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Evidence for five divergent thioredoxin *h* sequences in *Arabidopsis thaliana*

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ABSTRACT **Five different clones encoding thioredoxin homologues were isolated from *Arabidopsis thaliana* cDNA libraries. On the basis of the sequences they encode divergent proteins, but all belong to the cytoplasmic thioredoxins *h* previously described in higher plants. The five proteins obtained by overexpressing the coding sequences in *Escherichia coli* present typical thioredoxin activities (NADP$^+$–malate dehydrogenase activation and reduction by *Arabidopsis* thioredoxin reductase) despite the presence of a variant active site, Trp-Cys-Pro-Pro-Cys, in three proteins in place of the canonical Trp-Cys-Gly-Pro-Cys sequence described for thioredoxins in prokaryotes and eukaryotes. Southern blots show that each cDNA is encoded by a single gene but suggest the presence of additional related sequences in the *Arabidopsis* genome. This very complex diversity of thioredoxins *h* is probably common to all higher plants, since the *Arabidopsis* sequences appear to have diverged very early, at the beginning of plant speciation. This diversity allows the transduction of a redox signal into multiple pathways.**

Thioredoxins are small proteins (105–120 aa) that contain the strictly conserved sequence Trp-Cys-Gly-Pro-Cys (1). The reduced cysteine pair forms a very reactive center able to disrupt the disulfide bridge of target proteins. *In vivo*, thioredoxins are reduced by a thioredoxin reductase. Thioredoxins have been isolated from prokaryotes and eukaryotes, including vertebrates, invertebrates, and fungi (2, 3, 41). Higher plants present two independent thioredoxin systems: the chloroplastic system consists of two nuclear gene-encoded proteins, thioredoxins *m* and *f*, which are reduced by a ferredoxin-dependent thioredoxin reductase (4, 5), and regulates the dark/light-related cycles (6, 7). In addition, the presence of a thioredoxin *h* system that is reduced by an NADP-dependent thioredoxin reductase was demonstrated some years ago in heterotrophic plant tissues (8, 9), but the first sequences for plant thioredoxins *h* (10–13) and NADP-dependent thioredoxin reductase (14) were obtained only recently. In this paper, we show that the *Arabidopsis thaliana* genome encodes at least five very divergent thioredoxin *h* genes,§ a situation similar to the one of *Dictyostelium discoideum* (15) but very different from what is usually observed in other organisms. In addition, the sequences of three of them feature a variant active site, Trp-Cys-Pro-Pro-Cys, which does not alter their *in vitro* thioredoxin activity.

MATERIALS AND METHODS

Plant Material. *A. thaliana* (Landsberg erecta) plants were grown in a greenhouse. Tissues were frozen in liquid nitrogen and stored at −80°C. *A. thaliana* cell suspensions (a gift of Michele Axelos, Centre National de la Recherche Scientifique, Toulouse) were subcultured every 2 weeks by 10-fold dilution in B_5 medium (42) with sucrose (20 mg/ml) and 2,4-dichlorophenoxyacetic acid (1 μg/ml) and collected after 3 days of subculture (growth phase). Primary calli (seeds germinated on the callus medium) and established calli (the cell suspension grown on the callus medium) were grown for 1 month on the same medium solidified with 0.8% agar. *In vitro* plantlets were obtained from seeds germinated and cultivated *in vitro* for 1 month on the callus medium lacking 2,4-dichlorophenoxyacetic acid. Mesophyll protoplasts were isolated from fully expanded leaves from greenhouse plants.

DNA and RNA Isolation. Genomic DNA was extracted by the CsCl–BET procedure (16). Total RNA was isolated from leaves according to Shirzadegan *et al.* (17).

Southern and Northern Blots. For Southern blot analysis, samples (3 μg) of genomic DNA were digested with *Eco*RI or *Eco*RV restriction endonuclease. The DNA fragments were separated in 0.8% agarose gels and electroblotted to nylon membranes (Hybond-N; Amersham) in 20× SSC (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7). Blots were washed twice in 2× SSC at room temperature for 20 min. When needed, a stringent wash was made with 2× SSC/0.1% SDS at 65°C for 10 min.

For Northern blot analysis, samples (10 μg) of total RNA were electrophoresed in 1.2% agarose/formaldehyde gels, and the size-separated RNAs were blotted to Hybond-N membranes and hybridized with probes according to Amersham specifications.

PCR Preparation of DNA Probes. T7 and T3 primers were used for complete cDNA probes and specific oligonucleotide primers were used for 3′ probes. For TRX1–TRX5, we used, respectively, fragments of 115, 106, 72, 200, and 65 bp located 8, 67, 79, 22, and 62 bp downstream from the translational stop. Fragments were amplified in two steps. The first PCR volume was 50 μl, containing 1 ng of thioredoxin *h* pBluescript template DNA, 50 pmol of each primer, 1.5 mM $MgCl_2$, 120 μM each dNTP, and 3 units of *Taq* polymerase, covered with 25 μl of mineral oil. Initial denaturation at 93°C for 1 min was followed by 30 cycles of denaturation at 93°C for 3 min, annealing at 42°C for 2 min, and extension at 72°C for 2 min. The products of the first PCR were purified by agarose gel electrophoresis and use of a Geneclean II kit (Bio 101). The second PCR amplification was carried out with 0.6 pmol each of dGTP, dATP, and dTTP; 0.4 pmol of dCTP; and 50 μCi of [α-^{32}P]dCTP (Amersham catalogue no. PB10205; 1 Ci = 37 GBq) under the same conditions but for only 15 cycles.

cDNA Sequencing. cDNA libraries were originally prepared in pBluescript (Stratagene) by *Eco*RI–*Eco*RI or *Eco*RI–*Xho* I ligation. The nucleotide sequences of both strands were determined by the dideoxynucleotide chain-termination method (18) using Sequenase kits (United States Biochemical) and

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. Z35473–Z35476).

double-stranded DNA templates. Sequence analyses were done with DNA STRIDER 1.1 (19), alignments were achieved with LFASTA (20), and the phylogenetic tree was obtained by use of DARWIN (21).

RESULTS

Isolation of Five cDNAs Encoding Thioredoxin *h*. Using a tobacco probe (11), we isolated one *A. thaliana* clone encoding a thioredoxin *h* (13). Later, the *A. thaliana* cDNA sequencing program (22) led to the characterization of four expressed sequence tags showing similarities with the tobacco and *A. thaliana* thioredoxins *h* in the coding part of the tag. We have completed the sequencing of the clones, which were named TRX1 (Z14084), TRX2 (Z35475), TRX3 (Z35474), TRX4 (Z35473), and TRX5 (Z35476). Each one has an open reading frame of 110–138 aa. The deduced amino acid sequences have 60–70% identity with one another and with the two tobacco sequences, 45–50% with the *Chlamydomonas reinhardtii* thioredoxin *h*, 40% with the human thioredoxin, and 30% with the *Escherichia coli* and chloroplastic thioredoxins. They have no transit peptide, suggesting that they represent thioredoxins located in the cytosol. Fig. 1 shows a multiple alignment of the *A. thaliana* thioredoxin *h* proteins, other plant thioredoxin *h*-homologous molecules available in the GenBank and Protein Identification Resource (PIR) databases, and human and *E. coli* sequences as examples of vertebrates and prokaryotes. The N-terminal parts of the *E. coli* enzyme and thioredoxins *h* appear to be very different, with similarities detected only starting from Ile^{24} of the *E. coli* sequence. Two conserved regions are potentially important in all thioredoxins (1). The first is the protruding loop containing the redox-active cysteines in the consensus sequence Trp-Cys^{32}-Gly-Pro-Cys^{35} (residue numbers correspond to the *E. coli* sequence). This motif is almost completely conserved in thioredoxins (the only two exceptions being a prokaryotic sequence, *Clostridium nephridii* C2, and the partial sequence of spinach root thioredoxin *h*, both of which read Trp-Cys-Ala-Pro-Cys). Interestingly, the canonical sequence is replaced by Trp-Cys-Pro-Pro-Cys in three of the *A. thaliana* clones and in the partial maize sequence. The second conserved area is a flat hydrophobic surface of amino acids from different locations in the primary structure (Gly^{33}, Pro^{34}, Pro^{76}, Gly^{92}, and Ala^{93}) which is thought to mediate the interaction of thioredoxins with other proteins (1, 2, 23). In addition, other amino acids (Asp^{26}, Phe^{27}, Asp^{61}, Thr^{77}, Lys^{82}, and Gly^{84}) highly conserved in thioredoxins are present in the *Arabidopsis* sequences, suggesting that the five clones indeed encode thioredoxins. Some amino acids are common to higher plant thioredoxins *h* and animal thioredoxins (Lys^{48}, Asp^{59}, Met^{75}, and Lys^{105}) or found only in plant *h* (Val^{0}, Trp^{9}, Leu^{63}, and Ala^{74}) (numbered according to the *E. coli* sequence).

The Five Proteins Show Thioredoxin Activity *in Vitro*. The five open reading frames were inserted by PCR into pET vectors and the resulting constructions used to transform *E. coli* BL21 as described (14). The recombinant proteins were purified to homogeneity by a procedure to be described elsewhere. As frequently observed with thioredoxins of different sources, the production in *E. coli* was very successful, with yields ranging from 5 to 60 mg of purified protein per liter of culture after a 3-hr induction. The five proteins were active in the nonspecific insulin reduction test (data not shown). All five were also efficient in the activation of NADP^{+}–malate dehydrogenase (Table 1) in spite of the replacement of Gly^{33} by Pro in TRX3, TRX4, and TRX5. These three were, however, much less active in this reaction than chloroplastic thioredoxins *m* from either pea or spinach. Glutaredoxin, whose active site is Cys-Pro-Tyr-Cys, is totally inactive in this test. Further, all the proteins could be reduced with high efficiencies by the NADP–thioredoxin reductase from *Arabidopsis* (14), which preferentially reduces cytosolic thioredoxins. A notable exception is TRX4, which shows a higher K_m in this test, the V_{max} being unaffected.

Thioredoxin *h* Genes Have Diverged Very Early. We have produced a gene tree (Fig. 2), using DARWIN (21). In contrast to other programs which compare sequence pairs on the basis of alignment scores obtained by using a fixed PAM matrix, DARWIN determines the PAM matrix which optimizes the score and uses this PAM value and its variance as an estimation of the evolutionary distance between sequences. This approach allows a simple comparison of evolutionary distances, independent of sequence length. Applied to vertebrate thioredoxins, this method groups the mammalian thioredoxins together, while the chicken sequence is slightly more distant, suggesting that the method works well. This is confirmed by the association of the two available plant chloroplast thioredoxins *m* and the algal thioredoxins *m* within the prokaryotic sequences, whereas thioredoxin *f* forms a separate group of

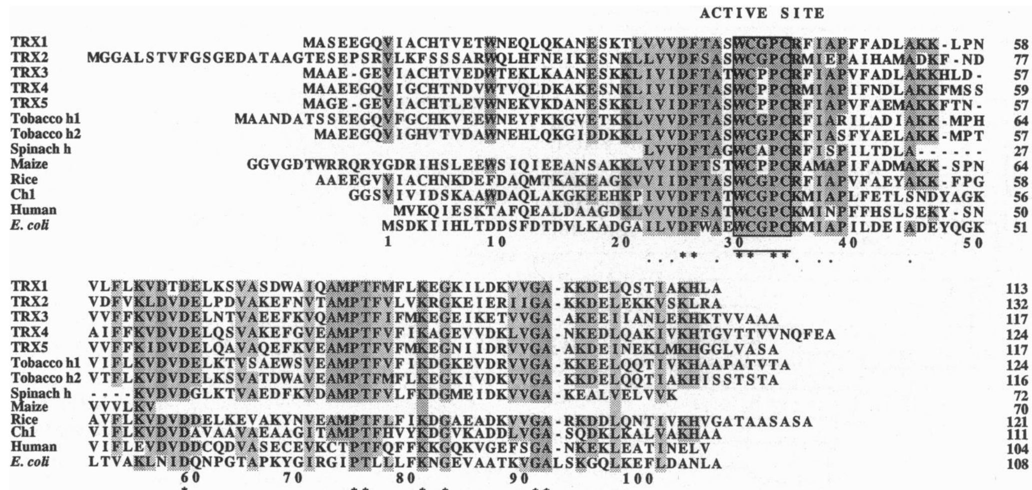

FIG. 1. Multiple alignment of plant thioredoxin *h* proteins. Identities between the predicted amino acid sequence of the *Arabidopsis* thioredoxin *h* proteins and the predicted amino acid sequences of tobacco thioredoxins *h*1 (P29449) and *h*2 (Z11803), the partial sequence of spinach thioredoxin *h* (PIR S15137), and the sequences of maize (U06108), rice (D21833), human (P10594), *C. reinhardtii* Ch1 (P80028), and *E. coli* (M54881) thioredoxins. Asterisks indicate amino acids conserved in all the sequences, dots indicate amino acids with similar biochemical characteristics, and dashes represent gaps introduced to optimize the alignments. Amino acid numbering is based on the *E. coli* sequence.

Table 1. Reactivities of the various recombinant thioredoxins from *Arabidopsis* with NADP$^+$-thioredoxin reductase (NTR) from *Arabidopsis* and with sorghum NADP$^+$-malate dehydrogenase (MDH)

Thioredoxin	NADP$^+$-MDH activation (DTT reductant) $S_{0.5}$, μM	Mutant NADP$^+$-MDH activation (NTR reductant) K_m, μM	DTNB assay (NTR reductant) K_m, μM
TRX1	14	4	2.0
TRX2	23	3	0.8
TRX3	24	2.1	0.7
TRX4	26	11	20
TRX5	11	1.2	1.6

The reactivity with the chloroplastic NADP$^+$-MDH was tested by the rate of activation of NADP$^+$-MDH by thioredoxins chemically reduced with dithiothreitol (DTT). This test, specific for chloroplastic thioredoxins, gave a half-saturation ($S_{0.5}$) of ≈5 μM with spinach thioredoxin *m* (24). The efficiency of reduction of thioredoxins by NTR was tested by the ability of reduced thioredoxins either to activate a mutant NADP$^+$-MDH (25) or to reduce 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (10). The NTR tests worked very poorly with chloroplastic *m*-type thioredoxin from pea, making it difficult to calculate K_m values.

different origin, as previously suggested (26, 27). All thioredoxins *h* are grouped together, near the vertebrate thioredoxins. In addition to the tobacco and *Arabidopsis* sequences obtained in our laboratory, we have used all thioredoxin *h* sequences available in GenBank or PIR in June 1994, including the partial spinach sequence obtained by protein sequencing, the maize expressed sequence tag (Dbest T155273), and a recently published (RICTH) rice sequence, as well as one unpublished wheat sequence (M. F. Gautier, Institut National de la Recherche Agronomique, Montpellier, personal communication). They include dicots and monocots and are clearly not grouped according to plant phylogeny. This indicates that the five *Arabidopsis* sequences diverged very early, even before the appearance of the monocots and dicots. This also suggests that most higher plants encode numerous thioredoxins *h* in their genome.

Thioredoxin *h* mRNA Levels are Developmentally Regulated. The presence and abundance of the mRNAs corresponding to the five clones TRX1–TRX5 were examined in various organs and tissues of *A. thaliana* by Northern blot analysis. Total RNA was isolated from agar-grown primary and established calli, cell suspensions isolated during the growth phase, freshly isolated mesophyll protoplasts, *in vitro* grown plantlets, and roots, stems, young leaves, mature leaves, siliques, flower buds, and flowers from greenhouse plants. Specific probes were prepared from cDNA encompassing the divergent 3′ noncoding sequences of the five cDNAs. Fig. 3 shows that they hybridized to mRNA species of approximately 1.5 kb for TRX1, 1.6 kb for TRX2, 0.9 kb for TRX3, 0.85 kb for TRX4, and 0.8 kb for TRX5. The five thioredoxin messages were abundant in the aerial plant organs such as young and mature leaves, siliques, flower buds, and flowers and less abundant in stems. In roots, mRNAs corresponding to TRX2, TRX4, and TRX5 were detected at very low levels, and TRX1 and TRX3 mRNAs were undetectable. In young plants grown *in vitro*, the five mRNAs were detectable at medium levels. In primary calli, only low levels of TRX2 and

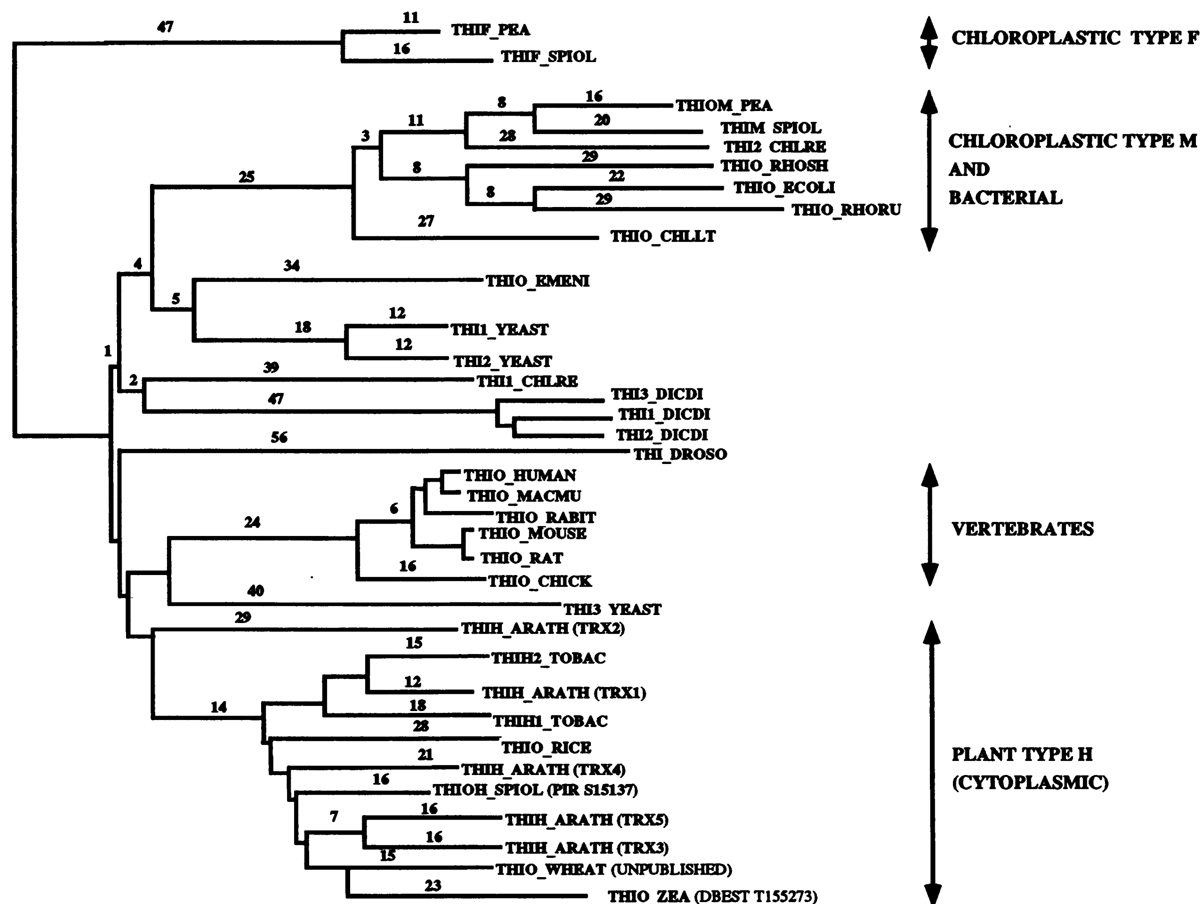

FIG. 2. Phylogenic tree of *A. thaliana* thioredoxins and related sequences from other organisms. The tree was obtained by use of DARWIN (21) and is based on the estimated PAM (accepted point mutation) distances between each sequence pair. Proteins are designated by their Swiss-Prot entry or by their accession number in PIR or Dbest, if they are not available in Swiss-Prot.

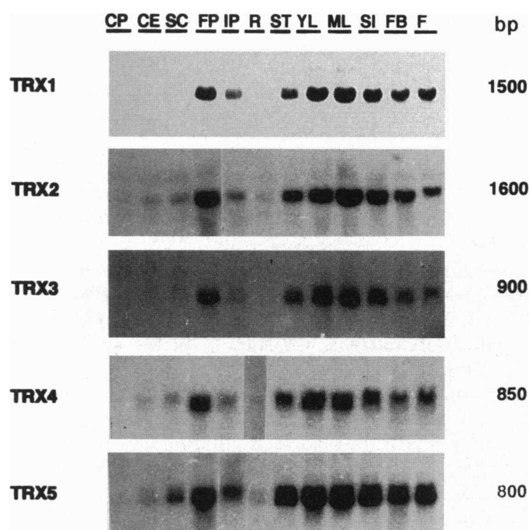

FIG. 3. Northern blot detection of five *A. thaliana* thioredoxin mRNAs. Samples (10 μg) of total RNA were isolated from the indicated sources: CP, primary callus (1 month old); CE, established callus (1 month old); SC, suspension-culture cells in growth phase (3 days old); FP, freshly isolated protoplasts; IP, *in vitro* grown plantlets (1 month old); R, roots; ST, stems; YL, young leaves; ML, mature leaves; SI, siliques; FB, flower buds; F, flowers from greenhouse grown plants. Blots were hybridized to α-^{32}P-labeled 3′ specific cDNA probes.

TRX5 were detected, whereas TRX2, TRX3, TRX4, and TRX5 were detected in established calli. All the transcripts were detected at low levels during the growth phase of the cell suspension. In freshly isolated protoplasts all the *A. thaliana* thioredoxin mRNAs were present at high levels, similar to those observed in leaves.

Genomic Organization of Thioredoxin *h* Sequences in *A. thaliana*. We performed Southern blot hybridizations on *Eco*RI and *Eco*RV digests of *A. thaliana* genomic DNA, using probes derived from 3′ noncoding sequence of the cDNAs to evaluate the number of genes encoding the different mRNAs. Only one band was obtained for each probe, suggesting the presence of only one gene for each mRNA. To investigate whether there are additional thioredoxin *h* genes in the *A. thaliana* genome, we performed similar hybridizations with

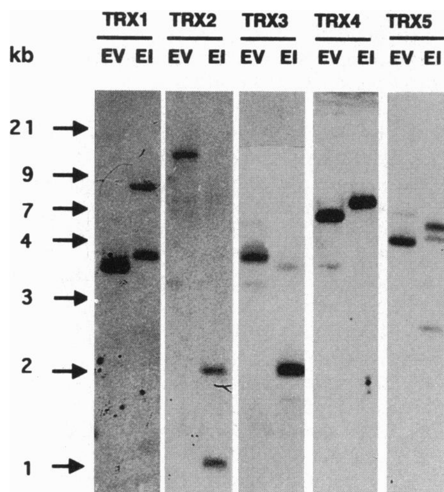

FIG. 4. Southern blot detection of thioredoxin genomic sequences. Samples (3 μg) of genomic DNA from *A. thaliana* were digested with *Eco*RI (EI) or *Eco*RV (EV), and the DNA fragments were separated in 0.8% agarose gels, transferred to a Hybond-N membrane, and hybridized with PCR-labeled cDNA probes. Molecular size markers were λ phage DNA fragments.

probes derived from the complete cDNAs (Fig. 4). The probes showed only weak or no cross-hybridization even at low stringency. However, the blots showed one strong additional band for the *Eco*RI digest of TRX1 and one for the *Eco*RI digest of TRX2, when compared with those obtained with the 3′ probes. This may be due to the presence of restriction sites present in introns (there are no *Eco*RI or *Eco*RV restriction sites within the cDNAs) or, alternatively, it may indicate the presence of additional thioredoxin *h* genes in the *A. thaliana* genome. Some faint bands may also reveal the presence of sequences related to thioredoxins.

DISCUSSION

The most spectacular result of this study is the presence of at least five very divergent thioredoxins *h* in the small genome of *A. thaliana.* We previously cloned two NADP–thioredoxin reductases from *A. thaliana* (14), confirming the complexity of the thioredoxin *h*/NADP–thioredoxin reductase equipment. Multiple thioredoxin genes have been reported only in fungi and higher plants. For example, the *D. discoideum* genome encodes at least three and perhaps up to five closely related thioredoxin genes (15), and *Saccharomyces cerevisiae* (28) has two related genes (YSCTRX1 and YSCTRX2) and one very distant thioredoxin (YCR83w) recently characterized by systematic sequencing. In spinach leaves two thioredoxins *h* have been characterized (8), and in tobacco two related nucleotide sequences have been described (12). The detection of only one gene in most prokaryotes and animals may be due to our limited knowledge of their genomes. Nevertheless, in spite of a considerable effort of random sequencing, the same human thioredoxin has been isolated independently in different experiments and is very closely related to the other vertebrate thioredoxins, suggesting that vertebrates have only one thioredoxin gene. On the other hand, an important sequencing program has been initiated in rice, leading to the isolation of one thioredoxin cDNA (RICTH) and of the corresponding gene (RICRTH). Amongs rice expressed sequence tags, two clones from a root library (RICSS396 and RICR24011A) and two from a callus library (RICC106901 and RIC23582A) are identical to the complete mRNA but two others (RICC28371 and RIC1378A) from callus present a good homology with plant thioredoxins *h*, including the Trp-Cys-Gly-Pro-Cys active site, but are nevertheless very divergent from the complete rice sequence and from each other. Although these clones should be characterized by a better and more complete sequencing, this supports our interpretation of the DARWIN tree that most higher plants present multiple and divergent thioredoxin genes.

This finding clearly raises the question of the function(s) of these different thioredoxins. Thioredoxin mutants in *E. coli* are not able to assimilate sulfate (29). The same phenotype has been observed in yeast after disruption of the two related thioredoxin genes. In addition, the double mutant shows reduced growth, due to a longer cell cycle with a particularly long S phase (28). These two yeast genes appear to be exchangeable, but recent studies suggest that *TRX2* plays a unique role after an oxidative stress (30). The recently characterized third gene has not been disrupted yet, and its function is unknown. In animals, high levels of thioredoxin mRNAs are frequently correlated with rapid cell proliferation (31), and it has been found that thioredoxin is an inducer of an interleukin 2 receptor (32). In addition, thioredoxin has been shown to participate in some developmental stages such as meiosis (3), embryo development (33), and pregnancy (34). The concept of redox regulation of transcription emerged from the study of Jun mutants and transcription factor NF-κB (35, 36), which bind DNA when they are in the reduced state, but not in their oxidized state. A reductant has been characterized as a bifunctional DNA repair/redox-active protein in insects, human, and *Arabidopsis* (37, 38). Clearly this protein should be

reactivated (reduced) and thioredoxin may play a role in the reduction cascade as shown *in vitro* (38).

In *Nicotiana tabacum*, in spite of some differences in the kinetics of appearance of the two mRNAs during *in vitro* culture (12), both thioredoxin *h*1 and *h*2 mRNAs are abundant in growing tissues but undetectable in mature nondividing cells (39), a situation similar to that observed in animals. Surprisingly, none of the five *A. thaliana* thioredoxin mRNAs follows the same rules: all are detectable in mature aerial organs. A possible explanation for this discrepancy is that the two tobacco thioredoxins and the five *Arabidopsis* thioredoxins may not be functional homologues, suggesting the presence of additional thioredoxin genes in the *A. thaliana* genome. Alternatively, thioredoxins may be related very indirectly to the developmental processes. In addition, it will be interesting to estimate the accumulation of the corresponding proteins, since mRNA accumulation is not necessarily correlated with protein accumulation.

The last important point is the demonstration that the variant sequence Trp-Cys-Pro-Pro-Cys is compatible with a thioredoxin *h* activity. This modified site is reminiscent of the glutaredoxin active site Cys-Pro-Try-Cys. Nevertheless the three variant sequences can be aligned with thioredoxins but not with glutaredoxins. Glutaredoxin sequences present a glutathione binding site (40), which seems to be absent from the three *Arabidopsis* sequences. In addition biochemical tests indicate that the five purified recombinant proteins are active in the dithiothreitol-dependent activation of $NADP^+$–malate dehydrogenase (although less efficiently than the chloroplastic thioredoxin *m* from pea or spinach), a reaction in which phage T4 glutaredoxin is totally inactive. This indicates that these proteins are indeed members of the thioredoxin family and not of the glutaredoxin family. Further, these proteins are reduced with high efficiencies by the specific reductase of cytosolic thioredoxins, whereas the chloroplastic thioredoxin *m* from pea is a very poor substrate for $NADP^+$–thioredoxin dehydrogenase. The data are in favor of an extrachloroplastic localization of all these thioredoxins. The presence of the same variant motif in the maize thioredoxin tag (Dbest T155273) suggests a very ancient origin of this motif and is an additional argument in favor of the hypothesis of divergence of the different thioredoxins *h* before the appearance of monocots and dicots.

In conclusion, the results presented in this paper and in our preceding work (14) show that *A. thaliana*, and most probably other higher plants, has a very complex NADP–thioredoxin reductase/thioredoxin *h* system. Since each individual thioredoxin characterized in this study allows the transduction of a redox signal, we infer each to be functional and we interpret this complexity to allow a greater versatility in the interaction with a diversity of substrates. In view of the recently discovered importance of redox regulation of various processes, including transcription, the presence of multiple cytoplasmic thioredoxins would allow plants to respond in different ways to changes in the energetic status of the cell.

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1. Holmgren, A. (1985) *Annu. Rev. Biochem.* **54**, 237–271.
2. Gleason, F. K. & Holmgren, A. (1988) *FEMS Microbiol.* **54**, 271–298.
3. Salz, H. K., Flickinger, T. W., Mittendorf, E., Pellicenapalle, A., Petschek, J. P. & Albrecht, E. B. (1994) *Genetics* **136**, 1075–1086.
4. Droux, M., Jacquot, J. P., Miginiac-Maslow, M., Gadal, P., Huet, J. C., Crawford, N., Yee, B. C. & Buchanan, B. B. (1987) *Arch. Biochem. Biophys.* **252**, 426–439.
5. Falkenstein, E., Vonschaewen, A. & Scheibe, R. (1994) *Biochim. Biophys. Acta* **1185**, 252–254.
6. Scheibe, R. (1991) *Plant Physiol.* **96**, 1–3.
7. Cséke, C. & Buchanan, B. B. (1986) *Biochim. Biophys. Acta* **853**, 43–63.
8. Florencio, F. J., Boihon, C. Y., Thomas, C. J. & Buchanan, B. B. (1988) *Arch. Biochem. Biophys.* **266**, 496–507.
9. Marcus, F., Chamberlain, S. H., Chu, C., Masiarz, F. R., Shin, S., Yee, B. C. & Buchanan, B. B. (1991) *Arch. Biochem. Biophys.* **287**, 195–198.
10. Decottignies, P., Schmitter, J. M., Dutka, S., Jacquot, J. P. & Miginiac-Maslow, M. (1991) *Eur. J. Biochem.* **198**, 287–294.
11. Marty, I. & Meyer, Y. (1992) *Plant Mol. Biol.* **17**, 143–147.
12. Brugidou, C., Marty, I., Chartier, Y. & Meyer, Y. (1993) *Mol. Gen. Genet.* **238**, 285–293.
13. Rivera-Madrid, R., Marinho, P., Brugidou, C., Chartier, Y. & Meyer, Y. (1993) *Plant Physiol.* **102**, 327–328.
14. Jacquot, J. P., Rivera-Madrid, R., Marinho, P., Kollarova, M., Le Maréchal, P., Miginiac-Maslow, M. & Meyer, Y. (1994) *J. Mol. Biol.* **235**, 1357–1363.
15. Wetterauer, B., Jacquot, J. P. & Véron, M. (1992) *J. Biol. Chem.* **267**, 9895–9904.
16. Dellaporta, S. J. (1983) *Plant Mol. Biol. Report.* **1**, 19–21.
17. Shirzadegan, M., Christie, P. & Seemann, J. R. (1991) *Nucleic Acids Res.* **19**, 6055.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
19. Marck, C. (1988) *Nucleic Acids Res.* **16**, 1829–1836.
20. Pearson, W. R. (1990) *Methods Enzymol.* **183**, 63–93.
21. Gonnet, G. M., Cohen, M. A. & Benner, S. A. (1992) *Science* **256**, 1443–1445.
22. Höfte, H., Desprez, T., Amselem, J., Chiapello, H., Caboche, M., *et al.* (1993) *Plant J.* **4**, 1051–1061.
23. Eklund, H., Cambillau, C., Sjöberg, B. M., Holmgren, A., Jörnvall, H., Höög, J. O. & Brändén, C. I. (1984) *EMBO J.* **3**, 1144–1449.
24. Jacquot, J. P., Issakidis, E., Decottignies, P., Lemaire, M. & Miginiac-Maslow, M. (1994) *Methods Enzymol.*, in press.
25. de Lamotte-Guery, F., Miginiac-Maslow, M., Decottignies, P., Stein, M., Minard, P. & Jacquot, J. P. (1991) *Eur. J. Biochem.* **196**, 287–294.
26. Kamo, M., Tsugita, A., Wiessner, C., Wedel, N., Bartling, D., Herrmann, R. G., Aguilar, F., Gardet-Salvi, L. & Schürmann, P. (1989) *Eur. J. Biochem.* **182**, 315–322.
27. Hartman, H., Syvanen, M. & Buchanan, B. B. (1990) *Mol. Biol. Evol.* **7**, 247–254.
28. Muller, E. G. D. (1991) *J. Biol. Chem.* **266**, 9194–9202.
29. Russel, M., Model, P. & Holmgren, A. (1990) *J. Bacteriol.* **172**, 1923–1929.
30. Kuge, S. & Jones, N. (1994) *EMBO J.* **13**, 655–664.
31. Ren, X., Bjornstedt, M., Shen, B., Ericson, M. L. & Holmgren, A. (1993) *Biochemistry* **32**, 9701–9708.
32. Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, H., Hamuro, J., Brown, N., Arai, K., Yokata, T., Wakasugi, H. & Yodoi, J. (1989) *EMBO J.* **8**, 757–764.
33. Natsuyama, S., Noda, Y., Yamashita, M., Nagahama, Y. & Mori, T. (1993) *Biochim. Biophys. Acta* **1176**, 90–94.
34. Tonissen, K., Wells, J., Cock, I., Perkins, A., Orozco, C. & Clarke, F. (1993) *J. Biol. Chem.* **268**, 22485–22489.
35. Meyer, M., Schreck, R. & Baeuerle, P. A. (1993) *EMBO J.* **12**, 2005–2015.
36. Hayashi, T., Ueno, Y. & Okamoto, T. (1993) *J. Biol. Chem.* **268**, 11380–11388.
37. Xanthoudalis, S., Miao, G., Wang, F., Pan, Y. C. E. & Curran, T. (1992) *EMBO J.* **11**, 3323–3335.
38. Babiychuk, E., Kushnir, S., Van Montagu, M. & Inze, D. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3299–3303.
39. Marty, I., Brugidou, C., Chartier, Y. & Meyer, Y. (1993) *Plant J.* **4**, 265–278.
40. Nikkola, M., Gleason, F. K., Saarinen, M., Joelson, T., Björnberg, O. & Eklund, H. (1991) *J. Biol. Chem.* **266**, 16105–16112.
41. Wetterauer, B., Véron, M., Miginiac-Maslow, M., Decottignies, P. & Jacquot, J. P. (1992) *Eur. J. Biochem.* **209**, 643–649.
42. Gamborg, O. L., Miller, R. A. & Ojima, K. (1968) *Exp. Cell. Res.* **50**, 151–158.