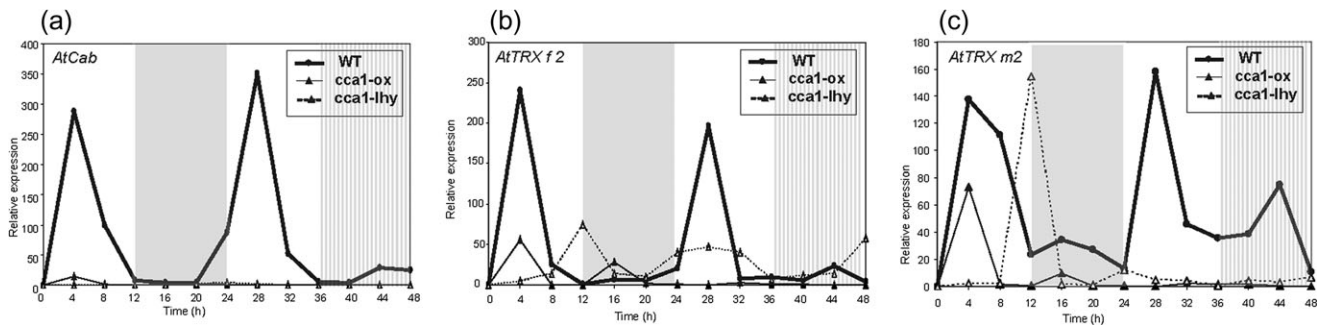


Fig. 6. Relative expression of *AtTRXCAB* (a), *AtTRX f2* (b), and *AtTRX m2* (c) genes in rosettes of *Arabidopsis* wild-type plants, plants overexpressing CCA1 (*cca1-ox*), and plants repressing CCA1 and LH1 (*cca1-lhy*) during the day/night cycle. Seeds were grown under a 12 h light/12 h night photoperiod and rosette samples were collected at 4 h intervals over a period of 48 h; during the second night, the light was kept on. Total RNA was extracted and analysed by real-time PCR using specific oligonucleotides. Clear areas in the graph indicate the day during the day/night periods, the grey area indicates the night, and the hatched area indicates subjective night. The graph shows the relative quantification of gene expression monitored after normalization by 18S rRNA expression as an internal control, considering the lowest value in each case. Numbers on the ordinates indicate the real time in hours at which samples were collected for RNA isolation.

peak appeared at the end of the first light phase (Fig. 6c). Meanwhile, the transcripts decreased rapidly.

Altogether, these results reflect a loss of oscillatory regulation of *AtCab2*, *AtTRX f2*, and *AtTRX m2* gene expression in both mutants, and strongly suggest that *AtTRX f2* and *m2* expression is controlled during the day/night cycle by CCA1 and LH1 transcription factors through the circadian mechanism.

Discussion

In the chloroplast, the light-dependent ferredoxin/TRX system is one of the most studied with the ability to reduce

and activate key enzymes of the Calvin cycle and proteins involved in different processes in plants (Buchanan, 1980; Balmer *et al.*, 2006b). This system appears to be one of the best candidates to avoid uncontrolled steps of carbon metabolism during the day/night cycle by anticipating its reaction to environmental light changes through the control of the circadian clock (Bläsing *et al.*, 2005; Yakir *et al.*, 2007).

The database developed by Harmer and colleagues (2000) showed that *A. thaliana* chloroplast TRX *f2* (*AtTRX f2*; At5g16400) and TRX-like 5 (At5g06690, WCRKC THIO-REDOXIN 1) were governed by circadian regulation, peaking at 6 h and 28 h in the light periods. Lemaire *et al.* (1999) were the first to observe that the expression of

Chlamydomonas TRX *h*, TRX *m*, ferredoxin, FTR, and PRK is under the control of the circadian oscillator. They suggested that TRX *h* might be an element in a light- and/or circadian-related transduction pathway. Research by Harmer *et al.* (2000) has shown that under circadian clock control are genes related to photosynthesis, carbon metabolism, sugar production and transport, as well as enzymes that participate in the regulation of sugar metabolism in higher plants (Pilgrim and McClung, 1993; McClung *et al.*, 2000; Schaffer *et al.*, 2001; Dodd *et al.*, 2005; Lu *et al.*, 2005). The up-regulation of these diverse genes near the end of the subjective day suggests that the clock plays an important role in allocating assimilated sugars to different pathways or to storage in the chloroplast.

Sequence alignment and the construction of a phylogenetic tree revealed high homology between *AtTRX f2* and *PsTRX f*, and between *PsTRX m1* and *AtTRX m2* (Fig. 7). Consistent with this was the finding that the orthologues *PsTRX f* and *AtTRX f2* share a common mechanism that directs transcript content to oscillate in a circadian manner during the day/night cycle. The oscillation of *PsTRX f* protein content suggests regulation at the transcriptional level. Similar results were found for *PsTRX m1* and *AtTRX m2* mRNA accumulation, which displayed a circadian pattern of expression, and transcriptional regulation was corroborated by the oscillation of *PsTRX m1* protein contents. None of the other chloroplastic *A. thaliana* TRXs (*f* or *m*) showed an oscillatory pattern of expression (Fig. 5). However, *AtTRX f1*, *AtTRX m1*, and *AtTRX m4* mRNA expression appears to be directly induced by light; further-

more, *AtTRX m3* does not display specific regulation under the day/night cycle. This TRX is highly expressed in flower petals during stage 15, whereas the other TRXs are in green tissues, cotyledons, rosettes, and leaves (Supplementary Fig. S1, Arabidopsis eFP Browser analysis, available at *JXB* online). These analyses provide proof of the regulation of *PsTRX f*, *PsTRX m1*, *AtTRX f2*, and *AtTRX m2* mRNA levels by the circadian clock, as has been observed for other photosynthetic genes.

Despite the challenges to understanding the circadian clock, it is well known that TOC1, CCA1, and LHY, the components of the core oscillator, comprise a negative feedback loop grounded in positive and negative transcriptional regulation (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Matsushika *et al.*, 2000; Strayer *et al.*, 2000). Light induces CCA1 and LHY expression and represses TOC1. CCA1 and LHY may directly activate clock-regulated genes that are expressed early in the day, while possibly repressing genes that are only expressed in the evening. CCA1 and LHY levels decrease during the day, a phenomenon that appears to weaken the repression of TOC1, whose levels increase towards the end of the day. Then TOC1 induces the expression of LHY and CCA1 (Wang and Tobin, 1998; Alabadi *et al.*, 2001). CCA1 and LHY have partially redundant functions in the control of period length, but are required to sustain the intensity and circadian rhythmicity (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Green and Tobin, 1999; Alabadi *et al.*, 2002; Mizoguchi *et al.*, 2002).

In wild-type *Arabidopsis* plants, CCA1 peaks 1 h after dawn and disappears at the end of the day, and this phase

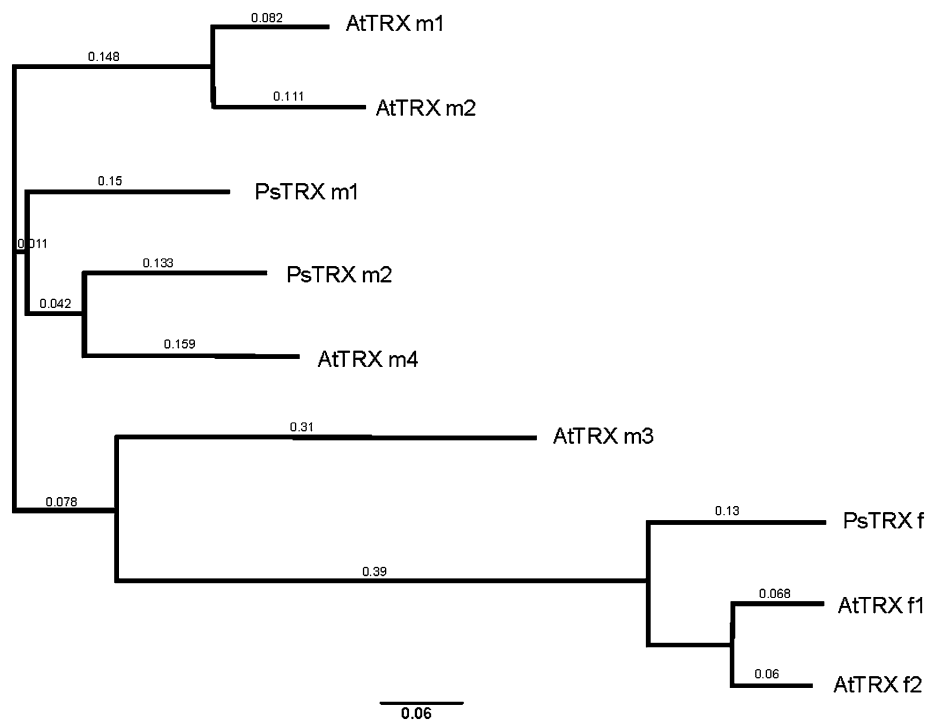


Fig. 7. CLUSTALX phylogenetic tree for chloroplastic TRXs *f* and *m* from pea (*Ps*) and *Arabidopsis* (*At*). Accession numbers are: *PsTRX f* (CAA45098), *PsTRX m1* (CAA53900), *PsTRX m2* (CAC69854), *AtTRX f1* (NP_186922), *AtTRX f2* (NP_197144), *AtTRX m1* (NP_849585), *AtTRX m2* (NP_192261), *AtTRX m3* (NP_179159), and *AtTRX m4* (NP_188155).

oscillation precedes that of CAB gene expression by ~3 h. Because the CAB gene encodes a chlorophyll *alb*-binding protein (Lhcb), this sequence suggests that CCA1 acts as a positive regulator of *Lhcb* RNA and may be responsible for the circadian rhythm of *Lhcb* genes early in the morning (Wang *et al.*, 1997; Green and Tobin, 1999). AtTRX *f2* and *m2* RNA peak 4 h after dawn, a phase relationship consistent with CCA1 driving the oscillation in their gene expression. The *Arabidopsis* microarray database (www.genestigator.ethz.ch) corroborates the data presented here by showing that all TRX *f* and *m* genes are induced by light, yet only *AtTRX f2* and *m2* are circadian regulated.

It has been shown that overexpression of CCA1 and inhibition of CCA1 and LHY in *Arabidopsis* plants disrupts the circadian mRNA accumulation profile of *AtTRX f2* and *AtTRX m2* mRNA (Fig. 6, and Supplementary Fig. S2 at *JXB* online). Therefore, if CCA1 is the main factor driving the rhythm of TRX expression, TRX mRNA levels would be expected to be up-regulated in CCA1-overexpressing plants (*cca1-ox*). However, in this mutant, *AtTRX f2* and *AtTRX m2* mRNA are 4- and 2-fold lower, respectively, during the first light phase than in wild-type plants, and the circadian rhythm under continuous light is lost. Wang and Tobin (1998) have shown that the constitutive expression of CCA1 in *Arabidopsis* plants abolished the circadian rhythm in the expression of the *Lhcb1* gene in continuous light. These results indicate that as with *Lhcb1* RNA, the circadian rhythm of *AtTRX f2* and *m2* RNA is mediated by the oscillation of the CCA1 protein. Moreover, in plants with repressed CCA1 and LHY, the decrease in *AtTRX f2* and *AtTRX m2* mRNA is 2- and 18-fold lower than in control plants, respectively, and the first peak is shifted toward the night phase. Alabadi and colleagues (2002) showed that plants lacking CCA1 and with reduced LHY functioning (*cca1-lhy-R* mutant) were able to sustain oscillations in both constant light and constant darkness. However, as with *AtTRX f2* and *AtTRX m2*, these plants continue to exhibit a degree of circadian function in light/dark cycles, indicating that the *Arabidopsis* circadian clock is not entirely dependent on CCA1 and LHY activities. In contrast to the earlier phase of activity seen in *cca1-lhy-R* plants compared with the wild type, the shifted peak in *AtTRX f2* and *AtTRX m2* mRNA suggests that the delays may reflect the need for an additional structural or energy requirement (Bvning and Moser, 1973).

Analysis of the promoter regions of the pea chloroplastic *f* and *m1* TRX genes identified the AATATCT sequence (Fig. 3) as similar to the EE, AAAATATCT, sequence described by Harmer *et al.* (2000). This element has been found to be over-represented in a cluster of 31 clock-regulated genes (Harmer *et al.*, 2000), and the importance of CCA1 in the regulation of gene expression by light and the circadian clock has been observed in microarray experiments (Michael *et al.*, 2008). The EE is known to be important for the rhythmic activity of several evening-phased promoters (Alabadi *et al.*, 2001; Michael and McClung, 2002).

When the functional relevance of the EE was tested in f444-GUS and m1874-GUS, it was shown that this element

confers circadian regulation with peak expression in light. The *AtTRX f2* regulatory region displayed a circadian-regulated motif at position -987, while *AtTRX m2* revealed several CCA1-binding sites at positions -178, -347, and -1868, and at 1003 in the antisense orientation, one circadian *cis*-motif (at position 528 in the antisense orientation) and two EEs (positions -1593, and 148 in the antisense orientation), suggesting that these motifs are important for oscillatory regulation. No functional EEs were detected in the promoter regions of the other TRXs, *AtTRX f1*, *AtTRX m1*, *m3*, or *m4*. However, among the putative regulatory elements of all *Arabidopsis* TRXs, the *f* and *m* promoters comprised several light-dependent elements such as GATA, GT1, and Ibox, which are conserved in the promoters of light-regulated genes such as LCHII type I Cab. Different bioinformatics analyses used to compare *AtTRX f2* and *m2* promoters did not reveal any relevant information that could help understand their circadian regulation, with the exception of the presence of circadian and EE *cis*-acting elements in the promoter sequences.

The present *in vitro* binding studies demonstrate that the CCA1 transcription factor binds *in vitro* directly to the EE of *PsTRX f* and *m1*, as has been shown in previous reports with other promoters (Alabadi *et al.*, 2001; Farre *et al.*, 2005). These results suggest that CCA1 is a regulator of TRX genes. The findings revealed that the A base located in the EE core is essential for binding to the CCA1 transcription factor, and therefore a single base pair difference suffices to specify the time of day when transcription occurs. Numerous studies have focused on motif element mutagenesis and mutant plants, and concluded that combinations of known and unknown motifs are necessary for the correct phasing of circadian genes (Matsushika *et al.*, 2002; Mizogushi *et al.*, 2002; Harmer and Kay, 2005).

The physiological relevance of the circadian regulation of chloroplast TRXs might be related to their redox regulation of light-dependent protein targets. The circadian clock appears as an additional regulatory mechanism that induces some TRX genes to anticipate the activation of protein involved in the regulation of photosynthesis, carbon fixation, and growth, and preventing the loss of balance between the supply and utilization of carbon (Smith and Stitt, 2007; Stitt *et al.*, 2007). Extending the night leads to an acute carbon limitation and catabolism of protein, lipids, and other sources of carbon (Usadel *et al.*, 2008). By comparing short- and long-period *Arabidopsis* mutants with wild-type plants, Dodd *et al.* (2005) showed that correct matching of the circadian clock period with that of the external light/dark cycle confers a substantial photosynthetic advantage, which in turn contributes to a higher chlorophyll content, and increased carbon fixation.

In conclusion, it was shown that *PsTRX f*, *PsTRX m1*, *AtTRX f2*, and *AtTRX m2* mRNA are controlled by the circadian clock, and new evidence is provided that elements of the clock regulate the expression of genes involved in the redox regulation of proteins, probably through direct interaction of CCA1 or LHY transcription factors with an EE of the promoter region of these genes. *PsTRX f* and

PsTRX m1 protein levels also oscillated during the day/night cycle, implying circadian regulation at the transcriptional level. However, *AtTRX f2* and *AtTRX m2* expression shows a circadian oscillation that is altered in lines that overexpress CCA1 as well as in those that repress CCA1 and LHY and have lower transcript levels. A complex mechanism entailing the interaction of proteins that are light and circadian clock dependent with other proteins induced by light only is envisaged. Thus at least two different but coordinated light-dependent pathways seem to be involved in the regulation of the expression of the genes analysed here.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Expression pattern of the TRX *f1*, *f2*, *m1*, *m2*, *m3*, and *m4* isoforms of *A. thaliana*. Data were obtained from the 'Arabidopsis eFP browser' (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>).

Figure S2. Relative expression of *AtTRXCAB* (a), *AtTRX f1* (b), *AtTRX f2* (c), *AtTRX m1* (d), *AtTRX m2* (e), *AtTRX m3* (f), and *AtTRX m4* (g) genes in rosettes of *Arabidopsis* wild-type plants, plants overexpressing CCA1 (*cca1-ox*), and plants repressing CCA1 and LHY (*cca1-lhy*) during the day/night cycle. Seedlings were grown under a 12 h light/12 h night photoperiod and rosette samples were collected at 4 h intervals over a period of 48 h; during the second night, the light was kept on. Total RNA was extracted and analysed by real-time PCR using specific oligonucleotides. Clear areas in the graph indicate the day during the day/night periods, the grey area indicates the night, and the hatched area indicates subjective night. The graph shows the relative quantification of gene expression monitored after normalization by 18S rRNA expression as an internal control, considering the lowest value in each case. Numbers on the ordinates indicate the real time in hours at which samples were collected for RNA isolation.

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