Cloning and characterization of thioredoxin h in the three-pistil line of common wheat

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ABSTRACT. Thioredoxin h (Trxh) is a ubiquitous protein that reduces disulfides in target proteins, and is itself reduced by NADPH-thioredoxin reductase. In the current study, the complementary DNA sequence and the genomic sequence of the three-pistil (TP) line of common wheat (*Triticum aestivum* L.) were obtained from spikes through reverse transcription-polymerase chain reaction (RT-PCR) and touchdown-PCR. Sequence alignment of amino acids of TPTrxh then allowed for predictions of its physicochemical properties, secondary structures, tertiary structures, and functional domains. Furthermore, the TPTrxh gene was overexpressed in *Escherichia coli* and its activity was demonstrated using a dithiothreitol-dependent insulin assay. The expression patterns of TPTrxh were analyzed through real-time RT-PCR in different tissues and across different developmental stages of young spikes. The complementary DNA of TPTrxh was found to be 411 bp in length, encoding 118 amino acids. Its genomic sequence was determined to be 2632 bp, possessing 3 exons and 2 introns. Functional domain analysis indicated that TPTrxh contained a WCGPC motif located at the end of the second β -fold and on the initial side of the second α -helix. The TPTrxh protein reduces intramolecular and intermolecular

disulfide bridges in target proteins. Young spikes contain higher levels of TPTrxh transcripts than do stems and leaves. The transcript levels in the young spikes (2-5 mm in length) of the Chinese Spring TP line increased 2.84-fold relative to those of young spikes (2-5 mm in length) of the Chinese Spring line. These data provide a basis for future research into the function of Trxh, and offer further insight into the molecular mechanism of the TP mutation in wheat.

Key words: Thioredoxin h; Three-pistil line; Real-time RT-PCR; Wheat

INTRODUCTION

Thioredoxins (Trxs) are small, ubiquitous proteins with a molecular mass of 12-13 kDa. These proteins contain a redox disulfide bridge that participates in thiol-disulfide reactions via 2 Cys residues found in a conserved active-site motif (CXXC) (Meyer et al., 2005). This motif is found in proteins of the Trx superfamily, such as Trxs and glutaredoxins, which function in reducing the intramolecular and intermolecular disulfide bridges in target proteins (Martin, 1995; Buchanan and Balmer, 2005). Several Trxs are found in plants, namely, the chloroplast Trxs (f, m, x, y, and CDSP32), the mitochondrial Trxo, and Trxh, which was previously thought to be present exclusively in the cytosol (Williams et al., 2000).

Trxh, which is found in most plant organs, is composed of an NADPH-Trx reductase module fused with a Trx module and uses NADPH as a reducing agent (Serrato et al., 2001; Reichheld et al., 2005). Trxh actively undergoes oxidation-reduction during development (Gobin et al., 1997; Rhazi et al., 2003; De Gara et al., 2003), reducing storage proteins and α -amylase inhibitors (Lozano et al., 1996; Yano et al., 2001). In addition, Trxh plays an important role in the mobilization of storage proteins at early stages of germination because it reduces and activates the seed-specific serine protease, thiocalsin (Besse et al., 1996). The accumulation of Trxh in the nucleus of aleurone and scutellum cells exposed to oxidative stress during seed maturation and germination (Serrato et al., 2001; Serrato and Cejudo, 2003) has led to the proposal that Trxh also interacts with nuclear factors.

The common wheat (*Triticum aestivum* L.) line three pistil (TP), which was selected by Peng (2003) from the “trigrain” wheat cultivar, is a valuable mutant for improving wheat yield potential by increasing grain number per spike and per unit area. In a previous study, an expressed sequence tag (EST) sequence was isolated from the suppression subtractive hybridization complementary DNA (cDNA) library for this line (Yang et al., 2011). The EST was tentatively designated as TPTrxh when BLASTx searches of the sequences against the GenBank database revealed that it is homologous to wheat Trxh. To date, most studies on Trx in wheat have focused on developing seeds (Gobin et al., 1997; Serrato and Cejudo, 2003). However, its role in TP wheat spikes is not clear.

In the current study, homologous primers were designed based on the wheat Trxh sequence, and then the TPTrxh gene was cloned from TP cDNA and genomic DNA. The sequence characteristics of the protein encoded by the cDNA were analyzed and compared with those of the Trxh in other crops. Furthermore, TPTrxh was overexpressed in *Escherichia coli* and its activity was demonstrated using a dithiothreitol (DTT)-dependent insulin assay.

The expression patterns of TPTrxh were analyzed through real-time reverse transcription-polymerase chain reaction (RT-PCR) in different tissues and developmental stages of young spikes.

MATERIAL AND METHODS

Plant materials

The common wheat TP line, Chinese Spring TP (CSTP), a near-isogenic line of the common wheat Chinese Spring that contains a TP gene (*Pis1*) derived from the TP mutant, and Chinese Spring (CS) were used in the current study. The common wheat TP line, near-isogenic line CSTP, and its recurrent parent, CS, were all grown in an experimental field at the China West Normal University, Nanchong, China. Young spikes were collected at the reproductive stage and examined under a stereomicroscope (Olympus SZX9). Flag leaves and spikes of TP were selected for DNA and mRNA extraction (spike size about 5 mm). The spikes of CSTP and CS at various developmental stages (2-5, 5-7, and 7-10 mm in length), flag leaves, and stems of CSTP at the time corresponding to when spikes were 2-5 mm in length, were selected for real-time PCR analysis.

DNA and RNA isolation

The genomic DNA was isolated from fresh flag leaves of TP, according to methods described in Porebski et al. (1997). The obtained DNA was dissolved in Tris-EDTA buffer and kept at -20°C. Total RNA was isolated from young spikes according to methods described in Manickavelu et al. (2007). The total RNA was dissolved in RNase-free double-distilled water, and kept at -70°C. The quality of DNA and RNA was confirmed through gel electrophoresis and the concentrations were calculated using a spectrophotometer at 260/280 nm.

Primer design, RT-PCR, cloning of cDNA sequence, and sequencing

The PCR primers were designed by Primer Premier 5.0 based on the mRNA sequence of TPTrxh from *T. aestivum* (AY072771 and AK330735). The specific primers of the cDNA sequence are as follows: TPTrxh-1F, 5'-CACCTTCGATCTCATCACCG-3' and TPTrxh-1R, 5'-CAAGAGGGGCATCACTCTTCC-3'.

Total RNAs were synthesized into first-strand cDNA using the Super Smart cDNA Synthesis Kit according to manufacturer instructions (Clontech, Palo Alto, CA, USA). Standard PCR amplification was performed separately with cDNA from the young spikes as the template. The PCR products were separated by electrophoresis on agarose gel along with a molecular size marker (DL2000, TaKaRa, Dalian, China), followed by sequencing of all amplified bands at Taihe Biotechnology Co., Ltd. (Beijing, China).

Cloning of the complete genomic sequence of TPTrxh

The PCR primers were also designed by Primer Premier 5.0 based on the cDNA and genomic sequences of the Trxh protein from the grain crops studied, including *T. aestivum*, *Oryza sativa*, and *Sorghum bicolor*. The specific primers of the genomic sequence are as fol-

lows: TPTrxh-2F, 5'-GATCTCATCACCGGGGAAGG-3' and TPTrxh-2R, 5'-ACACAAGAGGGGCATCACTC-3'.

The genomic sequence of the TPTrxh gene was amplified using touchdown-PCR under the following conditions: predenaturation at 94°C for 4 min, followed by one cycle at 94°C for 30 s, 68°C for 45 s, 72°C for 3 min, then decreasing the melting temperature from 68° to 63°C by 0.5°C every cycle for 10 cycles, and subsequently continuing the reaction for another 25 cycles at 63°C. The amplified fragment was also purified and sequenced by Taihe Biotechnology Co., Ltd.

Construction of the expression vector and overexpression of recombinant TPTrxh

PCR fragments corresponding to the TPTrxh polypeptide were amplified from the TPTrxh cDNA clone with the primer TPTrxh-3F: 5'-TGAATTCGGAAGGGGGATGG-3' (*EcoRI*) and TPTrxh-3R: 5'-GGTAAGCTTCTTCCCTTCTTAGGC-3' (*HindIII*).

The PCR was performed at 94°C for 4 min; 35 cycles of 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C; 10 min at 72°C. The amplified PCR product was cleaved using *EcoRI* and *HindIII*, then ligated into the corresponding site of the pET28a vector (Merck) at 16°C for 20 h. The recombinant plasmid construct was transformed into an *E. coli* BL21 (DE3) competent cell strain (Novagen) and used for induction by adding isopropyl β -D-1-thiogalactopyranoside at an OD₆₀₀ of 0.6 and culturing further for 4 h at 37°C, using the empty transformed BL21 (DE3) vector as the control. The recombinant protein samples were induced after 0, 0.5, 1.0, 2.0, 3.0, and 4.0 h.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The culture was centrifuged at 10,000 rpm for 5 min at room temperature after induction for 0.5, 1, 2, 3, and 4 h. The culture supernatant was concentrated with methanol and chloroform (3:1, v/v), and SDS-PAGE was performed to investigate protein production and purity using slab gels containing 12% (w/v) polyacrylamide on a Mini-Protein II Slab Cell Apparatus (Bio-Rad, Hercules, CA, USA). The protein samples were visualized by Coomassie brilliant blue R-250 staining.

Protein purification and activity assay

The recombinant proteins were purified by Ni-NTA affinity chromatography (Qiagen), according to manufacturer instructions. His-tagged TPTrxh activity was determined using a DTT-dependent insulin disulfide reduction assay (Cazalis et al., 2006).

Real-time RT-PCR

Using the near-isogenic line CSTP and its recurrent parent, CS, total RNA was isolated from spikes at various developmental stages (2-5, 5-7, and 7-10 mm in length). Total RNA was also isolated from the flag leaves and stems of CSTP at the time point corresponding to when spikes were 2-5 mm in length. The cDNA was synthesized using the PrimeScript Perfect Real-Time RT Reagent Kit (TaKaRa). The primers were designed using the Primer Express

2.0 software to amplify 110-bp fragments for the TPTrxh gene (TPTrxh-4F: 5'-TGCCACACC AAGCAAGAGTTC-3' and TPTrxh-4R: 3'-GGGCTATGACACGACAAGGAC-5').

Real-time expression assays were performed with SYBR Green dye (TaKaRa) using the BIO-RAD CFX-96 real-time PCR platform (Bio-Rad). Fold-changes of RNA transcripts were calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) with the wheat housekeeping genes actin (AB181991) and 18S (AY049040) as the internal controls (Yamada et al., 2009; Wang et al., 2010).

Data analysis

The sequence data were analyzed with the GenScan software (<http://genes.mit.edu/GENSCAN.html>). For homology analysis, wheat TPTrxh was compared with amino acid sequences of other species using BLAST 2.1 (<http://www.ncbi.nlm.nih.gov/blast/>). The open reading frame (ORF) of the DNA sequence was searched using the ORF finder software (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Basic parameters and secondary structure of the TPTrxh sequence were deduced using the PredictProtein software (<https://www.predictprotein.org/>). The tertiary structure of the TPTrxh sequence was simulated with the SWISS-MODEL software (<http://swiss-model.expasy.org/>). Multiple sequence alignment was performed using DNASTar Lasergene and DNAMAN 6.0 softwares.

RESULTS

Analysis of the cDNA of TPTrxh

One cDNA fragment was amplified from the TP line using the primers TPTrxh-1F and TPTrxh-1R (Figure 1). The sequencing results indicated that the length of the cDNA was 411 bp. The cDNA sequence shares a high homology with the Trxh1 (Accession No. AY072771) sequence from wheat seeds, which indicates that the cDNA encoding TPTrxh was isolated from the TP line. The TPTrxh cDNA sequence was submitted to GenBank (Accession No. JF769863). The 411-bp TPTrxh cDNA sequence contains a 30-bp 5'-untranslated region (5'-UTR) and a 25-bp 3'-UTR. A 357-bp ORF, containing 22.7% A, 24.9% C, 29.7% G, and 22.7% T, encodes 118 amino acids (Figure 2). Alignment analysis of the cDNA sequences between the TP and other grain crops, including *T. aestivum* (AY072771.1), *Hordeum vulgare* (AY245454.1), *O. sativa* (NM_001065604.1), *Zea mays* (EU970059.1), and *S. bicolor* (XM_002461523.1), was performed using the DNASTar Lasergene software. The homologies in the coding sequences of TP and the other grain crops were 100, 94.96, 76.69, 74.18, and 76.75%, respectively. The stop codon TAG was used in *Z. mays*, whereas TAA was used for TP and the other grain crops.

Analysis of the genomic sequence of TPTrxh

A DNA fragment was amplified with primers TPTrxh-2F and TPTrxh-2R (Figure 3). The length of the DNA fragment was 2632 bp (Accession No. JF769862). It was compared with the cDNA sequence and the DNA sequence of the TPTrxh amplified from TP using the DNASTar Lasergene software. The results indicated that the genomic sequence of TPTrxh possesses 3 exons and 2 introns, with lengths of 90, 123, 144, 2143, and 79 bp, respectively. The junction sequence of the exons and introns is consistent with the laws of gene composition

(GT-AG), which was also supported by a restriction map of the genomic and cDNA sequences (Figure 4). The G+C contents of intron 1 and intron 2 were 41.63 and 40.00%, respectively, but were substantially higher in exon 1 (61.11%), exon 2 (53.66%), and exon 3 (51.39%). Comparison of the genome sequences of TP with those of *O. sativa* and *S. bicolor* revealed that the lengths of the first and second exons of these 3 grain crops are the same, whereas the third exon of *O. sativa* is 12 bp longer than the others. However, the intron, the 5'-untranslated, and the 3'-untranslated sequences differed in length, especially the first intron of TP, which was twice the length of the others. Furthermore, alignment analysis comparing the coding regions between TP and the other grain crops showed that changes in the nucleotide coding sequence of the Trxh gene involved transition and transversion, but not insertions and deletions.

Figure 1. Reverse transcription polymerase chain reaction products of TPTrxh. Lane M = molecular ladder DL2000; lane 1 = amplified TPTrxh.

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1  CACCTTCGATCTCATCACCGGGGAAGGGGG  ATG GCC GCC GAG GAG GGA GCC GTG ATA GCG
1      M A A E E G A V I A
61  TGC CAC ACC AAG CAA GAG TTC GAC ACC CAC ATG GCT AAT GGC AAG GAG ACC GGC
11  C H T K Q E F D T H M A N G K E T G
115 AAG CTG GTG ATC ATT GAC TTC ACT GCT TCC TGG TGC GGT CCT TGT CGT GTC ATA
29  K L V I I D F T A S W C G P C R V I
169 GCC CCA GTC TTT GCT GAG TAC GGC AAG AAG TTC OCT GGC GCC ATT TTC CTG AAG
47  A P V F A E Y A K K F P G A I F L K
223 GTG GAC GTT GAC GAG CTG AAG GAC GTC GCT GAA GCA TAC AAC GTT GAG GCA ATG
65  V D V D E L K D V A E A Y N V E A M
277 CCG ACC TTC CTG TTT ATC AAG GAT GGT GCG AAG GTG GAC ACT GTT GTC GGT GGC
83  P T F L F I K D G A K V D T V V G G
331 AGG AAG GAT GAT ATC CAT ACC AAG ATA GTG GCC CTC ATG GGT TCT GCA TCT GCC
101 R K D D I H T K I V A L M G S A S A
385 TAA GAAGGGAAGAGTGATGCCCTCTTG
119 *
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Figure 2. Nucleotide sequence of cDNA encoding the thioredoxin (Trxh) and the amino acid sequence deduced from its open-reading frame. Nucleotides are numbered in the 5'-3' direction. The predicted amino acid sequence of the Trxh protein is shown under the nucleotide. (*) indicates the stop codon.

Figure 3. PCR products of complete genomic sequence of TPTrxh from the three-pistil line. *Lane M* = molecular ladder DL15000; *lanes 1, 2* = amplified TPTrxh complete sequences.

[illegible]

Figure 4. Genomic sequence of TPTrxh and amino acid deduced from 3 exons.

Analysis of the TPTrxh protein

Basic parameters of the TPTrxh protein

The composition and the physical and chemical characteristics of the amino acid sequence of Trxh in TP, *H. vulgare*, *O. sativa*, *Z. mays*, and *S. bicolor* were analyzed using the ProtParam software, according to methods described in Gasteiger et al. (2005). The results revealed that Trxh from different crops show slight variations in the length of the amino acid sequence, molecular weight, theoretical pI, and the number of negative and positive charged amino acids (Table 1). The Trxh sequences in these crops are all rich in Ala, Lys, and Val, whereas Asp and Gly are also abundant in TP and *H. vulgare*, and Glu is also abundant in *O. sativa*, *Z. mays*, and *S. bicolor*. The number of negatively charged amino acids was distinctly higher than that of positively charged ones, which indicates that Trxh is an acidic protein. The instability indices of Trxh from all crops were significantly less than 40 (range: 16.25-23.13), which implies that Trxh is a stable protein. The solubility and stability of Trxh can therefore be used to increase the solubility and stability of certain peptides or proteins.

Table 1. Analysis of compositions, physical, and chemical characters of the amino acid sequence of thioredoxin among the three-pistil line, *Hordeum vulgare*, *Oryza sativa*, *Zea mays*, and *Sorghum bicolor*.

Lines	Accession No.	Length of amino acid sequence	Molecular weight (kDa)	Theoretical pI	Negatively charged (%)	Positively charged (%)	Abundant amino acids (%)			Instability index
TP	AAL67139.1	118	12.69	5.29	14.41	11.02	Ala 14.41	Asp 7.63	Lys 9.32	18.71
							Gly 7.63			
							Val 10.17			
<i>H. vulgare</i>	AAP72290.1	118	12.75	5.09	15.25	11.02	Ala 12.71	Asp 8.47	Lys 9.32	20.43
							Gly 7.63			
							Val 10.17			
<i>O. sativa</i>	NP_001059069.1	122	13.16	5.17	15.57	12.3	Ala 17.21	Glu 8.20	Val 11.48	22.73
							Lys 10.66			
<i>Z. mays</i>	ACG42177.1	120	12.98	5.92	13.33	10.83	Ala 18.30	Glu 8.30	Val 12.50	23.13
							Lys 9.20			
<i>S. bicolor</i>	XP_002461568.1	118	12.9	5.79	14.41	11.86	Ala 16.10	Glu 10.17	Val 9.32	16.25
							Lys 10.17			

Prediction and analysis of the protein's functional sites

Primary structure analysis revealed that the molecular weight of the putative TPTrxh protein is 12.6937 kDa, with a theoretical pI of 5.29. Topology prediction shows that there is one N-glycosylation site, one casein kinase II phosphorylation site, one protein kinase C phosphorylation site, one amidation site, and a Trx family active site in the TPTrxh protein (Figure 5). Further analysis indicated that TPTrxh contains a third Cys residue (Cys11; Figure 5, triangle) on the top of the 2 Cys residues present at the active site (Cys 40 and 43; Figure 5, inverted triangles).

Structure of TPTrxh

As predicted by the PredictProtein Server program, the secondary structure of TPTrxh is mainly composed of α -helices (47.46%) and random coils (25.42%), as well as β -turns

TP	MAAEGAVIACHTKQEPDTHMANGKETGKLMIDFTASWOGPCRVIAPVFREYAKKFGAIF	62
<i>T. aestivum</i>	MAAEGAVIACHTKQEPDTHMANGKETGKLMIDFTASWOGPCRVIAPVFREYAKKFGAIF	62
<i>H. vulgare</i>	MAAEGAVIACHTKQEPDTHMANGKDTGKLMIDFTASWOGPCRVIAPVFREYAKKFGAIF	62
<i>O. sativa</i>	MAAEGVVIACHNKDEPDAQMTKAKEAGKVMIDFTASWOGPCRVIAPVFREYAKKFGAVF	62
<i>Z. mays</i>	MASEGCVVIACHTKADFDHMAKAKEAGKLMIDFTASWOGPCRFIAPLFVEHAKKFTQAVF	62
<i>S. bicolor</i>	MASEGCVVIACHTKAEFDAQMAKAKEAGKLMIDFTASWOGPCRFIAPLFVEHAKKFTQAVF	62
TP	LKVDVDELKDVAEAYNVEAMPTFLFIKDGAKVDTVWGGRKDDIHTKIIVLMG---SASA---	118
<i>T. aestivum</i>	LKVDVDELKDVAEAYNVEAMPTFLFIKDGAKVDTVWGGRKDDIHTKIIVLMG---SASA---	118
<i>H. vulgare</i>	LKVDVDELKDVAEAYNVEAMPTFLFIKDGAKVDTVWGGRKDDIHTKIIVLMG---SAST---	118
<i>O. sativa</i>	LKVDVDELKEVAEAYNVEAMPTFLFIKDGAEADKVVGARKDDLQNTIIVKHVGATAASASA---	122
<i>Z. mays</i>	LKVDVDELKEVAAYNVEAMPTFHFVKNGVTETVVGARKENLLAQIEKHCA---AAVFAA	120
<i>S. bicolor</i>	LKVDVDELKEVIAEYKIEAMPTFHFVKNGVTETIIVGARKDELLALIQKHTA---SASA---	118

—, N-glycosylation site; ■, casein kinase II phosphorylation site; ==, protein kinase C phosphorylation site; ~~, cAMP- and cGMP-dependent protein kinase phosphorylation site; ---, amidation site; □, thioredoxin family active site.

Figure 5. Comparison of the amino acid sequence of thioredoxin (Trxh) among the TP, *Triticum aestivum*, *Hordeum vulgare*, *Oryza sativa*, *Zea mays*, and *Sorghum bicolor*. TP = common wheat (*T. aestivum* L.) line “three pistils” (TP). Characteristic residues Cys11 (triangle) and Cys 40 and 43 (inverted triangles) of TPTrxh are also marked.

(8.47%) and extended strands (18.64%) (Figure 6A). The tertiary structure of TPTrxh was modeled using the structure of *H. vulgare* (PDB accession number 2vm1C) as a template. It contained 5 β -folds surrounded by 4 α -helices. The active site (WCGPC), the conserved structure of the Trx family (Svensson et al., 2007), was located at the end of the second β -fold and initial side of the second α -helix (Figure 6B).

Figure 6. A. Secondary structure of TPTrxh. H = α -helices, E = extended strands, T = β -turns, and C = random coils. B. Tertiary structure of TPTrxh modeled using the structure of *Hordeum vulgare* (PDB Accession No. 2vm1C) as a template.

Overexpression in *E. coli*, purification, and enzyme activity of the TPTrxh protein

SDS-PAGE was used for expression analysis of protein extracts from recombinants in *E. coli* BL21 (DE3) strains after induction with isopropyl β -D-1-thiogalactopyranoside (Figure 7). The results show that the fusion of the TPTrxh protein with an N-terminal His-tagged form caused the expected accumulation of a 16-kDa polypeptide. Recombinant TPTrxh was found in the soluble fraction after induction for half an hour, and the output of the induction continued after this point. The recombinant proteins were purified by Ni-NTA affinity chromatography after 4 h of induction (Figure 7). The recombinant TPTrxh activity was determined with a DTT-dependent insulin reduction assay (Figure 8).

Figure 7. Protein extracted from recombinant *Escherichia coli* BL21 (DE3) strains analyzed by SDS-PAGE and stained with Coomassie blue R250. The arrow indicates the recombinant protein bands induced by isopropyl β -D-1-thiogalactopyranoside after 0, 0.5, 1.0, 2.0, 3.0, and 4.0 h (lanes 3-8), respectively. Lane 1 = protein molecular weight marker (low, kDa); lane 2 = product of *E. coli* strains with the empty vectors used as control; lanes 9-10 = elution recovered the first and second column volumes - purified products; 16.8-kDa recombinant protein is marked by arrow.

Figure 8. Activity of TPTrxh determined with the DTT-dependent insulin reduction assay. The incubation mixture contained 100 mM potassium phosphate buffer, pH 7.1, 2 mM EDTA, 0.5 mg/mL insulin, 0.5 mM DTT, and 4 μ M TPTrxh as indicated. Assays in the absence of TPTrxh showing no activity were undertaken as controls.

Real-time PCR results

Real-time PCR was used to study the expression patterns of TPTrxh in different tissues across developmental stages of young spikes. As shown in Figure 9, TPTrxh transcripts accumulated to higher levels in the young spikes than in the stems and leaves. In the young CSTP spikes at stages 5-7 and 7-10 mm, the transcript levels were similar to the levels observed in CS. However, the transcript levels increased 2.84-fold in the young CSTP spikes at stages 2-5 mm compared with levels in CS at the same stages.

Figure 9. Quantitative RT-PCR analysis. Actin and 18S were used as internal controls. The transcript levels are shown as relative values. Using near-isogenic line Chinese Spring TP (CSTP) and its recurrent parent Chinese Spring (CS), total RNA was isolated from spikes at various developmental stages (2-5, 5-7, and 7-10 mm in length). Total RNA was also isolated from flag leaves and stems of CSTP at the corresponding time point that spikes were 2-5 mm in length.

DISCUSSION

In the current study, an EST sequence was isolated from the suppression subtractive hybridization cDNA library of TP wheat lines, which exhibited functions similar to those of Trxh. The EST was therefore tentatively designated as TPTrxh. The genome sequence, containing 3 exons and 2 introns, was cloned successfully from wheat for the first time. The first intron is twice the length of the others. Using RT-PCR, the ORF of TPTrxh was also obtained. Functional domain analysis indicated that TPTrxh is a Trxh protein and that it

contains a WCGPC motif. Although the nucleotide and amino acid sequences from different plants show some variation, the N-terminus of these proteins contains a conserved active-site motif. The non-conserved sequences of the TPTrxh protein may be responsible for the specific recognition of target proteins.

Trxh represents a large multigenic family, which can be classified into 3 subgroups based on analysis of their primary structure (Gelhaye et al., 2004). TPTrxh obtained from the TP wheat lines is classified into subgroup 1 (Yang et al., 2011). Members of subgroup 1 are abundant in the phloem sieve tubes of *Arabidopsis thaliana* and other plants (Ishiwatari et al., 1995; Balachandran et al., 1997; Reichheld et al., 2002; Santandrea et al., 2002). The TP mutant has 2 more pistils per floret compared to normal wheat. Therefore, TP mutants require relatively more nutrients during pistil development, which might induce overexpression of Trxh in young spikes (Yang et al., 2011). Overexpression of TPTrxh was observed in young spikes of CSTP at the 2-5 mm stage, just as the pistil primordia were protruding. Therefore, the overexpression of TPTrxh might be functionally important in determining the defining morphology of the TP trait.

Characteristics of programmed cell death (PCD), such as shrunken nuclei, were observed in a study of the degradation of pea carpels, and a DNA gradient was observed on gel electrophoresis (Orzaez and Granell, 1997). Similar observations were made with respect to the degradation of pistil primordial cells in the male inflorescence of *Z. mays* (Calderon-Urrea and Dellaporta, 1999). Therefore, the additional 2 pistils in TP mutants likely formed when 2 carpels that were not capable of degenerating in the normal way developed into complete pistils instead. Demura and Fukuda (1994) found that when the mesophyll cells of chrysanthemum were induced into tracheary elements, they accumulated a large amount of O_2^{2-} and H_2O_2 , which can easily damage cell membranes and induce subsequent PCD. A hypersensitivity reaction in plants due to oxidative bursts also results in the accumulation of O_2^{2-} and H_2O_2 (Mehdy, 1994; Lamb and Dixon, 1997). However, cell death has also been shown to be inhibited in the hypersensitivity reaction under hypoxic conditions (Mittler and Lam, 1996). Together, these observations suggest that reactive oxygen is most likely the common predisposing factor for PCD in plants. Peroxiredoxin reduces O_2^{2-} and H_2O_2 and may play a role in the regulation of, as well as in protection against, oxidative injury (Blokhina et al., 2003). Trxs are electron donors to peroxiredoxin, which are involved in protection against oxidative stress (Jung et al., 2002). Purification and activity analyses have demonstrated that TPTrxh is involved in redox reactions by reducing the intramolecular and intermolecular disulfide bridges in target proteins. Therefore, the induction of TPTrxh overexpression that is triggered by O_2^{2-} and H_2O_2 in young spikes during early developmental stages could protect against oxidative stress and prevent PCD in the pistil primordium, which is essential for the development of the TP trait.

In the current study, the cDNA and genomic sequences were obtained by RT-PCR and touchdown-PCR. Results indicated that the TPTrxh gene might be involved in the expression of the TP trait; however, the detailed function of TPTrxh needs further study.

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