Cloning and characterization of three thioredoxin \( h \) isoforms from wheat showing differential expression in seeds

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Abstract

Plants contain several genes encoding thioredoxin \( h \). In cereals, type-\( h \) thioredoxins are abundant in developing and germinating grains, but the mechanism regulating the expression of these genes and their specific function is poorly known. The cloning of three full-length cDNAs encoding thioredoxin \( h \), stated Trx\( h \)\(_1\), Trx\( h \)\(_2\) and Trx\( h \)\(_3\), from wheat (\( Triticum aestivum \) cv. Soissons) seeds is described here. TRX\( h \)\(_2\) and TRX\( h \)\(_3\) deduced proteins show high identity between them and with other thioredoxins \( h \) previously described from wheat, and contain exclusively the two Cys residues forming part of the active site. By contrast, TRX\( h \)\(_1\) shows a lower level of identity and contains an additional Cys residue. The three wheat thioredoxins were expressed in \( E. coli \) and their activity was demonstrated using both the DTT-dependent insulin assay and a coupled assay with recombinant NTR from wheat. Site-directed mutagenesis showed that the additional Cys residue of TRX\( h \)\(_1\) has a low effect on its activity but is essential for dimerization. Specific expression of the three thioredoxin genes was analysed by real-time RT-PCR in developing and germinating seeds and seedlings under stressed and unstressed conditions. An increase of Trx\( h \)\(_1\), Trx\( h \)\(_2\), and Trx\( h \)\(_3\) transcripts was detected at the beginning of the desiccation phase during seed development. Early after imbibition, Trx\( h \)\(_1\), but not Trx\( h \)\(_2\) or Trx\( h \)\(_3\), transcripts showed a transient increase. Treatment of wheat seedlings with salt or hydrogen peroxide caused a differential pattern of expression of the three Trx\( h \) genes between and within tissues, hence suggesting specific functions for these thioredoxins during germination and early seedling growth.

Key words: Development, germination, real-time RT-PCR, spikes, thioredoxin \( h \), wheat seed.

Introduction

Seed quality depends on a set of parameters including the structural organization of the storage compounds, protection of tissues against oxidative stress during seed desiccation and germination, or the activation of the metabolism of seed cells upon imbibition. For these processes, dithiol-disulphide exchange is of great importance because it is involved in the regulation of the activity of many seed enzymes and is required for the breakdown of reserve compounds (Wong et al., 1995, 2004). Some of the proteins involved in the dithiol-disulphide exchange have in their active site the most conserved redox motif CXXC; that is, two Cys residues separated by two residues.

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Abbreviations: DAA, days after anthesis; HAI, hours after imbibition; IPTG, isopropyl \( \beta \)-thiogalactoside; NTR, NADPH thioredoxin reductase; Trx\( h \), thioredoxin \( h \).

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usually the two Cys residues forming part of the active site. In
addition, a TRXh containing an additional Cys residue is
described, which was site-directed mutagenesis to address its possible function. It is shown that this new
thioredoxin presents slightly different kinetic properties and a different pattern of expression in seed and vegetative tissues.

Materials and methods

Plant material

Wheat (Triticum aestivum cv. Soissons) was grown outdoors (Experimental Station of Lamotte, Toulouse, France). Seeds were harvested at different days after anthesis (DAA), immediately frozen in liquid nitrogen and stored at −80 °C until used. After-ripening spikes were collected and stored at 4 °C. The fresh weight of 40 grains from the middle of the spikes was taken and the dry weight was deduced by oven-drying the grains at 80 °C until a constant weight was reached. Mature seeds were allowed to germinate in the dark under controlled, sterile conditions on filter paper soaked with water, 100 mM NaCl, or 10 mM H2O2, for up to 3 d. All reagents were of analytical grade and were purchased from Sigma.

Total RNA extraction and cDNA isolation

Plant tissues were dissected, frozen in liquid nitrogen, and ground with tungsten balls in a Mixer Mill MM300 (Qiagen). Total RNA from whole seeds at different stages of development or dissected tissues, as well as from spikes and mature leaves, was extracted with the RNasy plant minikit (Qiagen). RNA aliquots were treated with RNase-free DNase and cDNA synthesis was performed in a DNA thermocycler (GeneAmp PCR System 9600, Applied Biosystems). Total RNA samples (0.2 μg) from 12–30 DAA seeds were reverse-transcribed with Superscript II Reverse Transcriptase (Applied Biosystems) using an anchored 3’ primer (5’-GACTAGTTCTAGATCGCGAGCGCCT) generating cDNA collections from wheat seeds at different developmental stages. Samples of these cDNA collections were subsequently amplified with proof reading PH polymerase (Promega) by PCR using a 5’ nested primer (5’-GACCTAGTCTAGATCGCGAG) and 5’ consensus primers designed from plant thioredoxin h sequences from accessible databases. Fragments were PCR-screened on the basis of the corresponding WGCPC motif. Positive fragments were subcloned in pGEM vector and introduced in E. coli JM109. Sequencing of both DNA strands was performed according to standard methods on an ABI PRISM DNA sequencer (Applied BioSystems).

Expression of wheat thioredoxins h in E. coli

Thioredoxin h cDNAs were amplified with gene-specific oligonucleotides, which included an Ndel site (underlined) in the forward primer and an XhoI site (underlined) in the reverse one. The sequence of gene-specific primers is as follows: Trxh1 (5’-ATCATATGGCCGGCGTCGCCG; 5’-ATCTCTAGATTAAGCGGGCGGTAG); Trxh2 (5’-ATACATATGGCCGGCGACGAGG; 5’-ATCTCTAGATTAAGCGGGCGGTAG). The purified cDNA fragments were digested with Ndel and XhoI and subcloned into the expression vector pET16b (Novagen), producing plasmids pET-Trxh1, pET-Trxh2, and pET-Trxh3, respectively. After checking the correct sequences, these plasmids were introduced into E. coli BL21(DE3)pLyS S (Promega) as described in Hanahan (1983). Expression of the recombinant proteins was induced by addition of 1 mM IPTG for 4 h. The recombinant proteins were purified by Ni-NTA affinity chromatography (Qiagen) according to the manufacturer’s instructions.
Site-directed mutagenesis of wheat TRxh1

Site-directed mutagenesis was performed by PCR using as template pET-Trxh1 cDNA. The C43S mutant was produced with oligonucleotides F1 (5’-TTCGGTTGCTCCTCTGTCGTATAAGC) and R1 (5’-CTCTGTTGCTGGAATCACAGGAAAGCAGC-3’), which included a single change (underlined) to replace Cys43 by Ser. The C11S mutant was produced with oligonucleotides F1 (5’-AGCCGTGATAGCCTCACCAAGCAGA) and R1 (5’-CTCTGTTGCTGGAATCACAGGAAAGCAGC-3’), which included a single change (underlined) to replace Cys11 by Ser, using wild-type Trxh1 cDNA. To produce the double mutant C43SC11S, the same pair of oligonucleotides (F1-R1) was used with mutant C43S construct as template. In all cases, PCR (1 cycle at 94 ºC, 1 min; 16 cycles at 94 ºC, 30 s, 55 ºC, 1 min, 68 ºC, 14 min) was performed with Pfu polymerase (Promega). The PCR product was then digested with DpnI for 1 h at 37 ºC to eliminate the methylated template DNA. This DNA was then used to transform E. coli XL1-Blue. Resulting cDNAs were sequenced to check for the correct introduction of mutations and that no additional mutations occurred during the process.

Protein analysis and activity assay

Protein concentration was determined with the Bradford method using a protein assay kit (Bio-Rad). His-tagged thioredoxin activity was determined using the insulin-disulphide reduction assay using a protein assay kit (Bio-Rad). His-tagged thioredoxin activity was determined using the insulin-disulphide reduction assay using a protein assay kit (Bio-Rad). His-tagged thioredoxin activity was determined using the insulin-disulphide reduction assay using a protein assay kit (Bio-Rad).

Gene expression analysis

Total RNA (0.2 µg per sample), extracted as described above, was used to perform random hexamer-primed reverse transcription with M-MLV reverse transcriptase (Invitrogen) following the manufacturer’s instructions. Aliquots (1 µl) of each reverse transcription reaction were subjected to real-time quantitative PCR in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Amplifications were performed using the Quantitect SYBR GREEN reagent (Qiagen) in a final volume of 25 µl. The PCR conditions included 1 cycle at 50 ºC for 2 min, 1 cycle at 95 ºC for 15 min, and 40 cycles at 94 ºC for 15 s, 60 ºC for 30 s, and 72 ºC for 30 s. Agarose gel electrophoresis confirmed the homogeneity of the mRNA products. Gene-specific primers were designed with the Primer express software (Applied Biosystems), and are as follows: Trxh1 (5’-ACATGCTAAATGCAAGGAGA; 5’-CGTATGCTGAGCCACGACG); Trxh2 (5’-AGCTATCAGGAGAACGAGCGACG); Trxh3 (5’-TGTTGCAATGTTAGGTTG); Trx3 (5’-AGATCAGGAGAACGAGCGACG). Relative quantification for a given transcript was calculated with the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001). The relative amount of each seed Trxh transcript was normalized to the amount of 18S RNA transcript (internal control) in the same cDNA and the amount of Trxh2 in mature leaves (calibrator).

Results and discussion

Isolation and characterization of cDNAs encoding TRxh from wheat

After screening a collection of cDNAs generated with RNA from wheat seeds at different stages of development, three full-length cDNAs were identified putatively encoding TRxh, here stated Trxh1 (Accession number AY072771), Trxh2 (Accession number AF286593), and Trxh3 (Accession number AF420472). The deduced amino acid sequence of TRxh2 and TRxh3 showed a high identity (94.3%); both polypeptides are almost identical except at the N-terminus (Fig. 1A), which was larger in TRxh3 and contains a Pro residue (Fig. 1A, dot) not found in any other wheat TRxh. As shown in Fig. 1A, these thioredoxins also showed high similarity with previously reported thioredoxins h from wheat (Gautier et al., 1998); indeed, TRxh2 corresponds to a previously reported wheat cDNA stated TrxhB (Serrato et al., 2001). By contrast, TRxh1 is less similar to TRxh2 (54.6%) and TRxh3 (55.6%). TRxh1 belongs to a subclass of low molecular weight plant thioredoxins h, containing a third Cys residue (Cys11, Fig. 1A, asterisk) besides the two Cys at the active site.

The presence of the additional Cys residue was reported in type-h thioredoxins from poplar (Gelhaye et al., 2003) and barley (Maeda et al., 2003) and is found in thioredoxin h sequences from either dicots (Accession numbers AA042956, P29448, Q39363) and monocots (Accession number Q42443). As the rice phloem sap TRxh (Ishiwatari et al., 1995), TRxh1 lacks the Trp residue at the N-terminus (Fig. 1A, triangle), considered a thioredoxin h signature (Stein et al., 1995), which is replaced by Phe, it is still an aromatic amino acid. The structural model of TRxh1 (Fig. 1B) predicts a location for the additional Cys residue on a rather flexible part of the molecule and opens the possibility to additional interactions with Cys residues of target proteins. Phe17 is in the same position as its homologue Trp in the template (Fig. 1B), therefore no significant effect of this substitution on thioredoxin structure or activity is expected. In addition, the TRxh1 polypeptide presents an Arg residue at position 102 whereas TRxh2 and TRxh3 present Ile at this position (Fig. 1A, inverted triangle). Plant thioredoxins h have been clustered into three groups depending on the presence of the Arg, Ile/Met, or Asn residue at this position (Juttner et al., 2000; Maeda et al., 2003). Based on this classification, it is deduced that TRxh1 is grouped with the barley HvTRxh1 (Maeda et al., 2003) and the rice phloem sap thioredoxin (Ishiwatari et al., 1995). These thioredoxins are also characterized by the third Cys and Phe residues at the N-terminus as mentioned above. The identification of thioredoxins with the features of TRxh1 in monocots and dicots suggests that these genes evolved from an ancestral gene existing before the monocot/dicot diversification (Juttner et al., 2000).

Expression of TRxh1, TRxh2, and TRxh3 polypeptides in E. coli and characterization of TRxh1 mutants

The coding sequences of the three Trxh genes were subcloned in pET16b vector to produce the recombinant proteins in E. coli with a His-tag at the N-terminus. Recombinant TRxh2, TRxh1, and TRxh3 proteins were
found in the soluble fraction from the corresponding transformed E. coli cultures after induction with IPTG (Fig. 2A, lanes 1, 4, 7, respectively) and were purified by affinity chromatography on nickel column (Fig. 2A, lanes 3, 6, 9, respectively). SDS–PAGE analysis under non-reducing conditions showed that TRXh2 and TRXh1 appeared partially as dimers (Fig. 2B, lanes 1, 3). Despite the high sequence similarity with TRXh2, TRXh3 appeared exclusively in monomeric form (Fig. 2B, lane 2). Under reducing conditions all three TRXh were detected as monomers (Fig. 2B), thus showing that dimerization was produced by disulphide formation.

To analyse the effect of the third Cys residue of TRXh1 on activity and dimerization, different mutants were produced. To that end Cys43 and Cys11 were replaced with serine (C43S and C11S mutants), and the double mutant C43S-C11S was also produced. Under non-reducing conditions (–DTT) TRXh1 C43S mutant showed a lower level of dimerization than the wild type TRXh1 (Fig. 2C, lane 1 and 2). Under reducing conditions all three TRXh were detected as monomers (Fig. 2B, lane 2), thus showing that dimerization was produced by disulphide formation.

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The three recombinant thioredoxins TRXh1, TRXh2, and TRXh3 showed activity as determined with the DTT-dependent insulin reduction assay (Fig. 3A). Under these assay conditions TRXh1 showed slightly higher specific activity, whereas the rate of insulin reduction by TRXh2 and TRXh3 was indistinguishable. An important question concerning the activity of the different TRXh isoforms is its interaction with NTR, which catalyses the transfer of reducing power to TRXh in vivo. This question was addressed using the recombinant NTR from wheat (Serrato et al., 2002). TRXh1 showed a higher activity under these more physiological assay conditions (Fig. 3B). So, both activity assays showed that TRXh1 is the most efficient type-h thioredoxin in wheat. The effect of replacing the third Cys residue of TRXh1 on activity was then analysed using the more physiological NTR-based assay. As expected, mutation of active site Cys43, either alone (C43S

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**Fig. 1.** Alignment of wheat thioredoxins h and model structure of TRXh1. (A) Protein sequences deduced from wheat cDNAs, as indicated, were compared using the CLUSTALW software. The accession numbers are as follows: Trxh1, AY072771; Trxh2, AF286593; Trxh3, AF420472; pTAM, O64394; TrxhA, AJ009762. Characteristic residues Cys11 (asterisk), Phe17 (triangle), and Arg101 (inverted triangle) of TRXh1 and Pro20 of TRXh3 are marked. (B) Wheat TRXh1 structure was modelled using the structure of Chlamydomonas reinhardtii (PDB accession number 1tof) as a template. α-Helices are in red and β-strands are in blue. Cys11 and Phe17 residues are in black. The picture was generated by SPDB-Viewer. An overall structure quality was checked with the WHAT IF software.
mutant) or in combination with Cys11 (C43S-C11S mutant), completely abolished activity (Fig. 3C); by contrast, the Cys11 mutation, which completely abolished dimerization of TRXh1 (Fig. 2C), showed only partial effect on activity (Fig. 3C). So, the additional Cys residue of TRXh1 seems to be more relevant for dimerization than for activity, suggesting an important role for this residue in the ability of TRXh1 to interact with potential targets, in comparison with the other group of TRXhs containing exclusively the active site Cys residues.
Type-h thioredoxins are differentially expressed in wheat seeds

The different characteristics of the three wheat Trxh genes suggested that each of these thioredoxins, or at least TRXh1, might have a different function, which might be reflected in a different pattern of expression. Because all three TRXhS were similarly detected by the anti-TRXh antibody (results not shown), this possibility was addressed by analysing the accumulation of transcripts of the three Trxh genes during seed development and germination using real-time RT-PCR with gene-specific primers. Trxh1, Trxh2, and Trxh3 transcripts were detected at a rather low level during the early stages of seed development (Fig. 4A). At later stages, when the water content of the seed was below 40%, an increase of the three Trxh transcripts was observed, although Trxh1 accumulated at a lower level than Trxh2 and Trxh3. The pattern of expression of the Trxh genes was also analysed in spikes (Fig. 4B). Although the level of expression in this tissue was lower than in developing seeds, a slight increase was detected after 24 DAA (Fig. 4B) when the water loss in the corresponding seeds was below 50%, which corresponds to the phase of chlorophyll breakdown in spikes and their progressive desiccation (Carceller and Aussenac, 2001). In contrast to the seed, Trxh1 was the most abundant thioredoxin h transcript in spikes.

During seed development, the period between 10 and 20 DAA is characterized by a high activity of disulphide bond formation in gluten proteins (Gupta et al., 1996; Zhu and Khan, 1999; Aussenac and Carceller, 2000). Therefore, the expression of the Trxh genes, lower at these stages, seems not to be related to endosperm filling. By contrast, the accumulation of Trxh transcripts occurred towards the beginning of the maturation phase, when the transcriptional activity in the seed is lower, that is, Trxh transcripts accumulation is associated with desiccation in seeds and spikes. In a way, accumulation of Trxh transcripts follows the profile of genes for late embryogenesis abundant (LEA) proteins, which are expressed during the late stages of seed development, as well as in stress conditions (Delseny et al., 1994). Since it is generally admitted that LEA proteins play a role in the establishment of desiccation tolerance (Close, 1996), the question arises whether any of the wheat seed Trxh has a related function.

Differential expression of Trxh genes in response to abiotic stress

To test the differential expression of wheat Trxh genes in seed and vegetative tissues, wheat seeds were imbibed for 48 h and the content of the Trxh transcripts was analysed in dissected shoots, roots, scutellum, and the aleurone layer. Very early after imbibition (2–4 h), Trxh1 transcripts showed a marked and transient accumulation and then declined to the level of the Trxh2 and Trxh3 transcripts (Fig. 5).

In young seedlings, Trxh1 transcripts accumulated to a higher level in shoots, roots, and scutellum than in aleurone cells, which showed similar amounts of the three Trxh transcripts (Fig. 6A). Since Trxh transcript accumulation in developing seeds was raised during desiccation,
The different behaviour of the aleurone layer must be underlined because of the important role of these cells in seed germination. The starchy endosperm is a starch and protein storage tissue, whereas the aleurone cells store a large amount of lipids, mainly triglycerides, in oleosomes. This lipid reserve is used as the source of energy for the development of germination. The starchy endosperm is a starch and protein storage tissue, whereas the aleurone cells store a large amount of lipids, mainly triglycerides, in oleosomes.

Environmental stresses have a poor effect on Trx expression, except in the aleurone layer.

The oxidative stress treatment also promoted an increase of Trx1 and, to a lower extent, of Trx3 transcripts (Fig. 6B). The oxidative stress treatment also promoted an increase of Trx2 and Trx3 transcripts, but not of Trx1 in aleurone cells (Fig. 6C), showing no significant effect on the other tissues analysed. These results clearly show the difference in the expression profile of the thioredoxin h genes in aleurone compared with other tissues, which show the predominant expression of the Trx1 gene. Environmental stresses have a poor effect on Trx h genes expression, except in the aleurone layer.

The different behaviour of the aleurone layer must be underlined because of the important role of these cells in seed germination. The oxidative stress treatment also promoted an increase of Trx2 and Trx3 transcripts, but not of Trx1 in aleurone cells (Fig. 6C), showing no significant effect on the other tissues analysed. These results clearly show the difference in the expression profile of the thioredoxin h genes in aleurone compared with other tissues, which show the predominant expression of the Trx1 gene. Environmental stresses have a poor effect on Trx h genes expression, except in the aleurone layer.


