

# Cloning of thioredoxin *h* reductase and characterization of the thioredoxin reductase–thioredoxin *h* system from wheat

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Thioredoxins *h* are ubiquitous proteins reduced by NADPH–thioredoxin reductase (NTR). They are able to reduce disulphides in target proteins. In monocots, thioredoxins *h* accumulate at high level in seeds and show a predominant localization in the nucleus of seed cells. These results suggest that the NTR–thioredoxin *h* system probably plays an important role in seed physiology. To date, the study of this system in monocots is limited by the lack of information about NTR. In the present study, we describe the cloning of a full-length cDNA encoding NTR from wheat (*Triticum aestivum*). The polypeptide deduced from this cDNA shows close similarity to NTRs from *Arabidopsis*, contains FAD- and NADPH-binding domains and a disulphide probably interacting with the disulphide at the active site of thioredoxin *h*. Wheat NTR was expressed in *Escherichia coli* as a His-tagged protein. The absorption spectrum of the purified recombinant protein is typical of flavoenzymes. Furthermore, it showed NADPH-dependent thioredoxin *h* re-

duction activity, thus confirming that the cDNA clone reported in the present study encodes wheat NTR. Using the His-tagged NTR and TRXhA (wheat thioredoxin *h*), we successfully reconstituted the wheat NTR–thioredoxin *h* system *in vitro*, as shown by the insulin reduction assay. A polyclonal antibody was raised against wheat NTR after immunization of rabbits with the purified His-tagged protein. This antibody efficiently detected a single polypeptide of the corresponding molecular mass in seed extracts and it allowed the analysis of the pattern of accumulation of NTR in different wheat organs and developmental stages. NTR shows a wide distribution in wheat, but, surprisingly, its accumulation in seeds is low, in contrast with the level of thioredoxins *h*.

**Key words:** *Escherichia coli*, germination, thioredoxin mutant, *Triticum*.

## INTRODUCTION

Thioredoxins are ubiquitous proteins with molecular mass of 12–13 kDa, which are found in bacteria, unicellular eukaryotes, and plant and animal cells. These proteins have a conserved active site, WCGPC, containing a redox disulphide bridge [1]. Thioredoxins are able to reduce specific disulphide bridges in target proteins, hence regulating the activity of these proteins. Three thioredoxin types have been described in plants [2]. Two of them, thioredoxins *m* and *f*, encoded by nuclear genes, are located in the chloroplast and are reduced by ferredoxin thioredoxin reductase using photosynthetic reducing power. These thioredoxins reduce specific disulphide bridges and, in this way, regulate the activity of enzymes of the Calvin cycle. Therefore thioredoxins play an essential role in the control of photosynthetic carbon metabolism [3]. The third type, thioredoxin *h*, is found in the cell cytoplasm of most plant organs. Reduction of thioredoxin *h* depends on NADPH and is catalysed by NADPH–thioredoxin reductase (NTR) [2,4]. In the last few years, thioredoxin *h* systems specific to mitochondria have been identified in yeast [5], mammal cells [6,7] and *Arabidopsis thaliana* [8].

NTR is a flavoenzyme that catalyses the transfer of electrons from NADPH, via FAD and a redox-active disulphide, to the disulphide bridge of thioredoxins *h*. Like thioredoxins *h*, NTR is widely distributed in prokaryotic and eukaryotic cells; however, two groups of NTR have evolved with different molecular and kinetic characteristics [4]. Prokaryotes have a lower-molecular-

mass NTR, which consists of two identical subunits (approx. 35 kDa) forming a homodimer in its native form. The crystal structure of NTR from *Escherichia coli* contains three delineated domains in each of the subunits, the FAD, NADPH and central domain [9]. In the conversion into the reduced form, the NADPH domain rotates with respect to the FAD domain [10]. In contrast, NTR from mammal cells consists of a homodimer of subunits with a larger molecular mass (approx. 55 kDa). The human enzyme contains the unusual selenocysteine as the penultimate residue at the C-terminus [11,12], which is active in the catalysis [13].

A peculiar feature of the thioredoxin *h* system in plants is the large number of genes encoding for thioredoxin *h*, at least five in *Arabidopsis* [14], which are involved in multiple functions, as shown by functional complementation of yeast mutants [15]. In cereals, thioredoxin *h* is abundant in seeds. Since thioredoxin *h* inactivates inhibitors of hydrolytic enzymes [16] and activates serine proteases at early stages of germination [17], it has been proposed that they play an important role as a signal for cereal seed germination. Further analysis of thioredoxins *h* in germinating and developing wheat seeds revealed their accumulation in the nuclei of aleurone and scutellum cells [18], suggesting the possibility that these proteins interact with nuclear factors, as is the case in mammal and yeast cells [19,20]. The current evidence points to the hypothesis that the thioredoxin *h* system is an important component of monocot seed biology. However, the understanding of this system in seeds is limited, because no

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); IPTG, isopropyl  $\beta$ -D-thiogalactoside; NTR, NADPH–thioredoxin reductase; TRXh, thioredoxin *h*.

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description of cereal NTR has been reported so far. To our knowledge, the only NTR cloned from plants is from *A. thaliana* [21]. The crystal structure of this enzyme was established, so that the tertiary structure and kinetic parameters are known [22]. However, nothing is known about the pattern of expression of NTR in *Arabidopsis*. We were interested in determining the function of the NTR–thioredoxin *h* system in cereal seeds. For that purpose, we have isolated a full-length cDNA clone from wheat encoding a deduced polypeptide with close similarity to NTR. The protein, expressed in *E. coli*, is a flavoenzyme showing NTR activity. In addition, we have raised a polyclonal antibody against the wheat enzyme, which was used to analyse the pattern of expression of NTR in wheat plants and seeds.

## MATERIALS AND METHODS

### Plant material

Wheat (*Triticum aestivum* cv. Chinese Spring) plants were grown in a greenhouse under controlled conditions on a mixture of peat/vermiculite (3:1). Developing seeds were harvested at 18 days post anthesis (18 d.p.a.), frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used. Mature seeds were allowed to germinate at room temperature on filter paper soaked with water under sterile conditions for 4 days, and the aleurone layer, scutellum and starchy endosperm were carefully dissected. Shoots and roots for Western-blot analysis were dissected from 2–4-day-old seedlings, and mature leaves from 7-week-old plants. All reagents were of analytical grade and were purchased from Sigma.

### Wheat NTR cDNA cloning

A cDNA library (approx. 300000 plaque-forming units) constructed in  $\lambda$ gt11 with polyadenylated [poly(A)<sup>+</sup>] RNA from wheat aleurone cells was screened with anti-NTR polyclonal antibodies raised against the spinach enzyme (kindly provided by Dr F. J. Florencio) as described in [23]. The four positive clones obtained were purified and the sizes of their cDNA inserts were analysed. After subcloning in pGEM vectors (Promega), inserts were sequenced in both strands. To obtain the full-length sequence, the oligonucleotide (5'-GACCGCGACTGCCACCG-3') was designed to perform rapid amplification of cDNA ends using the Marathon cDNA synthesis kit (ClonTech) and total RNA from different wheat tissues. A full-length cDNA was obtained, cloned in pGEM vector and sequenced in both strands using the T7 Sequenase 2.0 DNA sequencing kit (USB, Cleveland, OH, U.S.A.).

### Expression of NTR in *E. coli* and production of polyclonal antibodies

Wheat NTR was expressed in *E. coli* as a His-tagged polypeptide. To that end, the coding sequence was amplified from the cDNA using the oligonucleotide 5'-GAGAGGATCCATGGAGGAGGCGGCCG-3', which added a *Bam*HI site (underlined) at the putative initial Met residue, and the oligonucleotide 5'-GAGAAAGCTTTAGTCAATCAGTCTTCCTCC-3', which added a *Hind*III site (underlined) at the 3'-end. The PCR fragment was then digested with *Bam*HI and *Hind*III and subcloned in pQE-30 expression vector (QIAexpress expression system; Qiagen). The resulting plasmid, termed pQE-TaNtr, was introduced in *E. coli* XL1-Blue, which was routinely grown in the presence of 2% (w/v) glucose to avoid expression of the wheat protein before induction. Expression of the recombinant protein was induced by incubation of transformed *E. coli* XL1-Blue cells for 3 h at  $30^{\circ}\text{C}$  in glucose-free Luria–Bertani medium supple-

mented with 0.1 mM isopropyl  $\beta$ -D-thiogalactoside (IPTG). The overexpressed protein was purified by  $\text{Cu}^{2+}$  affinity chromatography in pre-packed Hi-Trap affinity columns (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Aliquots of the purified protein were used to immunize rabbits at the Service for Animal Production (University of Seville, Spain). The immune serum was used to purify anti-NTR by Protein A chromatography before use. No signal was detected when Western blots were probed with the pre-immune serum.

### Construction of thioredoxin *h* C53S (Cys<sup>53</sup> → Ser) mutant and its expression in *E. coli*

The Cys<sup>53</sup> residue of thioredoxin *h* active site was mutated to serine. Site-directed mutagenesis was performed by PCR using the His-tagged TRXhA cDNA as template [18]. Two oligonucleotides were designed complementary to the sequence to be mutated, namely C53S-A (5'-CATGGTGTGGACCATCCC-CATCATGGCTC-3') and C53S-B (5'-GAGCCATGATGCG-GGATGGTCCACACCATG-3'), including a single change (underlined), which resulted in the replacement of the Cys<sup>53</sup> residue by Ser. The PCR (1 cycle at  $94^{\circ}\text{C}$  for 1 min; 16 cycles at  $94^{\circ}\text{C}$  for 30 s, at  $55^{\circ}\text{C}$  for 1 min and at  $68^{\circ}\text{C}$  for 14 min) was performed with *Pfu* polymerase (Stratagene). The PCR product was then digested with *Dpn*I for 1 h at  $37^{\circ}\text{C}$  to eliminate the methylated template DNA. This DNA was used to transform *E. coli* XL1-Blue. The resulting mutant cDNA was sequenced in both strands to check for the correct introduction of the mutation and that no additional mutations were generated during the process.

### Western-blot analysis

Plant tissues were frozen in liquid nitrogen and ground with a mortar and pestle. Crude extracts were prepared from the ground tissue with extraction buffer [50 mM Tris/HCl (pH 7.9)/0.2 mM EDTA/0.5 mM PMSF]. The homogenized mixture was centrifuged for 20 min at 15000 *g*. The resulting supernatant constituted the cell-free extract and was stored at  $-20^{\circ}\text{C}$ .

Protein samples were subjected to SDS/PAGE (10% gel) as reported in [24] and electrotransferred to a nitrocellulose sheet. The sheet was then soaked in 15 mM Tris/HCl (pH 7.4) containing 0.2 M NaCl (buffer A) and 5% (w/v) dry powdered milk at room temperature for at least 1 h. After overnight incubation at  $4^{\circ}\text{C}$  with Protein A-purified anti-NTR (diluted 1:1000 in buffer A), sheets were washed four times for 15 min each with buffer A containing 0.1% (v/v) Tween 20. Sheets were then incubated for 1 h in the same buffer containing affinity-purified goat anti-(rabbit IgG) alkaline phosphatase conjugate diluted to 1:30000. Membranes were subsequently washed in buffer A. The reaction was visualized by immersion in a developing solution [20 ml of 100 mM Tris/HCl (pH 9.5), 5 mM  $\text{MgCl}_2$ , 100 mM NaCl, 3 mg of 5-bromo-4-chloro-3-indolyl phosphate and 6 mg of Nitro Blue Tetrazolium].

### NTR and thioredoxin activity assays

NTR activity was determined by the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by the method of Holmgren and Björnstedt [25]. NTR activity was also assayed by its ability to reduce thioredoxin *h*, which reduces insulin disulphides using the turbidimetric assay method as described previously [18]. *E. coli* thioredoxin was purchased from Sigma.

## RESULTS AND DISCUSSION

### Isolation of a cDNA clone encoding NTR from wheat

A wheat cDNA library constructed with poly(A)<sup>+</sup> RNA isolated from aleurone cells of 1-day-imbibed seeds was screened with polyclonal antibodies raised against NTR from spinach leaves. Four positive clones were obtained and purified. All of them contained short inserts, approx. 300 bp, showing a high degree of similarity to the C-terminus of NTR from *A. thaliana* [21]. To obtain the full-length cDNA, an oligonucleotide was designed to perform rapid amplification of cDNA ends using RNA isolated from different wheat seedling tissues as template. A 1045 bp cDNA clone was obtained from root RNA (EMBL Nucleotide Sequence Database accession no. AJ421947). This cDNA contained a single open reading frame encoding a deduced polypeptide of 331 residues, with an expected molecular mass of 34.903 kDa and pI 5.79.

The sequence of the deduced polypeptide and the alignment with other NTR sequences is shown in Figure 1. The wheat enzyme shows the highest level of similarity to NTRs from *Arabidopsis*. It contains a putative FAD-binding domain formed by two motifs (marked with \* in Figure 1): the motif GxGxxA (residues 17–22, wheat numbering), at the N-terminal region of the protein, and the motif TxxxxVFAAGD (residues 283–293) at the C-terminus. These two motifs forming the FAD-binding domain are conserved in NTR from plants and unicellular eukaryotes. However, the motif GxGxxA is replaced by GxGxxG in the *E. coli* enzyme (Figure 1). An NADPH-binding domain (GxGxxA, residues 164–169) was also found in the wheat NTR (indicated by arrowheads in Figure 1), which is conserved in all the NTRs compared here. Finally, the active site of the wheat NTR (marked with a box in Figure 1) contains a disulphide bridge (residues 145 and 148, wheat numbering) potentially interacting with the thioredoxin *h* active site WCGPC [18]. The NTR active site presents a perfect match when compared with the more closely related NTRs, but the wheat enzyme shows a peculiarity, the conservative change of Thr (residue 143), which is replaced by the Ser residue in NTR from the other sources (Figure 1). In the *Arabidopsis* enzyme, this Ser residue and Asp<sup>139</sup> (*Arabidopsis* numbering) bind via a water molecule to N-5 of the isoalloxazine ring [22]. It is not expected that the Thr residue at that position in the wheat enzyme will have any significant effect on NTR activity. In summary, the protein deduced from the cDNA described in this report contains the characteristics of bacterial and plant NTRs.

A phylogenetic tree was constructed comparing NTR sequences from different sources (Figure 2). The wheat enzyme appears grouped with two NTRs from *Arabidopsis* (termed *A. thaliana* A and B respectively) in a branch including NTRs from unicellular eukaryotes. The third putative NTR identified in the *Arabidopsis* genome (termed *A. thaliana* C), although grouped with these NTRs, shows a weaker relationship. Therefore the phylogenetic analysis reveals a close relationship of dicot and monocot NTRs and a weak relationship with mammal NTRs. Of the two groups of NTR that have evolved [4], it is clear that the plant and lower eukaryotes belong to the bacterial-type group.

### Expression of wheat NTR in *E. coli* and characterization of the recombinant protein

To obtain definitive evidence that the wheat cDNA reported here encodes for an enzyme with NTR activity, the coding sequence was subcloned in pQE-30 expression vector, generating the plasmid pQE-TaNtr, so that wheat NTR was produced as a N-terminus His-tagged polypeptide. This approach was chosen to

facilitate the purification of the recombinant wheat NTR, avoiding possible contamination with the *E. coli* enzyme, which shows similar features and might be co-purified with the wheat enzyme. *E. coli* XL1-Blue was transformed with plasmid pQE-TaNtr and with pQE-30 vector as control. SDS/PAGE analysis of soluble proteins extracted from *E. coli* (pQE-TaNtr) that had been induced with IPTG shows the overexpression of a polypeptide with the expected molecular mass of 36.4 kDa, including the His tag (Figure 3A, lane 2), which was not present in *E. coli* cells transformed with the expression vector without insert (Figure 3A, lane 1). About half of the expressed protein was found in the pellet (results not shown), indicating that part of this protein is probably forming inclusion bodies. Since the soluble supernatant contained a significant amount of the wheat enzyme, we used this soluble extract as the starting material to purify the His-tagged NTR by Cu<sup>2+</sup> affinity chromatography. This procedure yielded a highly purified enzyme, based on SDS/PAGE analysis (Figure 3A, lane 3). In spite of losing part of the enzyme in inclusion bodies, the overall yield of this process was 1.6 mg/l of culture. To test whether the wheat NTR is an FAD enzyme, as suggested by the sequence comparison, we performed an absorption spectrum of the purified protein (Figure 3B). The spectrum is typical of flavoproteins showing characteristic absorption maxima at 270, 378 and 454 nm, clearly showing that the expressed protein is a flavoenzyme.

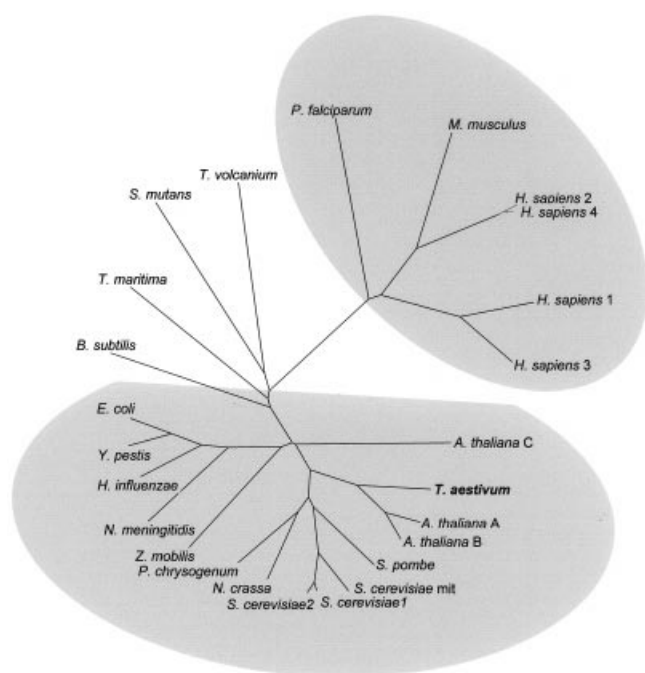
To confirm that this flavoenzyme corresponded to wheat NTR, we tested its ability to reduce wheat thioredoxin *h* *in vitro*. For that purpose, we used the turbidimetric insulin precipitation assay [25]. Wheat thioredoxin *h* (purified His-tagged TRXhA [18]) was able to reduce insulin, provided that NTR and NADPH were present in the reaction mixture (Figure 4). The velocity of the reaction, as shown by the increase in  $A_{650}$ , was dependent on the concentration of NTR at saturating concentrations of TRXhA and NADPH and did not occur in the absence of either NTR or NADPH. Therefore, these results confirm that the cloned cDNA encodes for wheat NTR, a flavoenzyme, which efficiently reduces wheat thioredoxin *h* using NADPH as electron donor. Furthermore, these results show that the wheat NTR–thioredoxin *h* system is efficiently reconstituted *in vitro* using purified His-tagged components. Further evidence that the wheat NTR–thioredoxin *h* is functional *in vitro* was obtained by the construction of the thioredoxin *h* mutant C53S, in which the Cys residue (Cys<sup>53</sup>) of the wheat TRXhA active site was mutated to the Ser residue. This mutation completely abolished the functionality of the system, as observed when the wild-type thioredoxin *h* was replaced by C53S mutant polypeptide in the insulin reduction assay (Figure 5).

### Kinetic parameters of wheat NTR

To determine the  $K_m$  for NADPH and NADH, we used the DTNB reduction assay [25]. Although wheat NTR was able to use NADH, the  $K_m$  of the enzyme for NADPH is three orders of magnitude lower (Table 1). Since NTR showed a similar  $V_{max}$  with both NADPH and NADH (Table 1), we conclude that, as expected, the *in vivo* electron donor for wheat NTR is most probably NADPH.

The turbidimetric insulin precipitation assay revealed that the  $K_m$  value of wheat NTR for wheat thioredoxin *h* was 7.6  $\mu$ M (Table 1), which is higher than 1  $\mu$ M previously reported for the *Arabidopsis* enzyme [21]. Sequence similarity and the *in vitro* characterization of the NTR–thioredoxin *h* system allow us to propose a reaction mechanism similar to those previously described for *E. coli* and *Arabidopsis* systems [22]. According to this model, it should be expected that wheat NTR is able to interact





**Figure 2** The phylogenetic tree of NTRs from different sources

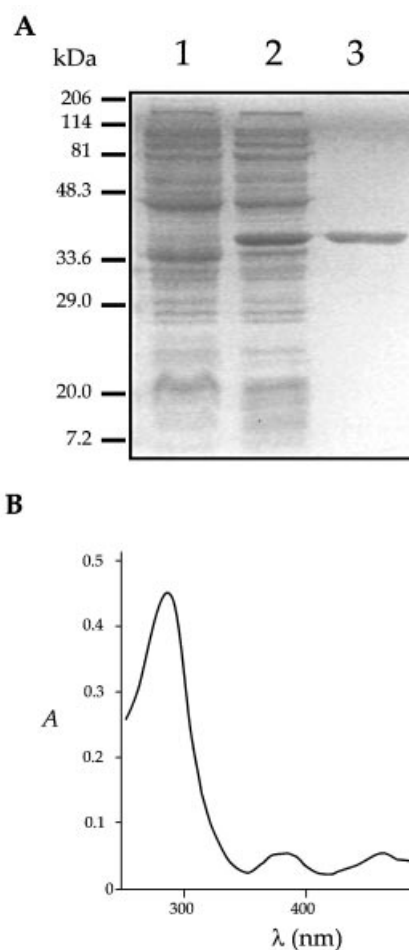
The phylogenetic tree was constructed using the program CLUSTALW ([www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)), with NTR sequences searched in EMBL and SWISSPROT databases. The accession numbers are: *A. thaliana* (A, AAB86519; B, CAB54874; C, AAB84351); *S. pombe* (CAA17692); *S. cerevisiae* (1, P29509; 2, AAA64747.1; mitochondrial, P38816); *N. crassa* (P51978); *P. chrysogenum* (P43496); *Zymomonas mobilis* (AF124757); *Haemophilus influenzae* (P43788); *Yersinia pestis* (NP\_404967); *E. coli* (P09625); *Neisseria meningitidis* (NP\_284253); *Bacillus subtilis* (NP\_391359); *Thermotoga maritima* (NP\_228678); *Streptococcus mutans* (JC2311); *Thermoplasma volcanium* (NP\_110989); *Plasmodium falciparum* (CAA60574); *Mus musculus* (AAH13688); *Homo sapiens* (1, XP\_049211; 2, AAH07489; 3, S66677; 4, NP\_006431).

and reduce *E. coli* thioredoxin. The results presented in Table 1 show that this is the case; however, wheat NTR shows a poor reactivity with the bacterial enzyme, as revealed by the higher  $K_m$  (36  $\mu\text{M}$ ) and lower  $V_{\text{max}}$ . The results reported here for the wheat NTR are in agreement with previously reported data for the *Arabidopsis* enzyme [21], confirming that, in spite of the structural relationship between plant and *E. coli* NTRs, the plant enzyme shows a poor reactivity with bacterial thioredoxin. These results further support the close relationship of *Arabidopsis* and wheat NTR.

#### Expression pattern of NTR in wheat

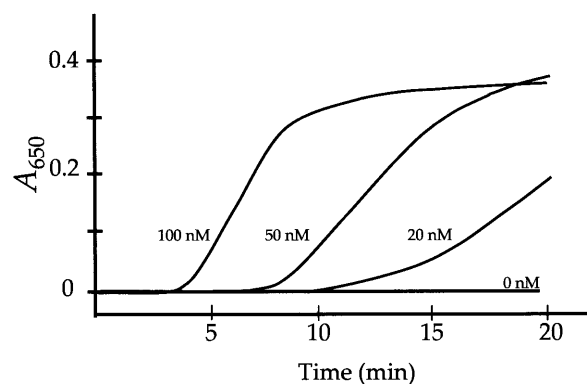
To analyse the pattern of accumulation of NTR in different wheat tissues and developmental stages, an antibody was raised against wheat NTR. This antibody efficiently detected amounts as low as 2 ng of the purified His-tagged enzyme in Western-blot analysis (Figure 6A). In protein extracts from mature wheat seeds, the antibody detected a single polypeptide with the expected molecular mass of 35 kDa (Figure 6A). It should be noted that the slightly different electrophoretic mobility is due to the His tag of the recombinant enzyme. According to the intensity of the band detected in the Western-blot analysis, we could estimate a content of approx. 1–2 ng/ $\mu\text{g}$  of protein extracts from mature wheat seeds.

The Western-blot analysis of the same amount of protein extracts from different tissues shows a high accumulation of



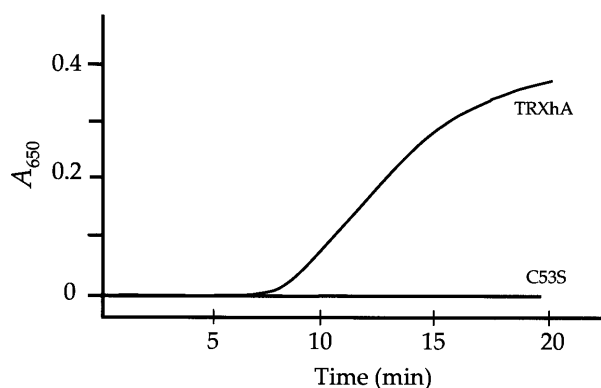
**Figure 3** Purification and absorption spectrum of wheat His-tagged NTR produced in *E. coli*

(A) Analysis of an SDS/PAGE gel stained with Coomassie Brilliant Blue R-250 that was loaded with the supernatant of extracts (20  $\mu\text{g}$  of protein) from pQE-30 without insert (lane 1), or pQE-TaNtr (lane 2) after IPTG induction. Lane 3, purified His-tagged TaNTR (1  $\mu\text{g}$  of protein) after  $\text{Cu}^{2+}$  affinity chromatography. (B) Absorption spectrum of the purified His-tagged TaNTR. The spectrum was recorded using a 5  $\mu\text{M}$  NTR solution in 100 mM K-phosphate buffer (pH 7.0) at room temperature.



**Figure 4** Insulin reduction assay of the wheat NTR–thioredoxin h system

The incubation mixture contained the indicated concentration of NTR, 3  $\mu\text{M}$  His-tagged TRXhA, 0.5 mg/ml insulin, 150 mM NADPH, 2 mM EDTA and 100 mM potassium phosphate buffer (pH 7.0). The basal line was also obtained when NADPH was omitted from the reaction mixture.



**Figure 5** Effect of replacement of the wild-type thioredoxin *h* by the C53S mutant on the activity of the NTR–thioredoxin *h* system

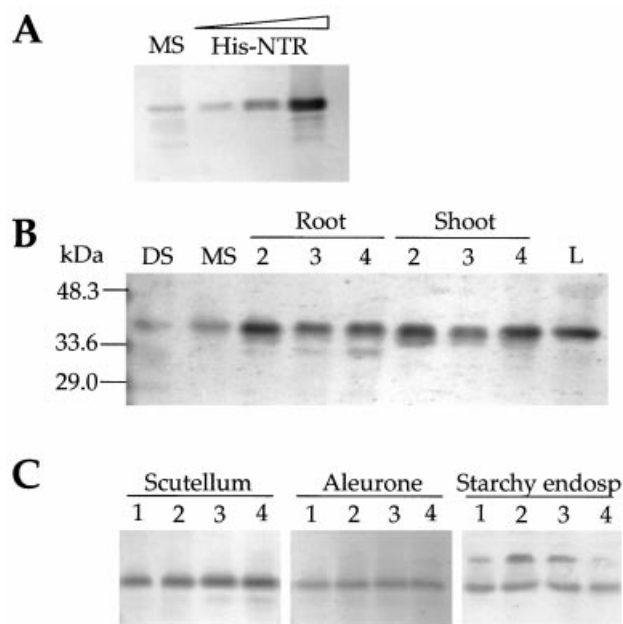
Assays were performed in 100 mM potassium phosphate buffer (pH 7.0) containing 50 nM NTR, 150 mM NADPH, 0.5 mg/ml insulin, 2 mM EDTA and 3  $\mu$ M of either wild-type or mutant C53S thioredoxin *h*, as indicated.

**Table 1** Kinetic parameters of wheat NTR

Wheat thioredoxin *h* corresponds to His-tagged TRXhA and was purified as described in [18]. *E. coli* thioredoxin was purchased from Sigma. The  $K_m$  and  $V_{max}$  values for thioredoxin were determined using the insulin reduction assay. The  $K_m$  and  $V_{max}$  values for NADPH and NADH were determined using the DTNB assay.

Substrate	$K_m$	$V_{max}$ ( $\mu$ mol $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$ )
NADPH	0.4 $\mu$ M	1.43
NADH	0.6 mM	1.38
Thioredoxin <i>h</i>	7.6 $\mu$ M	1.43
Thioredoxin ( <i>E. coli</i> )	36 $\mu$ M	0.42

NTR in vegetative tissues, in shoots and roots from 2–4-day-old seedlings, and in leaves from mature plants (Figure 6B). The content of NTR was lower in seeds, either in mature seeds (MS in Figure 6B) or in developing seeds harvested at 18 d.p.a. (DS in Figure 6B). An earlier report has described a relatively invariable content of NTR in the endosperm of wheat grains after germination [26]. We have analysed the content of NTR in the different tissues of germinating seeds; for that purpose, the aleurone layer, scutellum and starchy endosperm from seeds up to 4 days of imbibition were carefully dissected. Figure 6(C) shows a higher content of NTR in the scutellum, which slightly increased during the time after imbibition. The aleurone cells contained a lower content of NTR, which similarly increased after germination (Figure 6C). Surprisingly, the antibody detected two bands in protein extracts from the starchy endosperm. The band corresponding to the 35 kDa subunit showed a slight decrease, probably due to the proteolytic activity in the starchy endosperm during the time after imbibition [27]. It is not clear whether the upper band detected by these antibodies is actually NTR. This polypeptide shows a clear increase in starchy endosperm extracts from seeds after 2 days of imbibition, which then progressively disappeared (Figure 6C). Since this polypeptide was not detected in extracts from aleurone or scutellum cells, it is unlikely that it is synthesized and secreted from these tissues. It is equally unlikely that it is synthesized in the starchy endosperm, which is a dead tissue in seeds after germination [28].



**Figure 6** Western-blot analysis of NTR accumulation in wheat

(A) Protein extracts (15  $\mu$ g) from mature wheat seeds and increasing amounts (2, 10 and 50 ng) of the purified His-tagged TaNTR. (B) An equal amount of extracts (15  $\mu$ g of protein) from developing seeds at 18 d.p.a. (DS), mature seeds (MS), roots and shoots from 2–4-day-old seedlings, or leaves (L) from 7-week-old plants. Molecular-mass markers are shown on the left-hand side. (C) Protein extracts (15  $\mu$ g) from scutellum, aleurone layer or starchy endosperm dissected from seeds after 1–4 days of imbibition were subjected to SDS/PAGE, electro-transferred on to nitrocellulose sheets and probed with wheat anti-NTR antibodies.

Therefore this band may correspond to non-specific detection by the antibodies in extracts from the starchy endosperm or the union of NTR with another protein in the process of degradation.

The presence of several NTR genes in plants is not surprising. In *Arabidopsis*, at least three different genes encode putative NTRs (Figure 1). One of them, referred to as AtNTRB in Figure 1 (accession no. CAB54874), previously reported by Jacquot et al. [21], encodes for a protein of 35 kDa and shows a close similarity (76%) to the wheat NTR reported here. A second one, referred to as AtNTRA in Figure 1 (accession no. AAB86519), encodes for a mitochondrial NTR [8] and, therefore, is probably not detected in our Western-blot analysis. Finally, a third gene encoding a putative NTR, referred to as AtNTRC in Figure 1, was identified in *Arabidopsis* (accession no. AAB84351). As shown by the phylogenetic analysis, this gene shows a lower level of similarity (52%) and encodes a protein with a deduced molecular mass of 58.3 kDa. If the monocots contain a similar NTR gene, either it is not expressed in wheat or it has not been detected by the antibody used in the present study.

Interestingly, the Western-blot analysis of the accumulation of NTR shows a wide distribution of this enzyme in most wheat tissues as it occurs in thioredoxin *h*. Although it is remarkable that there is a low content of NTR in seeds, where thioredoxins *h* accumulate at high level [18], NTR accumulates in scutellum and aleurone cells of seeds after germination, in agreement with the increase in thioredoxins *h* in these important tissues of the germinating seeds [18]. These results suggest an important role of the NTR–thioredoxin *h* system for the success of germination. To date, three thioredoxins *h* have been described in wheat

[18,29], but it is likely that more thioredoxins *h* are present. To understand fully the role of the NTR–thioredoxin *h* system in seeds of cereals, it is necessary to determine whether they show any specificity of expression in the different organs and also to test the reactivity of each of these thioredoxins *h* with NTR.

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