Localization in Roots and Flowers of Pea Chloroplastic Thioredoxin *f* and Thioredoxin *m* Proteins Reveals New Roles in Nonphotosynthetic Organs[1][OA]

Juan de Dios Barajas-López, Antonio Jesús Serrato, Adela Olmedilla, Ana Chueca, and Mariam Sahrawy*

Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, 18008 Granada, Spain

Plant thioredoxins (TRXs) are involved in redox regulation of a wide variety processes and usually exhibit organ specificity. We report strong evidence that chloroplastic TRXs are localized in heterotrophic tissues and suggest some ways in which they might participate in several metabolic and developmental processes. The promoter regions of the chloroplastic *f* and *m1* TRX genes were isolated from a pea (*Pisum sativum*) plant genomic bank. Histochemical staining for β-glucuronidase (GUS) in transgenic homozygous Arabidopsis (*Arabidopsis thaliana*) plants showed preferential expression of the 444-bp *PsTRXf1* promoter in early seedlings, stems, leaves, and roots, as well as in flowers, stigma, pollen grains, and filaments. GUS activity under the control of the 1,874-bp *PsTRXm1* promoter was restricted to the leaves, roots, seeds, and flowers. To gain insight into the translational regulation of these genes, a series of deletions of 5′ elements in both *TRX* promoters were analyzed. The results revealed that a 126-bp construct of the *PsTRXf2* promoter was unable to reproduce the expression pattern observed with the full promoter. The differences in expression and tissue specificity between *PsTRXm1* and the deleted promoters *PsTRXm2* and *PsTRXm3* suggest the existence of upstream positive or negative regulatory regions that affect tissue specificity, sucrose metabolism, and light regulation. *PsTRXm1* expression is finely regulated by light and possibly by other metabolic factors. In situ hybridization experiments confirmed new localizations of these chloroplastic TRX transcripts in vascular tissues and flowers, and therefore suggest possible new functions in heterotrophic tissues related to cell division, germination, and plant reproduction.

Much progress has been made in recent years in discovering specific functions and protein targets of plant thioredoxins (TRXs). However, different TRXs can be active within the same organelle and may have redundant functions or act on different protein targets. This situation makes it difficult to determine their specificity and raises questions concerning their individuality (Balmer et al., 2004). The discovery of at least 20 different TRXs during sequencing of the Arabidopsis (*Arabidopsis thaliana*) genome has shown both the organization of this multigene family and the evolutionary pathways of the genes involved to be complex (Meyer et al., 2005).

TRXs are small (12–14 kD) proteins with a conserved redox-active site (WCXPC) involved in oxidoreductase activity with thiol-disulfide interchange reactions in all organisms (Holmgren, 1985). In its reduced form, TRX can function as a hydrogen donor or as a regu-

latory factor for various target proteins, such as metabolic enzymes, redox proteins, transcription factors, or mitogen-activated protein kinases (for review, see Arner and Holmgren, 2000; Schürmann and Jacquot, 2000). The bridge formed between two Cys of the TRX protein is reduced by ferredoxin via ferredoxin-TRX reductase (FTR) in the chloroplast, whereas cytosolic TRX is reduced by NADPH via NADPH-TRX reductase in other organelles and different parts of the plant.

Plant TRXs are organized in different groups depending on their localization. The fully sequenced Arabidopsis genome disclosed nine TRXs named *h*-type (heterotrophic) proteins localized in the cytosol, two mitochondrial TRX *o* proteins, and several chloroplastic TRXs encoded by four *TRX m* genes, two *TRX f* genes, one *TRX x* gene, and two *TRX y* genes. The specificity of TRX *h* is currently under review. However, several studies based on yeast complementation, proteomics, and protein target identification methods have indicated important new functions for these cytosolic TRXs and suggested a role for these proteins in different stress situations (Mouaheb et al., 1998; Wong et al., 2004; Traverso et al., 2007). Several potential targets for TRX *o* proteins have emerged recently, but no specific function has yet been discovered for them (Balmer et al., 2004). Among the chloroplastic TRX proteins, the *f* and *m* isoforms are the best described, and their function in carbon metabolism of the chloroplast has been well defined (Crawford et al., 1979; Mestres-Ortega and Meyer, 1999; Meyer et al., 2002; Collin et al., 2003, 2004). The chloroplastic *TRX m* is of prokaryote origin, whereas the chloroplastic *TRX f* and cytosolic *TRX h* share an intron

position and seem to have a common eukaryote ancestor (Sahrawy et al., 1996).

Research in pea (*Pisum sativum*) plants on the roles of TRXs has given rise to some confusion. To date, one TRX *f* and two TRX *m* (*m1* and *m2*) have been found in the chloroplast (López-Jaramillo et al., 1997; Pagano et al., 2000), and four TRX *h* are localized in the cytosol (Montrichard et al., 2003; Traverso et al., 2007). Both TRX *f* and TRX *m* regulate key enzymes that play an essential role in carbon fixation and sugar biosynthesis during photosynthesis. TRX *f* specifically activates photosynthetic Fru-1,6-bisphosphatase (FBPase), whereas malate dehydrogenase (NADP-MDH) is the target of TRX *m* (Bassham and Krause, 1969; López-Jaramillo et al., 1997). However, the distinction between the reactivity of the two chloroplastic isoforms with their target enzymes has not always been clear. Under certain assay conditions, TRX *f* can also activate NADP-MDH (Hodges et al., 1994; Geck et al., 1996), whereas FBPase can also be the target of TRX *m* (López-Jaramillo et al., 1997), but the efficiency of activation of the two targets differs. In consonance with their functions and localization, the biosynthesis of chloroplastic TRXs is induced by light (Carrasco et al., 1992).

A few years ago, it was reported that TRX *f* accumulates in roots as both a protein and a transcript (Pagano et al., 2000). This was the first finding of chloroplastic TRXs in a nonphotosynthetic organ. Recently, a TRX *m* isoform and the complete ferredoxin-TRX system were identified in the amyloplast of wheat (*Triticum aestivum*) endosperm (Balmer et al., 2006a). Several potential protein targets for chloroplastic TRX *m* have been identified with proteomics-based methods, but no new functions have been determined. Some studies have raised the possibility that chloroplastic TRXs participate in metabolic processes other than those related to photosynthesis in almost all stages of plant development. Despite extensive studies of the structure, function, and regulation of plant TRXs, however, little is known about the roles of plant TRXs. Few studies have focused on the search for new localization sites of chloroplastic TRXs or on the discovery of new roles in heterotrophic tissues.

Because the information provided by promoter regions could yield evidence of the sites of action and roles of different TRXs, we isolated the pea chloroplastic *TRX f* and *TRX m* promoters and analyzed their pattern of expression using the GUS reporter gene in Arabidopsis plants, which confirmed the presence of these TRXs in nonphotosynthetic organs. Database analysis of the chloroplastic *TRX* promoter regions identified several important cis-regulatory sequences related to tissue localization, light, and metabolite regulation, and serial deletions of these regulatory regions revealed that chloroplastic TRX *m* was finely regulated by the surrounding tissue and by exposure to light. We also developed an in situ hybridization method to detect chloroplastic *TRX* mRNA expression in any plant organ and structure.

We discuss how these results shed light on new sites of chloroplastic TRX action and analyze the regulation of these proteins in light of the cis-acting elements found in the promoter sequences. These considerations allow us to suggest possible new functions for chloroplastic TRXs in addition to their already well-known role in regulating carbon metabolism enzymes in the chloroplasts of green tissues.

RESULTS

Cloning and Structural Analysis of Pea *TRX f* and *TRX m* Promoters Revealed cis-Elements Related to Localization and Metabolic Regulation

We used the PCR-walking technique to isolate from several pea genomic DNA libraries two promoters corresponding to *TRX f* and *TRX m1* genes. To avoid confusion, *TRX m1* is designated *TRX m* throughout the text. The promoter of the *TRX f* gene is 444 bp upstream from the translation start site, whereas the promoter of the *TRX m* gene is 1,973 bp upstream from the ATG. The nucleotide sequences of the promoter fragment from *PsTRX f* and the 5′ flanking sequence from *PsTRX m* are presented in Figure 1, A and B. Although the *TRX f* promoter is shorter than expected, it contains the principal cis-acting elements that allow sufficient expression. RACE assays mapped the transcription start site to an A nucleotide 112 nucleotides upstream from the translation initiation codon of the *TRX m* gene (indicated as +1 in Fig. 1B). Despite several attempts, we were not able to determine the transcription start position of the *TRX f* gene. Bioinformatics analysis was undertaken to identify conserved motifs found in other eukaryotic promoters and to find putative cis-elements that might be essential in the regulation of *TRX f* and *TRX m* gene expression (Fig. 1). No typical TATA box was identified; however, for both *TRXs*, a putative TATA box was located at −31 bp from the transcription start site for the *TRX m* gene and −128 bp from the ATG for *TRX f*.

We classified the potential regulatory elements in the pea *TRX f* promoter into three categories: light-, tissue-, and carbon metabolism-dependent elements (Fig. 1A). Among the light-dependent elements were several GATA motif sequences and Ibox, as well as a GAAAAA motif at position −315, a GTAATA element at position −274, and a GTAATT sequence at position −190 (Lam and Chua, 1989; Gilmartin et al., 1990). Root tissue-specific elements (ATATT) were also found (Elmayan and Tepfer, 1995). The promoter sequence also contained four Dof-like elements (AAAG) related to the promoter of genes that encode enzymes involved in carbon metabolism (Yanagisawa, 2000).

Among the putative regulatory elements, the *TRX m* promoter comprised at least 16 different light-dependent elements, such as GATA, GT1, and Ibox (Fig. 1B). Several root tissue-specific cis-acting sequences (ATATT) were localized, as well as three CAAT box-like sequences at positions −7, −43, and −323 that have been found in promoters of genes encoding seed storage protein (Shirsat et al., 1989). The ATTTTA sequence is related

A

```
-394  5´-GTAAACAAATAAACCACATTGCGTGATTCATATATAATAACTATAAAATT
                PrTrxf1
-344  TTGCTCCATAAAATTATTTGTTGTTGTTTATATTTGCGATTGAAAGAAAC
-294  TGAAGTGAAAAAAAGAACGAGATATTCGAAGGGAATCTTGGCATCCAATTA
-244  TCCGGAAGAATGGTCAAAACTCTCCTAAAAAAATCAGAAAAGGGTAATTT
-194  GGTAATATTATATCCAAGCCTGGGCGATTTCACTAATTCCAACTTCTCTT
-154  TCATTCCTTTACGATTGTGTTTCATTGAATTTACTAACATCACCCTCATT
-144  TTCGCGATCAAAATAATTCCATACACAGACTTCAAGGGCAGCACCGTATT
                                           PrTrxf3
 -94  TTCAATCATCACACCCACTCTTCCTCCTCTTCACATCCACCACCACACTT
 -44  TCATATCTCAGTCCAATTCTCCACTCTTGGTCTTCCCCACATCAATG
                                          PrTrxf2
```

C

- 444 bp ——— | GUS | *PsTRXf1*::GUS (f1-GUS)

-126 bp ——— | GUS | *PsTRXf2*::GUS (f2-GUS)

D

- 1874 bp ▼ | GUS | *PsTRXm1*::GUS (m1-GUS)

- 1521 bp ▼ | GUS | *PsTRXm2*::GUS (m2-GUS)

- 503 bp ▼ | GUS | *PsTRXm3*::GUS (m3-GUS)

- 138 bp ▼ | GUS | *PsTRXm4*::GUS (m4-GUS)

+23 bp | GUS | *PsTRXm5*::GUS (m5-GUS)

B

```
5´ -                              GTCCTTGGTGCCTCTCAAACT  -1854
                                      PrTrxm1
AAATTTTTGTTAAAAGCTATGCATCCACTTTGAAATTTGTATCACGAACTACGATGTTTT  -1794
GATCCTTCATTTGGAGGTTGGTACGTAGGCACAAGTCCGAAGGTCTTGTCAAACACAAAA  -1734
ATATAATTAATGAATTCTCTTCTCATCCCCTCACTTTATTTGTTTGTAAACATCACTTTG  -1674
TACAAAATACATATGCACACAAAAGGGGCTCCCTAGGAGTACCTAGGACACTTTGGGTG  -1614
CTAACACCTTCCCTCTATGTAACCAACCCCCTTACCTGTAATCTCTGACATTTTATTAGT  -1554
TTTGATTTGAAAACTTCTTACTTTTGGGTTTTGTTCATACTTTTTCCCTTTTCCCTTGGA  -1494
                                       PrTrxm3
AACGAAAGAGACGCGGGGGCGACTCTTGTTATTTGATGCCTTGCTTATCCATAGCTCGAT  -1434
GATCATGAATTTACCGCTACAATCACTATATAAATATGTAGTAGTTTAAAATTGTTGT    -1374
ATTTTTAAAAATTGATAGACGTTTATTTTTTAAAATATATAAATATATATGCGCGGGTA    -1314
TGGGGCGTGGTGGGTACTAAGGTGCACATCTCTGCTTATTTAAGTGGGTAATTACCCGAG  -1254
CTCGTGCCTGTTCCCATTTTTATGGATTTTTACCCTACTAATTGTGGATACCCATTGGGG  -1194
ATGAGTCAAACTATCATCCTTAGAAGTAAGAAACATAAACACAAATATAAATAGAGAAAT  -1134
TCAGATTTTGTTATTCATATTAAGGGTTTGGATTTGGAAATTTGGATTACACGCAGAGAG  -1074
TAAAAACCAGACGAAAACGTCAACCTTAATCCTCAATTGTCCCAATTTTATACCAATTTAA  -1014
GTAGGAATTACATTTTTATTGGATAATCTAATGTAGCTTAACTATAATCTATTAAGCT    -954
ATTAAGGAGTTACGTGTATAAAAAATCATAAAAGTAAGATATGAGAAACTTATCAAAT    -894
ATGAGAGACGAATGTGCTTAATTTGAAGGCGTAGAAGTTGATGAAGTATTTGATTTGAAT  -834
GAACATAAGTACATATTGAAGCAAGATTGAAAAAAATGTTTGATTCAAATGAGCGAGAG   -774
TTTTGCTCTGCTCTTGAGAGAAGTTAAGAAGTTGGTTTCAAAAGAATGGAATGTGGAGG   -714
GAAAAATTGTATATGCACACTCGAATAAATGTGTCATAATATTAACATTCGAAGGTTGAA  -654
TCTGGGATTTCCAATCTTTGAACTGATTTTTTTACTTGTATTTGCCGTGTTTGCTAAGAT  -594
GGAGTTTGTTGTGATTAAAAATGAATTTAAATTTCAATGCTCCGGACTTATTTTTTACTT  -534
CTATTTCCACATGTTTCTGAGATAGAGTTTGTGTTTAGGGTACATTCTGATTTTATTTTT  -474
                                                  PrTrxm4
AGATTTTAAGAATATTTTTAAAAATTCCGTTATGGTGAATGAGTAATATATGAGGTGCAAT  -414
AAACAATGTTCAATAAAATCAAGAAATCCTCACCAATTTACTATGTAAAATTAACAAGAA  -354
CAACATGATCAAACCTTTCAAACCTAGATCCAATAGTTAATGAATTTTAACTAAATAATT  -294
AAATTCAAATAGTCAATAAACTTTTCATTAAGCTCGCATGGAGTTTCTAAATTTGATC    -234
TCTATTTTTTAAATCTAATTTGTGGAGTTTATTTACACCTCCCTACTAAAATGTGAAACA  -174
AAACCCCCCAGAGAAGAGAAGAGGATTTTGAGATAGTGTGGAGAAGTACTTTTTAGTGGG  -114
                                       PrTrxm5
GACAGAAAGGAGAGAGAGGATTGAAAACCTAAATAACATCCACATGAATCCAAGCCATCT  -54
CATAACCTCACAATCTCAAATAATATCCAGTTACAAACACACTTCACAATTTCATCATAC  +7
CCAACAAATTAAAAACCGCCACAACACTAGAAGCTTATCCACAACAAGTGCTGAAAACCC  +67
                  PrTrxm6
TCCCTTCATTTTCCCCAAACTATTCAGTAGAGTTACATACACAGAAAATAACATG - 3´  +112
                                          PrTrxm2
```
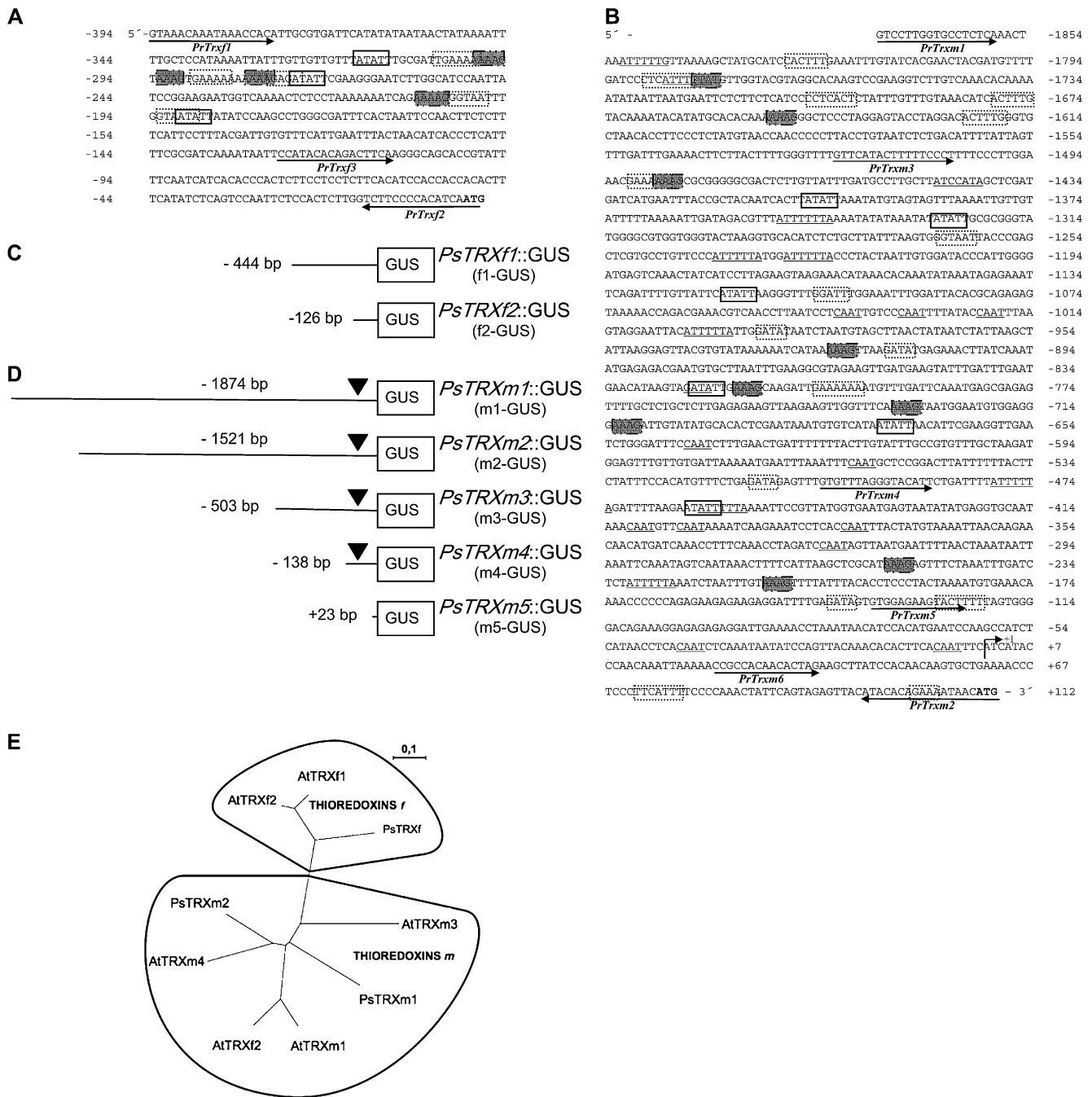
E

Figure 1. Sequence of the 5′ upstream region of the pea *TRX f* (A) and *TRX m* (B) genes, deleted promoter constructions of *TRX f* (C) and *TRX m* (D), and phylogenetic analysis (E). Nucleotides are numbered relative to the transcription start site indicated as +1 for the *TRX m* promoter and the ATG for *TRX f*. Putative cis-elements found in the database are indicated in boxes. Root-specific tissues are in white boxes, carbon metabolism-related elements are in shaded boxes, light-responsive elements are in dashed boxes, and elements related to seed development are underlined. C, Schema of the serial deletions of the promoter of the *TRX f* gene fused to the GUS reporter gene. D, Schema of the serial deletions of the promoter of the *TRX m* gene fused to the GUS reporter gene. E, ClustalX phylogenetic tree for chloroplastic TRX *f* and TRX *m* from pea (Ps) and Arabidopsis (At). Accession numbers are (in parentheses): PsTRXf (CAA45098), PsTRXm1 (CAA53900), PsTRXm2 (CAC69854), AtTRXf1 (NP_186922), AtTRXf2 (NP_197144), AtTRXm1 (NP_849585), AtTRXm2 (NP_192261), AtTRXm3 (NP_179159), and AtTRXm4 (NP_188155).

to the regulation of plant development and seed storage proteins. Finally, a number of sequences that bind Dof elements (AAAG) conserved in promoters of genes involved in sugar biosynthesis were identified in the *TRX m* promoter.

Our comparison of the two *TRX* promoters (Fig. 1) showed that, despite their different sizes, both contained similar cis-acting elements that determine their specific localization in the plant, their regulation by light, and their function during carbon metabolism, aspects that we considered important for the study of chloroplastic TRXs' localization and regulation. The bioinformatics analysis revealed, in addition, elements involved in processes of embryo and seed formation, protein storage, circadian clock, pollen localization, and hormonal regulation.

Because it was impossible to transform pea plants, we obtained Arabidopsis transgenic plants that carried the pea *TRX f* and *TRX m* promoters. Although this heterologous system is widely used in plant molecular biology, we decided to compare chloroplastic TRXs in both plant species at the protein and promoter sequence level to validate our results. A comparative phylogeny analysis of chloroplastic TRXs *f* and *m* from pea and Arabidopsis showed that PsTRX *f* was grouped with AtTRX *f1* and AtTRX *f2*, whereas the four Arabidopsis TRXs *m* were positioned together with PsTRX *m1* and PsTRX *m2* (Fig. 1E). These data confirmed the high similarity between both *f*-type and *m*-type TRX species: PsTRX *f* had 86% identity with AtTRX *f1* and 90% identity with AtTRX *f2*, whereas PsTRX *m1* shared 82%, 82%, 71%, and 82% identity with AtTRX *m1*, AtTRX *m2*, AtTRX *m3*, and AtTRX *m4*, respectively.

In The Arabidopsis Information Resource (TAIR) database, we examined the promoter regions of the two TRXs *f* and four TRXs *m* of Arabidopsis: AtTRX *m1* (AT1G03680), AtTRX *m2* (AT4G03520), AtTRX *m3* (AT2G15570), AtTRX *m4* (AT3G15360), AtTRX *f1* (AT3G02730), and AtTRX *f2* (AT5G16400). Bioinformatics analysis detected similar regulatory boxes in pea *TRX f* and *TRX m* promoters that were related to light, tissue, and carbon metabolism.

Promoter::GUS Fusion in Transgenic Arabidopsis Plants Revealed Expression in Green and Nonphotosynthetic Tissues

To examine the contribution of the 5′ region of chloroplastic *TRX* genes to the regulation of expression, different extensions of 5′ flanking sequences of *TRX f* and *TRX m* were transcriptionally fused to the *uidA* (GUS) reporter gene via the pBI101 vector, and designated *PsTRXf1*::GUS (*f1*-GUS) or *PsTRXf2*::GUS (*f2*-GUS) with the *TRX f* promoter, and *PsTRXm1*::GUS (*m1*-GUS), *PsTRXm2*::GUS (*m2*-GUS), *PsTRXm3*::GUS (*m3*-GUS), *PsTRXm4*::GUS (*m4*-GUS), or *PsTRXm5*::GUS (*m5*-GUS) with the *TRX m* promoter (Fig. 1, C and D). After transformation of Arabidopsis plantlets and generation of homozygous lines that expressed the promoter TRX::GUS fusion genes, we examined plantlets

for tissue-specific GUS activity. Enzyme activity was not detected in control plants transformed with an empty pBI101 vector.

PsTRX *f*::GUS Fusion

At early stages of germination in plants transformed with *PsTRXf1*, GUS was strongly expressed mainly in the cotyledons, hypocotyl, and meristematic ring (Fig. 2A). After 7 to 10 d of growth, GUS activity appeared in leaves, leaf primordia (Fig. 2B), and root apical meristems (Fig. 2C). Staining was also observed in the stomata and at the base of the trichome (Fig. 2D). In fully expanded plantlets (20 to 30 d old), flowers of transgenic plants that expressed the *TRX f* promoter::GUS fusion showed histochemical staining exclusively in anthers, pollen grains, filaments, and stigmas (Fig. 2E), and at the top and base of the siliques (Fig. 2F). Vascular tissues showed a weak, but clear, signal in leaves and sepals. The deletion of 267 bp of the promoter induced the complete loss of GUS expression in cotyledons (Fig. 2G) and fully developed plants (Fig. 2, H and I). Transgenic plants containing the empty control vector pBI101 showed no staining (Fig. 2, J and K).

PsTRX *m*::GUS Fusion

In the early stages of germination of plants transformed with the *TRX m* promoter::GUS fusion, staining was restricted to cotyledons (Fig. 3A). A few days later, transgenic plants displayed prominent GUS activity in leaves (Fig. 3B), as well as in regions of intense cell division, such as the apical meristem and procambium region, and in the root apical meristem and epidermis, including root hairs (Fig. 3, C and D). In flowers, we observed a specific signal in pollen grains and filaments as well as in stigmas (Fig. 3E). After 3 weeks of development, GUS staining appeared in the intersection of the secondary and primary stems and at the end of the siliques. Seeds inside siliques showed a weak, but localized, signal (Fig. 3F). Deletion of 1,877 bp of the *TRX m* promoter resulted in complete loss of GUS accumulation in all organs of *PsTRXm5*::GUS transgenic Arabidopsis plantlets (Fig. 3, N–P). The sequence comprising the region from position −138 to +23 bp of the 259-bp fragment (*PsTRXm4*::GUS) was sufficient to reproduce most features of the expression pattern seen with the complete promoter sequence and to maintain basal expression in cotyledons, hypocotyls, roots, and trichomes of seedlings. As plants matured, however, GUS expression became weaker in leaves (Fig. 3L) and almost disappeared in roots (Fig. 3M). In contrast to the results with the *PsTRXm1*::GUS fusion, plants transformed with the *PsTRXm2*::GUS and *PsTRXm3*::GUS fusions showed additional staining in the hypocotyl (Fig. 3G). Leaves of the plantlets accumulated high levels of GUS (Fig. 3, H and J), whereas expression in the roots decreased significantly (Fig. 3K). In flowers of *PsTRXm2*::GUS, *PsTRXm3*::GUS, and *PsTRXm4*::GUS plants, weak staining was detected in anther filaments, but staining was intense in stigmas
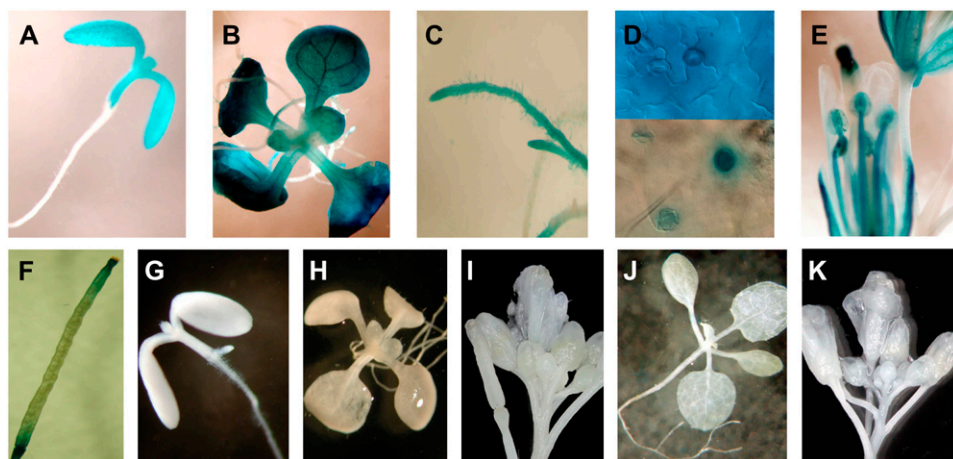
Figure 2. Histochemical localization of GUS expression under the control of the *PsTRXf1*::GUS promoter (A–F) and *PsTRXf2*::GUS deleted promoter (G–I) in transgenic Arabidopsis plants. A, Seven-day-old seedling. B, Fifteen-day-old plant grown in tissue culture, showing strong GUS expression in the cotyledons, hypocotyls, and leaves. C, GUS expression in the root apical meristem of a 15-d-old plant. D, Stomata showing expression in the guard cells. E, Flowers of 21- to 30-d-old plant showing stained pollen grains, filaments, and stigmas. F, Siliques showing GUS activity at the top and bottom of developing siliques. G (seedling), H (15-d-old plant), and I (flower), Deleted Ps*TRX f2*::GUS promoter (*f2*-GUS) showing complete loss of expression. J and K, Vector pBI101 in control transgenic plants. J, Fifteen-day-old plant. K, Twenty-one-day-old plant.

(Fig. 3I). These results were confirmed in two independent reporter lines. The intensity of GUS activity in the leaves of transgenic plants containing the *TRX f* or *TRX m* promoter decreased as plant senescence progressed. Transgenic plants containing the empty control vector pBI101 showed no staining (Fig. 3Q).

Figure 4 shows a cross section of a GUS-stained Arabidopsis leaf expressing the *PsTRXm1*::GUS and *PsTRXf1*::GUS promoters. *TRX f* (Fig. 4, A and B) and *TRX m* (Fig. 4, C and D) were located in the chloroplast of mesophyll cells and in stomata. Surprisingly, a strong signal was also seen in the vascular tissue of the leaf and in the tracheids, as observed in transgenic plants.

Semiquantitative PCR Expression of GUS Revealed Light-Dependent Regulation

In Arabidopsis plants carrying the *PsTRXf1*::GUS construct and subjected to a 12-h-light/12-h-dark photoperiod, GUS RNA expression displayed a peak at the middle of the day that decayed by 33% during the dark period in comparison to the light period (Fig. 5A). This light-dependent expression disappeared in plants carrying the shorter fragment of the *TRX f* promoter (*PsTRXf2*::GUS), and the level of messenger was similar during the day and night phases (Fig. 5A). We also found high GUS expression in transgenic plants under the control of the *PsTRXm1*::GUS promoter; expression was most intense after 8 h of light and decreased during the dark period by 31% (Fig. 5B). The pattern of expression was similar in plants bearing the *PsTRXm2*::GUS: high expression during the light period, which decreased by 33% during the night. Surprisingly, the *PsTRXm3*::GUS and *PsTRXm4*::GUS promoters yielded similar levels of expression during the day and the night, with

decreases during the dark period of only 10% and 4%, respectively, in comparison to the light period. Light-induced expression disappeared completely in plants with the *PsTRXm5*::GUS promoter. Unexpectedly, with the exception of *PsTRXm5*::GUS, serial deletions of the *TRX m* promoter induced a progressive increase in GUS mRNA expression in response to light. In plants with the *PsTRXm4*::GUS promoter, mRNA expression was up to 5-fold as high as the expression of *PsTRXm1*::GUS mRNA.

In Situ Hybridization Revealed New Sites of Action in Reproductive Organs and Vascular Tissues

To determine the patterns of expression of the *TRX f* and *TRX m* pea chloroplastic genes, we conducted in situ hybridization with a digoxigenin-labeled antisense RNA probe specific to each of the chloroplastic TRX cDNA sequences in different pea tissues. Leaf cross sections revealed *TRX f* and *TRX m* mRNA throughout the parenchyma and mesophyll layers (Figs. 6A and 7A). However, *TRX f* expression was more intense in collenchyma cells and regions adjacent to the leaf midvein and around the xylem vessel and phloem sieve tube, as observed in cross sections of stained leaves (Fig. 6, A and B). In contrast, intense *TRX m* signals were detected in the mesophyll cells of the leaf (Fig. 7B) and low expression was detected in the parenchyma (Fig. 7A). Again, we were able to detect a strong signal around the vascular tissues, phloem, and xylem, and neither of the TRXs seemed to be expressed in the epidermis. In root cross sections, we found localized expression of *TRX f* in the vascular tissue (Fig. 6D) and vascular tissue triarchs (Fig. 6E). When the same tissues were assayed with sense probes, no specific reaction
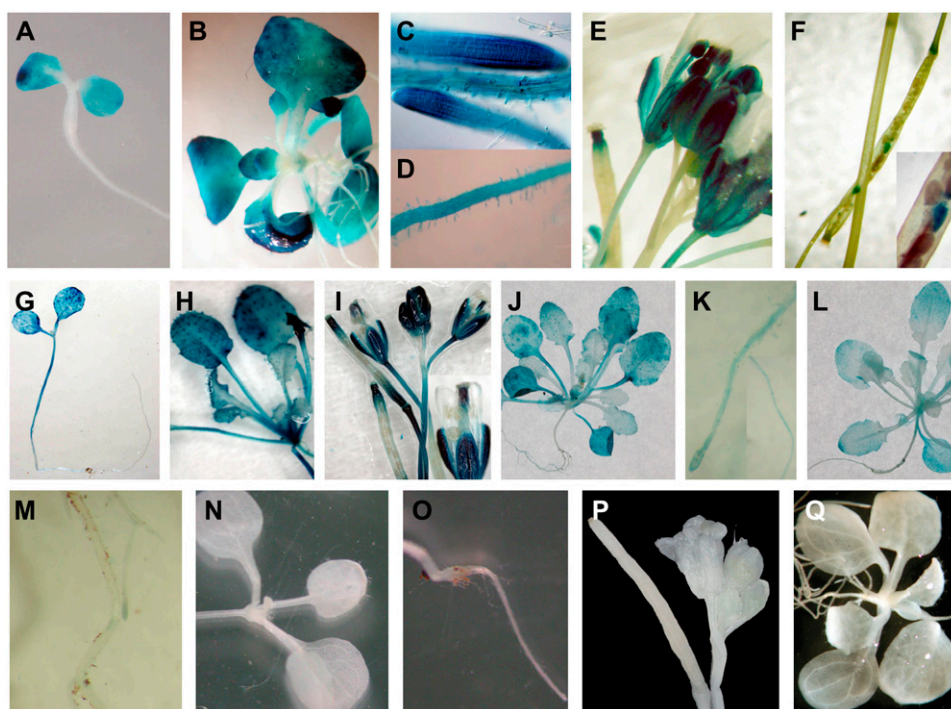
Figure 3. Histochemical localization of GUS activity under the control of the *TRX m* promoter. A to F, GUS expression in transgenic Arabidopsis plants carrying the *PsTRXm1*::GUS fusion construct. A, GUS staining localized in the cotyledons of 7-d-old seedlings. B and C, Leaves and roots from a 15-d-old plantlet. C and D, GUS activity localized in root apical meristem and root hairs of 15-d-old plantlet. E, GUS staining localized in flowers of a 21-d-old plant. F, GUS expression in seeds in the silique. G to I, GUS expression under the deleted promoter *PsTRXm2*::GUS. G, Additional staining in the hypocotyl of 7-d-old seedlings. H, GUS activity throughout the aerial part of a 15-d-old plant. I, Weaker GUS signals are visible in flowers, with the exception of strong staining in the stigma of a 21- to 30-d-old plant. J and K, Aerial part and root of a 15-d-old plant showing GUS expression under the deleted promoter *PsTRXm3*::GUS. The pattern of expression is similar to that detected in *PsTRXm2*::GUS transgenic plants. L and M, Aerial part and root of a 15-d-old plant, showing GUS activity under the deleted promoter *PsTRXm4*::GUS. The signal in this transgenic line is similar to that found with the previous deleted construct. Assays with the *PsTRXm5*::GUS fusion yielded no expression, as shown in N (10-d-old seedlings), O (root of a 15-d-old plant), and P (flowers of a 21- to 30-d-old plant). Q, Vector pBI101 in control transgenic plants.

was detected (Fig. 6, C and F). Pea seeds also accumulated *TRX f* messenger in the outer membrane of the coat seed and in a region of active cell division that likely corresponded to radicle protrusion (Fig. 6, G and H). A strong signal was detected in ovules, pollen grains, and connective tissue when flower longitudinal sections were hybridized with a *TRX f* antisense probe (Fig. 6J). However, no clearly defined structure could be identified inside the pollen (Fig. 6M). Two intense signals appeared inside the ovule. One area of expression was localized in the middle of the embryonic sac, an area corresponding to the endosperm tissue which, soon after fertilization, starts to undergo cell division at a high rate to provide nutrients. The second area was localized in the external part of the ovule and might be related to the developing integument, which grows to cover the nucella (Fig. 6, K and L). Sense experiments confirmed that the probes used were specific for their hybridization (Fig. 6, I, N, and O).

In root cross sections, a strong signal was evident in vascular tissues (Fig. 7D). By comparing the results with those in sense probe-treated tissue (Fig. 7F), we detected *TRX m* mainly in the xylem, with only weak reactivity in the phloem. This pattern of expression was also seen in the secondary vascular tissue toward lateral roots. Observations in longitudinal sections of roots confirmed that *TRX m* was expressed in the epidermis of the differentiation zone (Fig. 7E). We also observed that *TRX m* was widely expressed in reproductive organs (Fig. 7G) and was prominent in pollen grains (Fig. 7H), tapetal cells of the anthers, ovules, and connective tissue. The two signals in pollen grains appeared to be localized in the generative cell and tube cell, the latter of which is involved in the process of forming the pollen tube for pollination.

In later stages of flowering, the signal in pollen grains disappeared and the embryo displayed a strong reaction localized in the middle of the embryonic sac, a site of intense cell division during endosperm synthesis (Fig. 7, H, K, and L). The vascular tissue of reproductive organs also expressed TRX *m*, as seen in the pedicel (Fig. 7I). No signal was observed upon hybridization with the *TRX m* sense probe in leaves (Fig. 7C), roots (Fig. 7F), or flowers (Fig. 7J).
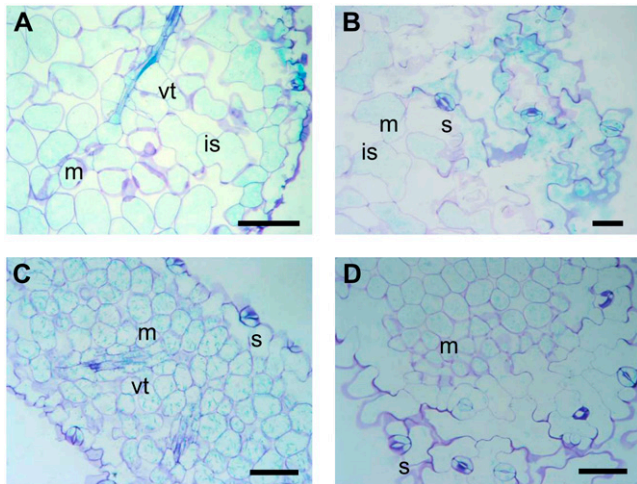
Figure 4. Semithin cross sections of leaves from GUS-stained plants transformed with *f1*-GUS (A and B) and *m1*-GUS (C and D). Bars = 50 µm. GUS staining is observed in mesophyll cells, vascular tissue, and stomata. m, Mesophyll; s, stomata; vt, vascular tissue; is, intercellular spaces.

Pea Tissue Expression Detected *FTR* and *TRX f* and *TRX m* in Different Tissues

The cDNA obtained by reverse transcription (RT) of RNA extracted from pea leaves, stems, roots, seeds, and flowers was subjected to PCR with specific oligonucleotides corresponding to the *TRX f*, *TRX m*, and *FTR* genes. As expected, all three genes were expressed in leaves and stems (Fig. 8A; Table I); additionally, a significant level of mRNA expression was also detected in seeds as well as in nonphotosynthetic tissues, such as roots, and in flowers. The *TRX m* gene was highly expressed in leaves, seeds, and flowers. Expression of *TRX f* was clearly positive, but comparatively weaker, and appeared mainly in stems, leaves, and flowers. The level of *FTR* mRNA was higher in leaves than in other tissues. In all cases, we detected the presence of the three genes in roots and flowers, indicating a possible role for chloroplastic TRXs in heterotrophic tissues.

Western Blotting Identified TRX *f* and TRX *m* Protein in Root and Flower Tissues

Immunoblot analyses were performed with a pea anti-TRX *m* and anti-TRX *f* serum. Seeds, flowers, leaves, roots, and pod extracts from pea showed a clear band corresponding to TRX *m* recognized by the antibody, with a molecular mass of 14 kD (Fig. 8B). Similar results were obtained with anti-TRX *f* serum (Fig. 8B). Chloroplastic TRXs *m* and *f* were detected in all tissues analyzed (Table I).

Table I summarizes the sites of expression and level of expression observed with four different techniques that yield information on all steps of gene expression. The findings show clearly that the localization as well as the level of expression were similar in organs assayed.

DISCUSSION

Since the discovery of chloroplastic TRXs in non-photosynthetic organs, significant efforts have been focused on the search for protein targets for all plant chloroplastic TRXs in an attempt to assign specific functions to each protein. To attain better insights into the potential role of chloroplastic TRXs, we first analyzed the spatial and temporal distribution of these proteins by inserting fusions of the GUS gene with the pea *TRX f* and *TRX m* promoters into Arabidopsis plants to trace the localization and pattern of gene expression of pea *TRXs f* and *m*. The results obtained were informative for new sites of action and are potentially useful in assigning different roles to chloroplastic TRXs.

During germination of *PsTRXf1*::GUS transgenic plants, the pattern of GUS expression showed that *TRX f* is localized in areas of intensive cell division and
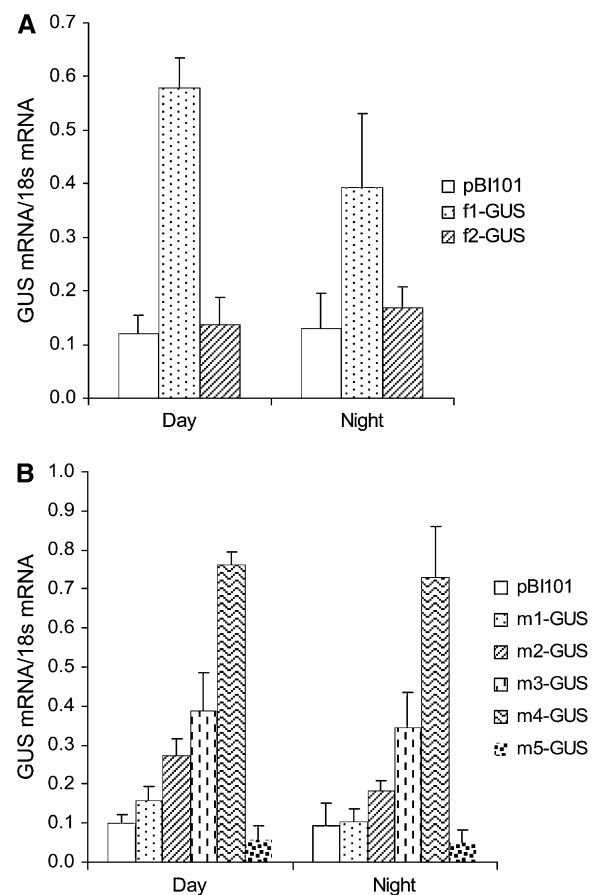

Figure 5. Light/dark regulation of GUS mRNA expression under *TRX f* and *TRX m* promoters. Transgenic plants carrying complete promoter constructs (*f1*-GUS or *m1*-GUS) or a truncated promoter (*f2*-GUS, *m2*-GUS, *m3*-GUS, *m4*-GUS, or *m5*-GUS) were subjected to a 12-h-day/12-h-night photoperiod, and mRNA levels of the GUS-encoding gene were determined in leaves by semiquantitative RT-PCR as described in "Materials and Methods." A, GUS expression under the *TRX f* promoter. B, GUS expression under the *TRX m* promoter. Samples were harvested at midday and midnight from 20-d-old plants. Values are the average of three determinations of two cDNA preparations.
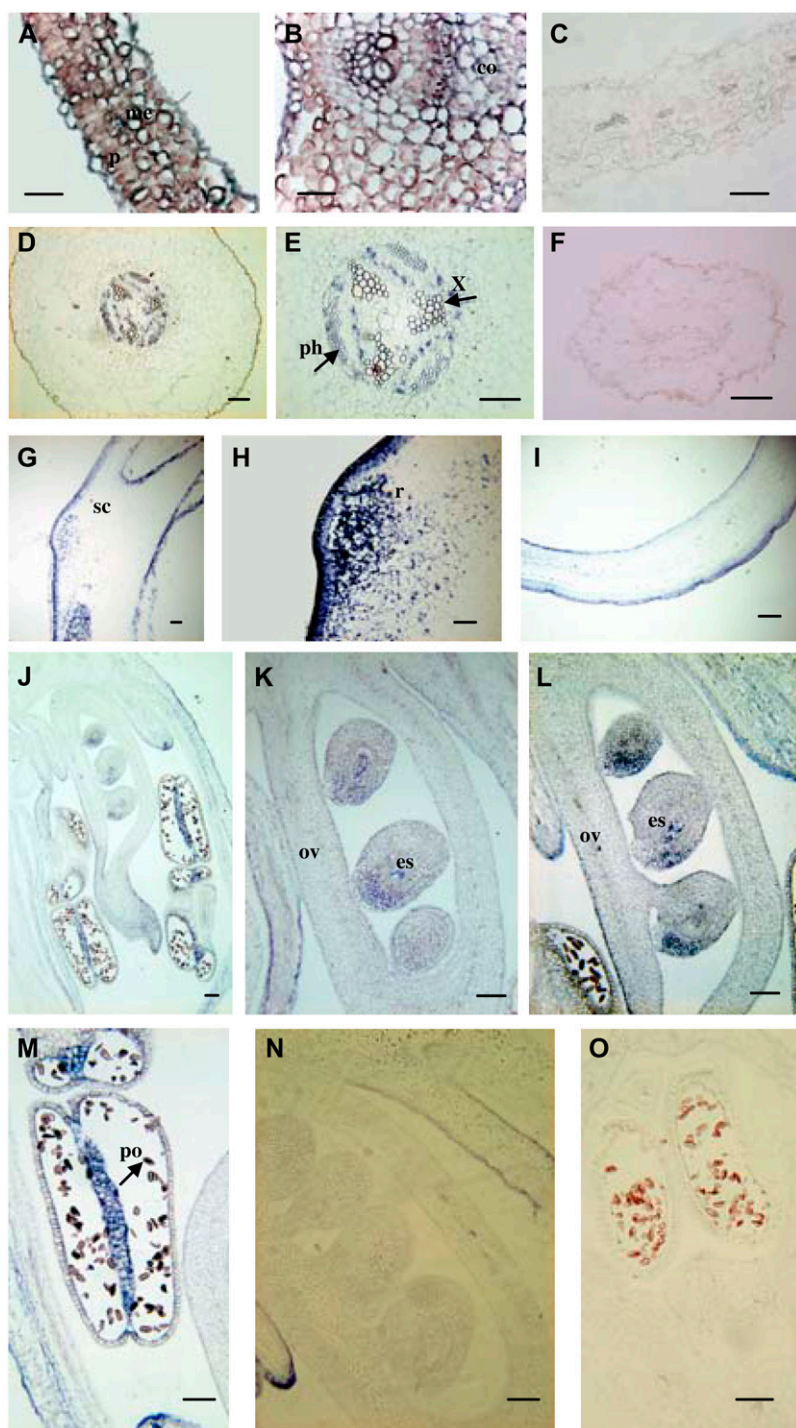
Figure 6. Localization of *TRX f* mRNA in pea plants by in situ hybridization with the *TRX f* antisense probe. Bars = 100 μm. A and B, Cross and longitudinal sections of a leaf. C, Leaf cross section hybridized with sense probe. D, Root cross section. E, Root cross section at higher magnification showing positive reaction around the vascular area. F, Root cross section hybridized with the sense probe. G and H, Longitudinal section of pollen grains. I, Control sample of pollen grains hybridized with sense probe. J to M, Longitudinal section of flowers at different stages. J, Developing flower. K and L, Details of developing embryos. M, Pollen grains in the anthers. N and O, Control samples of flower sections hybridized with the sense probe. me, Mesophyll; p, parenchyma; co, collenchyma; x, xylem; ph, phloem; sc, seed coat; r, radicle; ov, ovary; es, embryonic sac; po, pollen.

confirmed previous studies that found *TRX f* mRNA in roots. These data suggest that pea TRX *f* might be involved in cell proliferation. We observed that, at later stages of growth, the intensity of GUS expression decreased at the beginning of senescence, indicating that chloroplastic TRX *f* is not related to the senescence process. In contrast, however, Laloi et al. (2004) found that *AtTRX h5* was overexpressed in old leaves; these

authors accordingly related this gene to senescence rather than to cell division. We have also detected TRX *f* in guard cells of stomata. Among the numerous proteins localized in guard cells, ADP-Glc pyrophosphorylase (AGPase), which is redox regulated, seems to be reduced by TRX *f* (Ballicora et al., 2000). Surprisingly, however, we detected GUS staining in stigmas, as well as in pollen grains in floral organs. Cytosolic TRXs have

Figure 7. Localization of *TRX m* in pea plants by in situ hybridization with the *TRX m* antisense probe. Bars = 100 μm. A and B, Cross and longitudinal sections of a leaf. C, Leaf section hybridized with the sense probe. D, Cross section of a root. E, Longitudinal section of a root. F, Sense control of *TRX m* mRNA in roots. G, Longitudinal section of a flower. H, Pollen grain. I, Vascular tissue of the pedicel. J, Longitudinal section of a flower showing the sense control. K to M, Higher magnifications of longitudinal sections of ovaries showing details of developing embryos. me, Mesophyll; p, parenchyma; co, collenchyma; vt, vascular tissue; e, epidermis; al, anther locus; po, pollen; ov, ovary; es, embryonic sac.
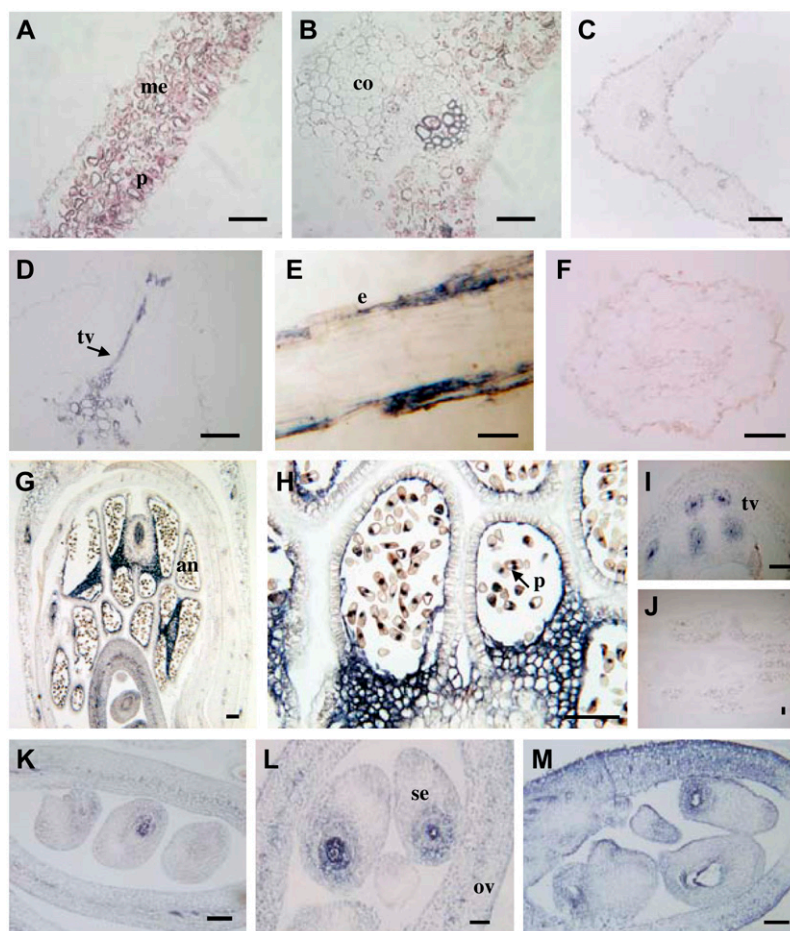
been isolated from pollen and were reported to take part in the self-incompatibility process in pollen-pistil recognition of *Brassica oleracea* (Bower et al., 1996; Cabrillac et al., 2001); however, the role of AtTRX *h* in pollen is still poorly understood in Arabidopsis, where self-incompatibility does not occur. Some authors have shown that the allergic response to grass pollen is linked to TRX *h*, which may thus represent a novel family of cross-reactive allergens that might contribute to the symptoms of baker's asthma (Weichel et al., 2006). In contrast to TRX *h*, however, no previous studies have found chloroplastic TRXs to be related with allergies. Despite the presence of Arabidopsis chloroplastic TRX *y1* (Collin et al., 2004) and TRX *m3* (Mestres-Ortega and Meyer, 1999) in flower buds, no previous studies have mentioned the existence of TRX *f* in reproductive organs.

Analysis of the *TRX f* promoter identified three cis-elements specific to root localization (ATATT) and at least five light-regulated motifs that are important for spatially regulated expression during normal plant development. Consistent with a regulatory role of these cis-acting sequences is our finding that GUS activity was not induced in transgenic plants with the truncated 149-bp promoter.

As with the *TRX f* promoter, in seedlings and 15-d-old Arabidopsis plants, the entire *TRX m* promoter directed strong GUS activity in regions of intense cell division, such as the cotyledons and leaf apical meristem. In fully developed transgenic plants, the intensity of GUS activity decreased as senescence progressed; however, GUS staining became stronger in the apical root meristem, at the ends of the siliques, and in flowers, pollen grains, and seeds. Like TRX *f*, the activity of TRX *m* may be directly related to the redox regulation of processes in tissues with high proportions of cells undergoing division. Serial deletions of 5′ fragments showed that a 259-bp segment of the *TRX m* proximal promoter (*PsTRXm4*::GUS) was sufficient to confer a pattern of localization of GUS expression similar to that seen with *PsTRXm1*::GUS. It therefore appears likely that the remaining 5′ flanking sequence of *PsTRXm5*::GUS (97 bp) that harbors the putative TATA box and two light-dependent elements is largely repressed in Arabidopsis transformants.

In contrast to our findings with the *PsTRXm1*::GUS fusion, experiments with plants carrying *PsTRXm2*::GUS or *PsTRXm3*::GUS (lacking the −1,874 and −1,521 and −1,874 and −503 sequences, respectively) led to staining of the hypocotyl and cotyledons, indicating
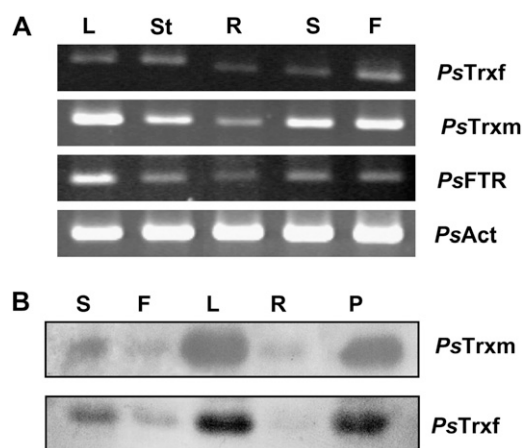
Figure 8. A, Tissue distribution of *TRX f*, *TRX m*, and *FTR* mRNA isolated from pea leaves (L), stems (St), roots (R), seeds (S), and flowers (F). Absolute mRNA levels of all genes were determined by semiquantitative RT-PCR. cDNA was standardized by reference to an actin standard. B, Western blotting analysis of different pea tissue extracts. PsTrxm, Antibodies against pea TRX *m*. PsTrxf, Antibodies against pea TRX *f*. Twenty micrograms of protein were loaded in each lane from tissue extracts of seeds (S), flowers (F), leaves (L), roots (R), and pods (P).

the existence of regulatory cis-elements between nucleotides −1,874 and −503 that repressed expression in the hypocotyl. This finding reflected a tissue-dependent mechanism of control of gene expression. Deletion of this region may also have contributed to the high level of expression of *PsTRXm2* and *PsTRXm3*::GUS in comparison to *PsTRXm1*::GUS. Taken together, these results indicate that the region between −1,874 and −503 bp harbors positive and negative regulatory elements that coordinate the tissue-specific regulation as well as the intensity of *TRX m* expression. Removing the sequence between −503 and −138 bp to obtain *PsTRXm4*::GUS also led to expression of the *TRX m* promoter in the hypocotyl and cotyledons and to higher GUS activity. Although we had expected removal of light-regulatory motifs to decrease GUS expression when plants were subjected to light, serial deletions of 5′ fragments in the *TRX m* promoter led to an increase in the light-induced level of GUS mRNA, such that *PsTRXm4*::GUS expression was more than 5-fold as high as *PsTRXm1*::GUS expression, and PsTRXm2::GUS expression was twice as high as GUS *PsTRXm1*::GUS expression. Even more interesting, however, was the

finding that the loss of GUS expression did not correlate with the transition to darkness. The loss of light regulation suggests that the fragment between −1,521 and −138 bp, which harbors eight light-related cis-elements, is required for the controlled increase and decrease in expression when plants are subjected to light-to-dark transition.

Nevertheless, it is also possible that chloroplastic *TRX*s are regulated not only by light, but also by other nutritional or hormonal factors that might influence their patterns of expression. These factors could explain why expression was not completely lost during darkness. Because chloroplastic TRXs regulate enzymes involved in carbon metabolism, it is likely that sugars are an essential factor that controls the transcription of the *TRX*s. Analysis of the *TRX f* and *TRX m* gene promoters fused to the GUS reporter gene revealed that environmental factors and tissue-specific control of *TRX* gene expression require complex integration of multiple cis-acting regulatory elements with different trans-acting proteins. However, accurate analyses of the promoters involved will be necessary to identify overrepresented motifs that might mediate organ-specific expression and the response to light and other stimuli. Currently, we are working on the research of expression pattern under different nutritional and light conditions.

Both TRX *f* and *m* are found in heterotrophic tissues and, like some of the cytosolic TRXs, chloroplastic TRX proteins might be involved in the redox control of cell proliferation during growth, as observed with the tobacco (*Nicotiana tabacum*) TRX *h1* (Brugidou et al., 1993; Reichheld et al., 1999). No specific role has been established for this physiological process. However, in recent years, a number of new protein targets linked to regulatory disulfide protein TRXs have been identified, and these targets have helped to elucidate their function in chloroplasts (Motohashi et al., 2001, 2003; Balmer et al., 2003). Among them are the proteins involved in nitrogen metabolism, the C4 cycle, the translation process, fatty acid biosynthesis, and the 2-Cys peroxiredoxin stress-related protein. Proteins that might be related to the specific expression of chloroplastic TRX *f* and TRX *m* in areas of high cell division have been grouped together with proteins involved in folding and assembly (70-kD heat shock protein) and plastid division (FtsZ protein), and with proteins that play a role in DNA replication and transcription (ATP-dependent DNA helicase; Balmer et al., 2003).

Table I. *Semiquantitative data for TRX f and TRX m expression in different Arabidopsis and pea tissues and organs, determined with four techniques*

These data summarize the observations of transgenic plant lines and pea tissues. +++, High; ++, moderate; +, low; nd, not determined.

	Leaf		Root		Flower		Seed		Fruit	
	TRX *f*	TRX *m*	TRX *f*	TRX *m*	TRX *f*	TRX *m*	TRX *f*	TRX *m*	TRX *f*	TRX *m*
GUS staining (Arabidopsis)	+++	+++	++/+	++/+	+++	+++	++	nd	++	++
ISH method (pea)	+++	+++	++	++	++	++	++	++	nd	nd
mRNA expression (pea)	++	+++	+	+	++	+++	++	+++	nd	nd
Western blotting (pea)	+++	+++	+	++	++	++	++	++	+++	++

Our findings in roots and flowers prompted a more detailed study of these tissues. We carried out in situ hybridization experiments that showed, as expected, that most *TRX f* and *TRX m* transcripts were expressed in the mesophyll cells of the leaves and around the vascular tissues. Hybridization experiments also confirmed the presence of *TRX f* and *TRX m* in roots, where TRXs were intensely expressed around the xylem and phloem. Because nutrients, water, ions, proteins, lipids, hormones, and other substances are transported through the xylem and phloem, it is difficult to identify a specific protein target in these tissues. Nevertheless, the role of chloroplastic TRXs is likely related to the complex transport system in vascular tissue, where these proteins may be involved in redox regulation of some membrane transporters. Support for this hypothesis is provided by the finding with proteomic methods that the plastidial ADP-Glc transporter (Brittle-1 protein) was a potential protein target of the spinach (*Spinacia oleracea*) TRX *m* (Balmer et al., 2006a).

TRX expression in developing pollen grains and embryos seems to correspond to a developmental requirement, as well as to nutritional status. Within the pollen grain, *TRX m* was detected as two distinct signals, probably situated in vegetative and generative cells. The pollen grain cells contain several organelles, endoplasmic reticulum, and plastids with starch used in pollen tube formation and storage substances such as lipids, proteins, and vitamins. The precise function of TRXs in floral organs is not yet clear, but it appears evident that chloroplastic TRXs are involved in redox regulation of some processes related to ovule and pollen grain maturation, fertilization, and embryo formation. The occurrence of both TRXs in the developing embryo is likely related to a role in the process of cell division during seed formation. In seeds, chloroplastic *TRXs* have been detected in an area of very active cell division, indicating an important function during germination. Montrichard and colleagues (2003) reported a differential pattern of expression of pea cytosolic *TRX h3* and *TRX h4* in early seedlings and seeds and suggested that these proteins were closely linked to germination. As found for other TRX *h* subtypes, chloroplastic TRXs might play a role in activating enzymes involved in storage protein mobilization during early seedling growth, as well as in carbohydrate metabolism.

To confirm these new sites of localization, we carried out immunological studies with antibodies against TRX *f* and TRX *m*. This approach detected protein in leaves, roots, pods, seeds, and flowers, and the amount of protein correlated with the level of mRNA expression in the same tissues (Table I). Recently, TRX *m* was detected with spinach TRX *m* antibodies in the endosperm amyloplast of wheat seeds (Balmer et al., 2006a). The question is thus whether the role of chloroplastic *TRX* expressed in reproductive organs and in roots is similar to that of the protein involved in regulating enzymes of the Calvin cycle in green tissue or in storage protein mobilization during seed germination (Kobrehel et al., 1992; Besse et al., 1996; Wong et al., 2002; Marx et al.,

2003), or whether the *TRX* signal we observed is involved in self-incompatibility control in pollen-pistil recognition (Bower et al., 1996; Cabrillac et al., 2001), as has been observed for *TRX h*. Although our findings thus far in roots and flowers are insufficient to explain the specific role of TRXs in these parts of the plant, they nonetheless offer intriguing clues as to the possible functions of these proteins.

In relation to the role of plastid TRX in heterotrophic tissues during carbon metabolism, previous research reported low levels of FBPase and MDH transcripts and proteins in seeds and roots, but no enzyme activities were detected in these organs (Pagano et al., 2000). However, the fact that roots, pollen grains, and ovaries contain plastids and amyloplasts, which store starch and other important components, suggests that the role of TRX *f* and TRX *m* in roots and floral organs may lie in the redox regulation of enzymes involved in starch and carbohydrate metabolism. Additional support for the role of plastidial TRX in nonphotosynthetic tissues comes from the earlier finding that redox activation of AGPase in potato (*Solanum tuberosum*) tubers by chloroplastic TRX is part of a general mechanism for the regulation of starch synthesis in response to carbon status (Ballicora et al., 2000). Furthermore, Sparla and colleagues (2006) found transcripts of TRX-regulated β-amylase in leaves, roots, flowers, pollen, and seeds.

These findings suggest that TRX redox regulation is not a mechanism of regulation that occurs exclusively in photosynthetic tissues, but is rather a system common to every plastid in the plant (Tiessen et al., 2002, 2003; Geigenberger et al., 2005). In nonphotosynthetic organs, ferredoxin may not be reduced by light through the ferredoxin-TRX system, as is the case in chloroplasts, but in studies that used novel and improved methods, Balmer and colleagues (2006a) identified a complete ferredoxin-TRX system in amyloplasts from wheat starchy endosperm that involves ferredoxin, ferredoxin-NADP reductase, and FTR. As a mechanism of regulation, these authors suggested that light might be recognized as a thiol signal in the chloroplast, where it allows synthesis of sugar to proceed during photosynthesis. Balmer and colleagues further proposed that the synthesized sugar was then transported to sink organs, such as the seeds, and hydrolyzed by Glc-6-P dehydrogenase and 6-phosphogluconate dehydrogenase to generate NADPH, a thiol signal needed to reduce ferredoxin with ferredoxin-NADP reductase in the amyloplast (Balmer et al., 2006a). The reactions taking place in the amyloplast would thus be indirectly controlled by photosynthesis.

Because roots and floral organs also contain amyloplasts and starchy substances, we propose that they might also be equipped with a complete ferredoxin-TRX system. We detected the presence of pea *FTR* together with *TRX f* and *TRX m* mRNAs in leaves, stems, roots, flowers, and seeds, and suggest that in nonphotosynthetic tissues FTR is probably the enzyme that reduces TRXs. However, it will be necessary to identify other components of the ferredoxin-TRX system in roots and

flowers before more can be said about the redox mechanisms through TRXs in the chloroplast of these organs. A search of Genevestigator microarray gene expression databases revealed similar patterns of expression of Arabidopsis chloroplastic *TRXs* in the tissues and organs assayed in our experiments (Zimmermann et al., 2004) and thus supported the new localizations we found for these proteins.

In relation with plastidial TRX functions, the identification of potential protein targets in wheat endosperm amyloplasts has helped to determine the possible roles of the disulfide protein in heterotrophic organs. These proteins are classified according to the process in which they are involved (Balmer et al., 2006b). Among 289 proteins, we found proteins involved in starch and carbohydrate metabolism, lipid and amino acid biosynthesis, amino acid metabolism, and protein assembly and folding. In addition to AGPase, other protein targets have been biochemically linked to TRX (e.g. acetyl-CoA carboxylase [Sasaki et al., 1997], cyclophilin [Motohashi et al., 2003], peroxiredoxin BAS1 [König et al., 2002], protein disulfide isomerase [Danon and Mayfield, 1994; Kim and Mayfield, 2002], and thiosulfate sulfurtransferase [Ray et al., 2000]). In nonphotosynthetic tissues, chloroplastic TRXs appear to be related with carbohydrate and lipid metabolism and amino acid synthesis, DNA replication and translation, protein folding, and protein storage mobilization, rather than with photosynthesis.

In conclusion, this work provides strong evidence that TRX *f* and TRX *m* proteins are localized in roots, vascular tissues, seeds, and pollen grains and embryos in reproductive organs, and seems to participate in all stages of plant development. In addition, our results strongly suggest that chloroplastic TRX *f* and TRX *m* are involved in cell division and plant reproduction. By analyzing *TRX f* and *TRX m* promoters we were able to localize several functional domains necessary for light-dependent expression and negative regulation of tissue-specific expression of *TRX m* in the hypocotyls of Arabidopsis plants. Future work will concentrate on (1) analyzing the specific function of these proteins in nonphotosynthetic tissues to elucidate their role in different organs; (2) identifying specific protein targets for each plastidial TRX in plant parts that have yet to be studied, especially in heterotrophic tissues and flowers; and (3) assigning definite physiological functions to these proteins in different stages of development and growth of the plant. It is hoped that our results will contribute to the understanding of the physiological roles of other TRXs in different parts of the plant.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum* var. Lincoln) leaves, stems, roots, flowers, and seeds were used in protein, DNA, and RNA extraction and for in situ hybridization experiments. Plants were grown in a green cabinet for 15 d, first in vermiculite, and then in compost until flowering.

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia and transgenic seeds were germinated in compost and vermiculite (compost:vermiculite [2:1]) under controlled environmental conditions: 140 μmol photons m^{-2} s^{-1} photosynthetically active radiation; 23°C temperature; 16-h-light/8-h-dark photoperiod, and daily watering with a nutrient solution for Arabidopsis. For in vitro germination, we used Murashige and Skoog medium described by Murashige and Skoog (1962) supplemented with vitamins and the appropriate antibiotic (kanamycin) for the transgenic lines.

Isolation of Promoter DNA

The promoters of the *TRX f* and *TRX m* genes were isolated by PCR walking according to the protocol described by Devic et al. (1997). Approximately 2.5 μg of pea genomic DNA were digested overnight with 50 units of each enzyme at a final volume of 100 μL. Seven restriction enzymes (*Eco*RV, *Hind*III, *Sca*I, *Pvu*II, *Dra*I, *Hpa*I, and *Stu*I) to create blunt-ended fragments were used independently for library construction. The adaptor was ligated to genomic DNA overnight at 16°C in the presence of 10 units of T4 DNA ligase (Promega) in a final reaction volume of 20 μL. Adaptor primers were AP1, 5'-GGATCCTAATACGACTCACTATAGGGC-3'; and AP2, 5'-CTATAGGGC-TCGAGCGGC-3'. For nested PCR reactions, Long Expand Taq polymerase mix (Boehringer) was used in combination with oligonucleotides specific for *TRX f* and *TRX m*. For the *TRX f* promoter, we used F1, 5'-ACCTCTGTAGACC-AACCCTCTTCG-3'; and F2, 5'-GGTGCCGATCCATTTAGGGGAGGTG-3'. For the *TRX m* promoter, we used M1, 5'-GGAAGACAATGAGGTCTTG-GTGTGG-3'; and M2, 5'-ACTCTTAAACAAGCTCTCAAGGGCC-3'.

The primary PCR cycling parameters were seven cycles at 94°C for 1 min and 72°C for 4 min, then 32 cycles at 94°C for 1 min and 65°C for 4 min, and the oligos used were AP1 and F1 for *TRX f* and AP1 and M1 for *TRX m*. For the second PCR, the program was five cycles at 94°C for 1 min and 72°C for 4 min, then 25 cycles at 94°C for 1 min and 65°C for 4 min, and the oligos were AP2 and F2 for *TRX f* and AP2 and M2 for *TRX m*. The fragments obtained were subcloned into the plasmid pGEM-T Easy vector (Promega) for sequencing.

Database-Assisted Promoter Analysis

Putative cis-regulatory sequences were sought with bioinformatics approaches using two databases that identified transcription factor binding sites or cis-acting sequences in plant promoters: PLACE (http://www.dna.affrc. go.jp/htdocs/PLACE; Higo et al., 1999) and Plant CARE (http://sphinx.rug. ac.be:8080/PlanCARE/index.html; Rombauts et al., 1999). We also analyzed the promoter regions of the two TRXs *f* and four TRXs *m* of Arabidopsis located in the TAIR database. To analyze the Genevestigator databases for microarray gene expression, we used the tools at https://www.genevestigator. ethz.ch (Zimmermann et al., 2004).

RNA Extraction and Transcription Start Sites

The transcription start point was determined by reverse transcriptase-mediated RACE-PCR with a FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instructions. Total RNA (12 μg) extracted from pea leaves was isolated with TRIzol (BD) and used for RT. 5'-RACE-PCR was done in two steps with nested gene-specific inner and outer primers (F1 and F2 for *TRX f*; M1 and M2 for *TRX m*) and nested primers from the 5'-RACE adaptor provided in the kit. Annealing temperatures for the reactions were 60°C (outer) and 65°C (inner). For sequencing, the PCR products were cloned into pGEM-T Easy vector (Promega).

RT and Semiquantitative PCR Analysis

First-strand cDNA was synthesized from 2.5 μg of total RNA using Super-Script III reverse transcriptase (Invitrogen) and oligo(dT)$_{12-15}$ primer. The reaction was incubated at 50°C for 1 h. To analyze expression, we used the pea actin gene as a housekeeping gene. The PCR reaction conditions were 2 min at 94°C, 30 cycles at 92°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 5 min. The specific oligonucleotides were 5'-TGATAAAACCGTCGTCCTCGAT-3' for Trxf-F, 5'-ATTTCCTCATCTTCCCCTCAGC-3' for Trxf-R, 5'-GTTTC-ACTTCGCTGGTGTTGC-3' for Trxm-F, 5'-CTTTCTCAGACAGAGTAGCC-3' for Trxm-R, 5'-CAAGCTTCAACCTTCGCCGTC-3' for FTR-F, 5'-TGGCA-CTCCTTTCTCTCTCTC-3' for FRT-R, 5'-AATGGTGAAGGCTGGATTTG-3' for Act-F, and 5'-AGCAAGATCCAAACGAAGGA-3' for Act-R.

GUS Reporter Gene Construction and Expression in Transgenic Arabidopsis Plants

Full-length fragments were amplified by PCR between positions ATG and −444 for *TRX f* and between ATG and −1,874 for *TRX m* with oligonucleotides bearing appropriate restriction sites (SalI and BamHI), and subcloned into the pBI101 vector to produce the constructions *PsTRXf1* and *PsTRXm1*. Genomic fragments of 444 bp (*TRX f*) and 1,973 bp (*TRX m*) were used to construct a series of 5′ deletions of the two chloroplastic *TRX* promoters fused to the GUS reporter gene. One 5′ deletion fragment for the *TRX f* promoter extending from positions ATG to −126 (*PsTRXf2*) and four 5′ deletion fragments for the *TRX m* promoter from positions ATG to −1,521 (*PsTRXm2*), ATG to −503 (*PsTRXm3*), ATG to −138 (*PsTRXm4*), and ATG to +23 (*PsTRXm5*) were also produced by DNA amplification with Long Expand Taq polymerase mix. To obtain the deleted promoter fragments by PCR, we used the following primers (Fig. 1): prTrxf1, 5′-GT**GTCGAC**GTAAACAAATAAACCACAT-3′; prTrxf2, 5′-GT*GGATCC*CATTGATGTGGGGAAGA-3′; prTrxf3, 5′-GT**GTCGAC**CCA-TACACAGACTTCAA-3′; prTrxm1, 5′-GT**GTCGAC**CTCCTTGGTGCCTCT-CAA-3′; prTrxm2, 5′-GT*GGATCC*CATGTTATTTTCTGTGTAT-3′; prTrxm3, 5′-GT**GTCGAC**GTTCATACTTTTTCCCT-3′; prTrxm4, 5′-GT**GTCGAC**G-TGTTTAGGGTACATTC-3′; prTrxm5, 5′-GT**GTCGAC**GTGTGGAGAAGTA-CTT-3′; and prTrxm6, 5′-GT**GTCGAC**CCGCCACAACACTAG-3′. Restriction sites are shown in bold (SalI) and italics (BamHI).

PCR products were ligated in-frame to the GUS reporter gene in the binary vector pBI101 using the restriction sites SalI and BamHI. Seven constructs were generated: *PsTRXf1*::GUS (444 bp), *PsTRXf2*::GUS (126 bp), *PsTRXm1*::GUS (1973 bp), *PsTRXm2*::GUS (1642 bp), *PsTRXm3*::GUS (624 bp), *PsTRXm4*::GUS (259 bp), and *PsTRXm5*::GUS (89 bp; Fig. 1). The promoter region of all constructs was confirmed by sequencing. Plasmids were introduced into *Agrobacterium tumefaciens* C58C1Rif and used to transform Arabidopsis plants by the floral-dip method (Bechtold et al., 1993). Seeds from infiltrated plants were sterilized for 1 min with 70% ethanol and 5 min with a mixture of 5% sodium hypochlorite and 2.5% Tween 20, and then rinsed three times with sterile water and germinated on Murashige and Skoog medium with the addition of 50 μg mL^{-1} kanamycin. Seedlings were grown in a light cabinet at 23°C and a 16-h-light/8-h-dark photoperiod, and the kanamycin-resistant plantlets were transplanted individually to sterile compost. Primary transformants were selfed and the homozygous seeds from the second or third generations (T2 or T3) were used for later experiments after cultivation under similar conditions. Arabidopsis plants carrying the pBI101 vector without promoter were used as a negative control for GUS expression. For the GUS assay, plants were grown in Murashige and Skoog medium supplemented with Suc, agar, and kanamycin in a light cabinet or in sterile compost in a greenhouse under a 16-h-light/8-h-dark regime. Samples were taken either from in vitro experiments or from soil every 4 h during the day and every 8 h during the night.

GUS Expression Analysis

Histochemical staining for GUS in transgenic Arabidopsis plants was performed according to standard procedures with minor modifications (Jefferson et al., 1987). Histochemical in situ localization of GUS expression was performed on five independent transgenic lines of the *PsTRXf1*::GUS and *PsTRXm1*::GUS constructs, and on two independent lines of each of the deletions. One kanamycin-resistant plant for the pBI101 binary vector was used as a control.

Localization of *TRX f* and *TRX m* Expression

Histochemical staining for GUS was performed at 37°C by incubating the material in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide as a substrate, 50 mM potassium phosphate, pH 7, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% (v/v) Tween 20, and 0.1% (v/v) Triton X-100. After staining, plants were incubated in 70% (v/v) ethanol at 37°C overnight. Plant materials used were either early seedlings grown on plates or different growth stages of the rosette and flowers of 4- to 5-week-old plantlets grown in soil. Pigments were extracted from stained tissues with ethanol:acetic acid (1:1). After extensive washing, the clarified tissues were stored in 50% (v/v) glycerol until photodocumentation. Micrographs were taken with an Olympus AX-70 microscope. Stained leaves were embedded in Unicryl (British BioCell International); semithin sections were obtained with a Leica RM2164 microtome. Sections were counterstained with toluidine blue or safranine dyes and observed with a Zeiss Axioplan microscope equipped with a CCD and micrographs were taken with a Canon PowerShot S40 camera.

In Situ Hybridization

In situ hybridization was performed according to earlier methods (Ferrandiz et al., 2000). The plasmids *pTrxf* and *pTrxm*, containing the open reading frame of *TRX f* and *TRX m*, respectively, were used to generate sense and antisense probes for in situ hybridization. Sense and antisense probes were labeled with digoxigenin-11-UTP (Roche Molecular Biochemical) according to the manufacturer's instructions. For the sense probe, *pTrxf* and *pTrxm* were linearized with *Sac*II (*Ksp*I); for the antisense probes, *pTrxf* was linearized with *Sal*I and pTrxm was linearized with *Sac*I. The linearized probes were transcribed with either T7 RNA polymerase (*TRX f* sense and *TRX m* antisense) or SP6 RNA polymerase (*TRX f* antisense and *TRX m* sense). Tissue samples were fixed in FAE solution (ethanol:acetic acid:formaldehyde:water at 50:5:3.7:41.3 [v/v/v/v]), dehydrated, embedded in paraffin, and sectioned at 10 μM. Sections were hybridized with the different probes overnight at 50°C and incubation with the antibody and color detection were done according to the manufacturer's instructions (Roche). Micrographs were taken with an Olympus BX51 microscope and an Olympus DP50 camera.

Protein Extraction and Western Blotting

Flowers, seeds, leaves, roots, and pods were homogenized with 25 mM Tris-HCl, pH 7.5, and 5 mM MgCl$_2$ (1:3 [w/v]). After 20 min of centrifugation at 12,000g, the supernatant was removed and the protein concentration determined with the Bradford assay (Bio-Rad), with bovine serum albumin as the standard (Bradford, 1976). Electrophoresis in polyacrylamide gel was performed according to Laemmli (1970), proteins were transferred to a nitrocellulose membrane, and western blotting was performed as in Towbin et al. (1979), using polyclonal antibodies against pea-leaf chloroplastic TRX *m* diluted 1:1,000 or TRX *f* diluted 1:1,000 (v/v). Bound antibody was visualized with enhanced chemiluminescence (ECL system; Amersham Biosciences).

ACKNOWLEDGMENTS

LITERATURE CITED

Arner ES, Holmgren A (2000) Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem **267:** 6102–6109

Ballicora MA, Frueauf JB, Fu Y, Schürmann P, Preiss J (2000) Activation of the potato tuber ADP-glucose pyrophosphorylase by thioredoxin. J Biol Chem **275:** 1315–1320

Balmer Y, Koller A, del Val G, Manieri W, Schürmann P, Buchanan BB (2003) Proteomics gives insight into the regulatory function of chloroplast thioredoxins. Proc Natl Acad Sci USA **100:** 370–375

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. Proc Natl Acad Sci 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. J Exp Bot **57:** 1591–1602

Balmer Y, Vensel WH, Tanaka CK, Hurkman WJ, Gelhaye E, Rouhier N, Jacquot JP, Manieri W, Schürmann P, Droux M, et al (2004) Thioredoxin links redox to the regulation of fundamental processes of plant mitochondria. Proc Natl Acad Sci USA **101:** 2642–2647

Bassham JA, Krause GH (1969) Free energy changes and metabolic regulation in steady-state photosynthetic carbon reduction. Biochim Biophys Acta **189:** 207–221

Bechtold N, Ellis J, Pelletier G (1993) In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Ser III Sci Vie **316:** 1194–1199

Besse I, Wong JH, Kobrehel K, Buchanan BB (1996) Thiocalsin: a thioredoxin-linked, substrate-specific protease dependent on calcium. Proc Natl Acad Sci USA **93:** 3169–3175

Bower MS, Matias DD, Fernandes-Carvalho E, Mazzurco M, Gu T, Rothstein SJ, Goring DR (1996) Two members of the thioredoxin-h family interact with the kinase domain of a Brassica S locus receptor kinase. Plant Cell **8:** 1641–1650

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brugidou C, Marty I, Chartier Y, Meyer Y (1993) The *Nicotiana tabacum* genome encodes two cytoplasmic thioredoxin genes which are differently expressed. Mol Gen Genet **238:** 285–293

Cabrillac D, Cock JM, Dumas C, Gaude T (2001) The S-locus receptor kinase is inhibited by thioredoxins and activated by pollen coat proteins. Nature **410:** 220–223

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*. Physiol Plant **84:** 236–242

Collin V, Issakidis-Bourget E, Marchand C, Hirasawa M, Lancelin JM, Knaff DB, Miginiac-Maslow M (2003) The Arabidopsis plastidial thioredoxins: new functions and new insights into specificity. J Biol Chem **278:** 23747–23752

Collin V, Lamkemeyer P, Miginiac-Maslow M, Hirasawa M, Knaff DB, Dietz KJ, Issakidis-Bourguet E (2004) Characterization of plastidial thioredoxins from Arabidopsis belonging to the new y-type1. Plant Physiol **136:** 4088–4095

Crawford NA, Yee BC, Nishizawa AN, Buchanan BB (1979) Occurrence of cytoplasmic *f*- and *m*-type thioredoxins in leaves. FEBS Lett **104:** 141–145

Danon A, Mayfield SP (1994) Light-regulated translation of chloroplast messenger RNAs through redox potential. Science **266:** 1717–1719

Devic M, Albert S, Delseny M, Roscoe TJ (1997) Efficient PCR walking on plant genomic DNA. Plant Physiol Biochem **35:** 331–339

Elmayan T, Tepfer M (1995) Evaluation in tobacco of the organ specificity and strength of the rol D promoter, domain A of the 35S promoter and the 35S^2 promoter. Transgenic Res **4:** 388–396

Ferrandiz C, Gu Q, Martienssen R, Yanofsky MF (2000) Redundant regulation of meristem identity and plant architecture by FRUITFULL, APETALA1 and CAULIFLOWER. Development **127:** 725–734

Geck MK, Larimer FW, Hartman FC (1996) Identification of residues of spinach thioredoxin *f* that influence interactions with target enzymes. J Biol Chem **271:** 24736–24740

Geigenberger P, Kolbe A, Tiessen A (2005) Redox regulation of carbon storage and partitioning in response to light and sugars. J Exp Bot **56:** 1469–1479

Gilmartin PM, Sarokin L, Memelink J, Chua NH (1990) Molecular light switches for plant genes. Plant Cell **2:** 369–378

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic Acids Res **27:** 297–300

Hodges M, Miginiac-Maslow M, Decottignies P, Jacquot JP, Stein M, Lepiniec L, Crétin C, Gadal P (1994) Purification and characterization of pea thioredoxin *f* expressed in *Escherichia coli*. Plant Mol Biol **26:** 225–234

Holmgren A (1985) Thioredoxin. Annu Rev Biochem **54:** 237–271

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

Kim J, Mayfield SP (2002) The active site of the thioredoxin-like domain of chloroplast protein disulfide isomerase, RB60, catalyzes the redox-regulated binding of chloroplast poly(A)-binding protein, RB47, to the 5′ untranslated region of psbA mRNA. Plant Cell Physiol **43:** 1238–1243

Kobrehel K, Wong JH, Balogh A, Kiss F, Yee BC, Buchanan BB (1992) Specific reduction of wheat storage proteins by thioredoxin *h*. Plant Physiol **99:** 919–924

König J, Baier M, Horling F, Kahmann U, Harris G, Schürmann P, Dietz KJ (2002) The plant-specific function of 2-Cys peroxiredoxin-mediated detoxification of peroxides in the redox-hierarchy of photosynthetic electron flux. Proc Natl Acad Sci USA **99:** 5738–5743

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

Laloi C, Mestres-Ortega D, Marco Y, Meyer Y, Reichheld JP (2004) The Arabidopsis cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. Plant Physiol **134:** 1006–1016

Lam E, Chua NH (1989) ASF-2: a factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in Cab promoters. Plant Cell **1:** 1147–1156

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

Marx C, Wong JH, Buchanan BB (2003) Thioredoxin and germinating barley: targets and protein redox changes. Planta **216:** 454–460

Mestres-Ortega D, Meyer Y (1999) The *Arabidopsis thaliana* genome encodes at least four thioredoxins *m* and a new prokaryotic-like thioredoxin. Gene **240:** 307–316

Meyer Y, Reichheld JP, Vignols F (2005) Thioredoxins in Arabidopsis and other plants. Photosynth Res **86:** 419–433

Meyer Y, Vignols F, Reichheld JP (2002) Classification of plant thioredoxins by sequence similarity and intron position. Methods Enzymol **347:** 394–402

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 Physiol **132:** 1707–1715

Motohashi K, Kondoh A, Stumpp MT, Hisabori T (2001) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci USA **98:** 11224–11229

Motohashi K, Koyama F, Nakanishi Y, Ueoka-Nakanishi H, Hisabori T (2003) Chloroplast cyclophilin is a target protein of thioredoxin. Thiol modulation of the peptidyl-prolyl cis-trans isomerase activity. J Biol Chem **278:** 31848–31852

Mouaheb N, Thomas D, Verdoucq L, Monfort P, Meyer Y (1998) In vivo functional discrimination between plant thioredoxins by heterologous expression in the yeast *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA **95:** 3312–3317

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant **15:** 473–497

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. J Exp Bot **51:** 1299–1307

Ray WK, Zeng G, Potters MB, Mansuri AM, Larson TJ (2000) Characterization of a 12-kilodalton rhodanese encoded by glpE of *Escherichia coli* and its interaction with thioredoxin. J Bacteriol **182:** 2277–2284

Reichheld JP, Vernoux T, Lardon F, Van Montagu M, Inzé D (1999) Specific checkpoints regulate plant cell cycle progression in response to oxidative stress. Plant J **17:** 647–656

Rombauts S, Déhais P, Van Montagu M, Rouzé P (1999) Plant CARE, a plant cis-acting regulatory element database. Nucleic Acids Res **27:** 295–296

Sahrawy M, Hecht V, López-Jaramillo J, Chueca A, Chartier Y, Meyer Y (1996) Intron position as an evolutionary marker of thioredoxins and thioredoxin domains. J Mol Evol **42:** 422–431

Sasaki Y, Kozaki A, Hatano M (1997) Link between light and fatty acid synthesis: thioredoxin-linked reductive activation of plastidic acetyl-CoA carboxylase. Proc Natl Acad Sci USA **94:** 11096–11101

Schürmann P, Jacquot JP (2000) Plant thioredoxin systems revisited. Annu Rev Plant Physiol Plant Mol Biol **51:** 371–400

Shirsat A, Wilford N, Croy R, Boulter D (1989) Sequences responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. Mol Gen Genet **215:** 326–331

Sparla F, Costa A, Lo Schiavo F, Pupillo P, Trost P (2006) Redox regulation of a novel plastid-targeted β-amylase of Arabidopsis. Plant Physiol **141:** 840–850

Tiessen A, Hendriks JH, Stit M, Branscheid A, Gibon Y, Farré 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. Plant Cell **14:** 2191–2213

Tiessen A, Prescha K, Bransheid A, Palacios N, McKibbin R, Halford NG, Geigenberger P (2003) Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating

post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. Plant J **35:** 490–500

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

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 Physiol **143:** 300–311

Weichel M, Glaser AG, Ballmer-Weber BK, Schmid-Grendelmeier P, Crameri R (2006) Wheat and maize thioredoxins: a novel cross-reactive cereal allergen family related to baker's asthma. J Allergy Clin Immunol **117:** 676–681

Wong JH, Cai N, Balmer Y, Tanaka CK, Vensel WH, Hurkman WJ, Buchanan BB (2004) Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. Phytochemistry **65:** 1629–1640

Wong JH, Kim YB, Ren PH, Cai N, Cho MJ, Hedden P, Lemaux PG, Buchanan BB (2002) Transgenic barley grain overexpressing thioredoxin shows evidence that the starchy endosperm communicates with the embryo and the aleurone. Proc Natl Acad Sci USA **99:** 16325–16330

Yanagisawa S (2000) Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize. Plant J **21:** 281–288

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis Microarray Database and Analysis Toolbox. Plant Physiol **136:** 2621–2632