

The Level of Expression of Thioredoxin is Linked to Fundamental Properties and Applications of Wheat Seeds

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ABSTRACT Work with cereals (barley and wheat) and a legume (*Medicago truncatula*) has established thioredoxin *h* (Trx *h*) as a central regulatory protein of seeds. Trx *h* acts by reducing disulfide (S–S) groups of diverse seed proteins (storage proteins, enzymes, and enzyme inhibitors), thereby facilitating germination. Early *in vitro* protein studies were complemented with experiments in which barley seeds with Trx *h* overexpressed in the endosperm showed accelerated germination and early or enhanced expression of associated enzymes (α -amylase and pullulanase). The current study extends the transgenic work to wheat. Two approaches were followed to alter the expression of Trx *h* genes in the endosperm: (1) a hordein promoter and its protein body targeting sequence led to overexpression of Trx *h5*, and (2) an antisense construct of Trx *h9* resulted in cytosolic underexpression of that gene (*Arabidopsis* designation). Underexpression of Trx *h9* led to effects opposite to those observed for overexpression Trx *h5* in barley—retardation of germination and delayed or reduced expression of associated enzymes. Similar enzyme changes were observed in developing seeds. The wheat lines with underexpressed Trx showed delayed preharvest sprouting when grown in the greenhouse or field without a decrease in final yield. Wheat with overexpressed Trx *h5* showed changes commensurate with earlier *in vitro* work: increased solubility of disulfide proteins and lower allergenicity of the gliadin fraction. The results are further evidence that the level of Trx *h* in cereal endosperm determines fundamental properties as well as potential applications of the seed.

Key words: metabolic regulation; molecular physiology; seed biology; preharvest sprouting; seed germination; thioredoxin *h*; wheat allergenicity.

INTRODUCTION

Research during the past two decades has witnessed the emergence of *h*-type thioredoxin (Trx *h*) as a central regulator of seed germination. Thus, in cereals, extensive evidence indicates that disulfide proteins stored in the dry seed are reduced to the sulfhydryl state by Trx *h* following the addition of water (Kobrehel et al., 1992; Gobin et al., 1996; Lozano et al., 1996; Cho et al., 1999; Yano et al., 2001; De Gara et al., 2003; Maeda et al., 2003; Marx et al., 2003; Rhazi et al., 2003; Wong et al., 2003; Maeda et al., 2004, 2005; Shahpiri et al., 2008; Zahid et al., 2008). Trx-linked reduction taking place during germination increases both the solubility of grain proteins and their susceptibility to proteolysis, thereby facilitating emergence and growth of the new seedling (Jiao et al., 1992, 1993; Gautier et al., 1998; Serrato et al., 2002; Maeda et al., 2003; Wong

et al., 2003, 2004a, 2004b; Faris et al., 2008). Further, overexpression of Trx *h* in the starchy endosperm of barley seeds led to an increase in pullulanase activity (Cho et al., 1999) and accelerated germination and release of α -amylase (Wong et al., 2002). A recent study extended the role of Trx *h* to germination in a dicot, the legume, *Medicago truncatula* (Alkhalfioui et al., 2007).

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Results of the earlier experiments with transgenic barley raised the question of the effects of manipulation of the Trx *h* gene on the properties of wheat grain. This question has been addressed in the present work using two approaches, namely the overexpression and underexpression of Trx *h* genes in the starchy endosperm. These experiments yielded positive results with both approaches: increased solubility and lower allergenicity of the gliadin storage protein fraction with overexpressed lines (Trx *h5*) (*Arabidopsis* numbering), and reduced rate of germination and mitigation of preharvest sprouting with an underexpressed counterpart (Trx *h9*). In addition to extending fundamental knowledge, the current findings suggest that manipulation of Trx *h* genes can enhance both the quality and yield of cereal grain. Certain of the results presented have been previously reported in preliminary communications (Zhou et al., 2006; Buchanan et al., 2007; Guo et al., 2007; Ren et al., 2007).

RESULTS AND DISCUSSION

Experiments with Overexpressed Trx *h*

Overexpression of Trx *h*

A gene construct similar to that earlier found successful with barley (Cho et al., 1999) was used in the present transformation experiments. The construct consisted of the B₁-hordein promoter, B₁-hordein signal sequence, wheat Trx *h* (AAF88067), and a 3' nos terminator (Figure 1). Due to its amino acid similarity to *Arabidopsis* Trx *h5* (59%, see Supplemental Figure 1A and 1B), the wheat Trx gene used for overexpression is referred to here as 'wheat Trx *h5*'. Western blot analysis (Figure 2)

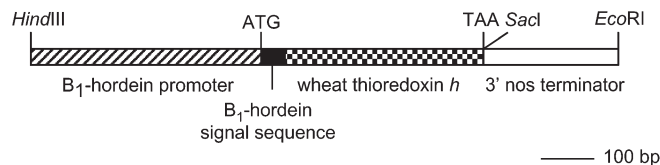


Figure 1. Plasmid pdBhssWTRXhN3-8 Used to Transform Wheat Overexpressing Trx *h* in Endosperm.

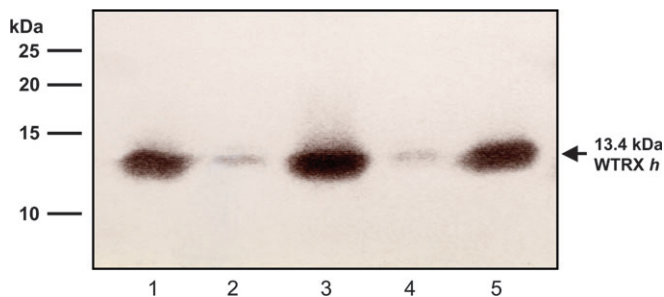


Figure 2. Overexpression of Trx*h5* in Wheat Seeds Revealed by Western Blot.

Lane 1: Anza cultivar heterozygote overexpressing Trx *h5*. Lane 2: Nontransgenic Anza control. Lane 3: Yecoroa Rojo heterozygote cultivar overexpressing Trx *h5*. Lane 4: Nontransgenic Yecoro Rojo control. Lane 5: Purified Trx *h* from wheat germ.

revealed that this construct resulted in 60 and 30-fold increases in Trx *h5* expression with the Yecoro Rojo cultivar relative to that observed in companion null segregant lines and nontransgenic controls, respectively (Table 1). Parallel experiments with heterozygous seeds of a second cultivar (Anza) revealed an enhanced expression of 10-fold relative to the wild-type nontransgenic line. These overexpression levels are similar to those found previously with transgenic barley (Cho et al., 1999).

Allergy Studies

The dog has been widely used as a model for human allergy in a range of studies, including the hypersensitive response to food (Frick, 1996; Buchanan et al., 1997; Ermel et al., 1997; del Val et al., 1999; Buchanan and Frick, 2002; Teuber et al., 2002; Frick et al., 2005). This model proved to be effective in studies relating the effect of Trx to the allergenicity of disulfide food allergens assessed by both skin tests and feeding challenges (e.g. wheat and milk). In earlier work with wheat, we applied canine skin tests (1) to assess the relative allergenicity of the Osborne protein fractions of grain (albumins, globulins, gliadins, and glutenins), and (2) to determine the effect of Trx-catalyzed reduction on the allergenicity of each (Buchanan et al., 1997). In that work, gliadins proved to be the strongest allergens, followed by glutenins, albumins, and globulins. Further, reduction by Trx mitigated the allergenicity associated with both the gliadins (α -, β -, and γ -types) and glutenins—but gave less consistent results with the minor fractions (albumins and globulins).

In the current study, we compared the allergenicity of homozygous transgenic Yecoro Rojo overexpressing Trx *h5* in the protein body with the corresponding null segregant (Table 2). In 28 different trials, the gliadin fraction of the transgenic line proved to be 3-fold less allergenic than the null segregant counterpoint (student's *t*-test = 0.023). The effect of overexpressed Trx *h5* on the other fractions (albumin/globulins,

Table 1. Densitometric Scanning of Western Blots Shows Relative Levels of Trx *h* in Transgenic and Null Segregant Wheat Seeds.

Line	Ratio of Trx <i>h</i> HT:NT	Ratio of Trx <i>h</i> HZ:NS
Yecoro Rojo 1	60 times	30 times
Anza 21	10 times	Not available

HT, heterozygote; NT, non-transgenic; HZ, homozygote; NS, null segregant.

Table 2. Overexpression of Trx *h* Mitigates Allergenicity of Gliadin Fraction from Wheat Seeds.

Protein fraction	Seed status	Number of trials (n)	Minimal protein (ng)		<i>p</i> -value	
			Average	STD	Sign rank	Student's <i>t</i>
Gliadin	HZ	29	8318	0.00	0.033	0.023
	NS	29	2518	0.02		

glutenins) was not significant (data not shown). In a recent case with human sera, the allergenic response to gliadins was also shown to be mitigated by reduction with Trx, itself reduced with NADPH and NADP-thioredoxin reductase (NTR) (Waga et al., 2008). Treatment of a salt-soluble wheat fraction with Trx showed similar mitigation effects (Matsumoto et al., 2007). It should be noted that a similar salt fraction from wheat endosperm in one of our studies contained several types of gliadins (Wong et al., 2004a).

Effect on Solubility

Earlier work has revealed that, among other changes, Trx-linked reduction increases the solubility of wheat seed proteins, including gliadins (Wong et al., 2004a). In view of the above allergenicity data, it was of interest to determine whether the decrease in allergenicity was accompanied by a change in gliadin solubility. The results in Table 3 show this to be the case: Trx *h5* overexpression in the protein body increased the solubility of the gliadin fraction by 14% (vs. 25% with 2-mercaptoethanol). Other experiments revealed a similar increase in the aqueous solubility of chloroform-methanol soluble (CM) proteins (data not shown). The results in Tables 2 and 3 jointly suggest that Trx *h5* enhances solubility and reduces the allergenic response to gliadin proteins, likely by changing epitopes exposed to IgE. As described above, results from other laboratories are in accord with this conclusion. Additional work is required, however, to determine whether the effect of Trx *h5* can be enhanced and extended to other major wheat allergens such as the glutenins, and also whether the overexpression of Trx is effective in decreasing the allergenicity of products such as bread and pasta. Future work should also consider the to-be-confirmed report that Trx *h*, itself, may have allergenic properties (Buchanan and Frick, 2007; Limacher et al., 2007).

Experiments with Underexpressed Trx *h*

The findings of the effects observed with Trx *h5* overexpressed in barley grain raised the question of the effect of lowering the expression of Trx *h*. Thus, in the present wheat study, experiments have been carried out with the goal of assessing the effect of underexpressing a member of this gene family on germination and the activity of associated enzymes. In general, as seen below, the germination effects were opposite to those obtained with Trx *h5* overexpression in barley (Cho et al., 1999; Wong et al., 2002). In the present experiments with

wheat, the relevant parameters were also monitored in developing grain. It is noted that the Trxs were differentially targeted in the two studies: the overexpressed Trx to the protein body, the underexpressed counterpart to the cytosol.

Underexpression of Trx

An expression vector, pBS–Gli–AntiTrx, including the entire coding sequence of an atypical Trx (*PTrx*) from the grass, *Phalaris coerulescens* (mRNA ID AF159388) (Juttner et al., 2000), was used in the transformation experiments (Figure 3). The gene codes for a protein highly similar to a wheat Trx *h* (AAN63622.1), sequence identity 95% vs. 65% for closest *Arabidopsis* Trx *h* (NP_187483 or Trx *h9*). Due to its amino acid similarity to *Arabidopsis* Trx *h9* (Supplemental Figure 1A and 1B), the targeted gene is referred to below as wheat Trx *h9*. Figure 4 shows that the *P. coerulescens* antisense *PTrx* gene, which was directed to the cytosol, effectively down-regulated endogenous wheat Trx *h9* in seeds from nine independent events (Supplemental Figure 2). Each of the nine transgenic lines showed significant down-regulation of Trx *h9* relative

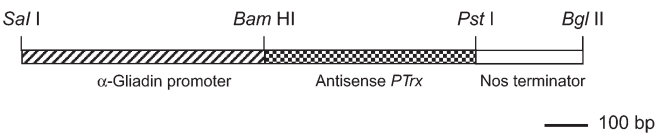


Figure 3. Plasmid pBS–Gli–AntiTrx Used to Transform Wheat Under-expressing Trx *h* in Endosperm. The antisense *PTrx* gene was from *P. coerulescens* (see Supplemental Figure 1C).

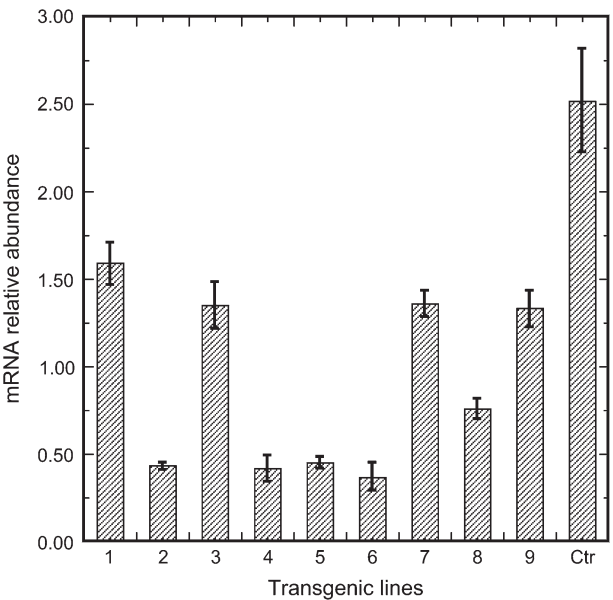


Figure 4. Relative Abundance of Trx *h9* mRNA in Germinating Transgenic Wheat Seeds from Nine Independent Transformation Events. The measurement was made 10 h after water addition.

Table 3. Gliadin Solubility Is Increased in Transgenic Wheat Seeds Overexpressing Trx *h*.

Extraction	Volume of immunoblot		% increased solubility HZ/NS $\times$ 100
	HZ	NS	
Buffer	9970	8721	114
Buffer + 2-ME	26975	21489	125

to the nontransgenic control. With four lines, expression was less than 20% that of the control. One of these lines, no. 5, was used in the experiments described below.

Further comment on the antisense experiments is in order. Supplemental Figure 1C shows that the *P. coeruleus* antisense gene (*PTrx*, AF159388) is quite similar to the wheat *Trx h9* counterpart (AF438359). This close similarity minimized the effect that *PTrx* might have on other Trx-related genes in wheat (Supplemental Figure 1A and 1D). It is also apparent that *Trx h9* differs markedly from other wheat Trxs, thereby helping to insure silencing specificity of the gene by *PTrx*.

Changes in Developing Grain

The effect of *PTrx* antisense was also followed biochemically using the insulin reduction assay. When measured in developing seeds, Trx activity was maximally decreased at 30 d post anthesis, but then the effect was progressively lost. Thus, 30 d after anthesis, the level of Trx activity was decreased 25% in the homozygote relative to the null segregant (Figure 5A). As the seed matured (days 50–70), this difference became progressively less and essentially disappeared at the end of the monitoring period. Further, relative to the null segregant, the decrease in Trx expression also led to a decrease in α -amylase activity that, again, disappeared when the transgenic seed reached maturity (Figure 5B). Finally, there was an ongoing lowering of pullulanase activity (up to 20% 70 d post anthesis) in the homozygote relative to the null segregant (Figure 5C). The results suggest that, while effecting a limited lasting decrease in pullulanase activity, the level of *Trx h9* has little effect on the activity of either Trx itself or α -amylase in mature seeds.

Effect on Seed Germination

Earlier experiments with transgenic barley revealed that *Trx h5* overexpressed in the endosperm accelerated the rate of germination by about 1 d. Based on this finding, it might be expected that underexpression of *Trx h* would have an opposite effect, namely to suppress germination. As shown in Figure 6, this expectation was fulfilled, albeit with a different *Trx h* isoform: germination of transgenic seeds with antisense *PTrx* was impeded for at least 6 d following the addition of water. Even on day 7, there still appeared to be a statistical difference between the transgenic and null segregant lines. In short, lowering the level of *Trx h9* by antisense technology inhibited germination significantly. Eight other independent lines underexpressing *Trx h9* showed similar effects. However, there were no adverse effects on total crop yield (data not shown). Moreover, similar effects on germination were observed with another white wheat cultivar (Yumai 70) underexpressing *Trx h9* (data not shown).

It is noted that the expression of Trx was suppressed to a greater extent during germination than during grain development. The extent of decrease of Trx activity in the homozygote ranged from 20 to 40% as germination progressed (Figure 7A). In future research, it will be of interest to determine whether *Trx h* isoforms other than *h9* are affected under

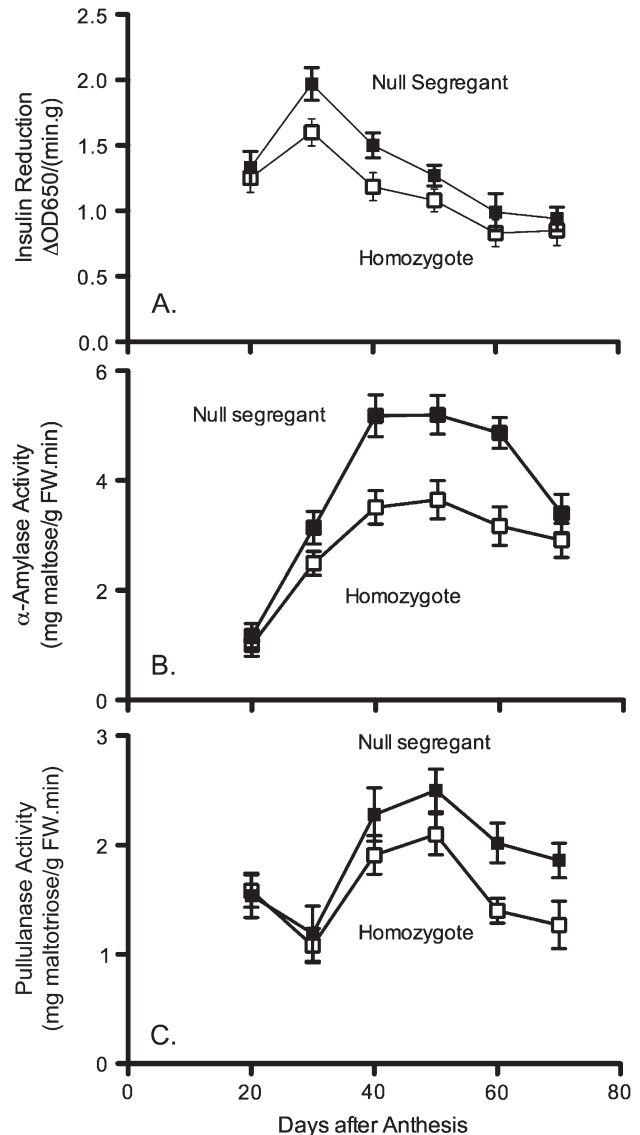


Figure 5. Activity changes of Trx and Associated Enzymes in Developing Wheat Seeds Underexpressing *Trx h9*.

(A) *Trx h* activity in developing wheat seeds underexpressing *Trx h9*.

(B) α -Amylase activity in developing wheat seeds underexpressing *Trx h9*.

(C) Pullulanase activity in developing wheat seeds underexpressing *Trx h9*.

these conditions. Based on mRNA sequence comparisons of the different forms of wheat *Trx h*, this would seem unlikely (Supplemental Figure 1D).

Changes in Associated Enzymes

The formation of enzymes functional in starch degradation, viz. α -amylase and pullulanase, has long been known to be linked to germination in cereals (Chrispeels and Varner, 1967; Jacobsen and Varner, 1967; Fincher, 1989). Thus, with transgenic barley having an increased level of *Trx h5* and an accelerated rate

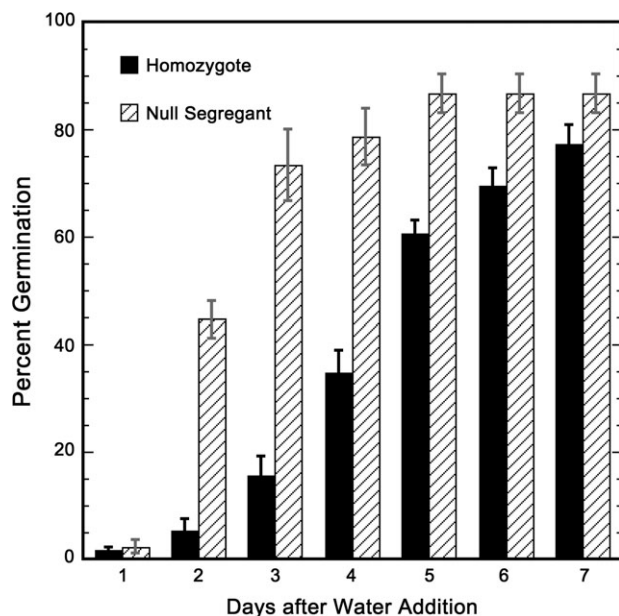


Figure 6. Germination of Wheat Seeds Underexpressing Trx *h9*.

of germination, the onset of α -amylase activity was expedited, and the activity of pullulanase increased throughout the germination period. The experiments with Trx *h9* underexpressed in wheat, in general, followed a path opposite to that observed previously with the overexpressed Trx *h5* in barley. That is, relative to the null segregant, the appearance of α -amylase activity of the homozygote was delayed by 2 d but then reached the level of the null segregant (Figure 7B). Pullulanase activity was marginally suppressed in the homozygote for 2 d after water addition, but then rose to a level roughly equal to that of the control (Figure 7C). These events were paralleled by Trx activity that was initially lower and remained static in the homozygote underexpresser, but progressively increased in the null segregant (Figure 7A). The results shown in Figure 7B and 7C are consistent with the conclusion that α -amylase functions first (early stage of starch breakdown, peak at 1 d) and is followed by pullulanase for starch debranching (day 2). Overall, the present experiments support and extend the earlier work with barley and strengthen the link between Trx *h* and germination. As noted above, decrease in the activity of both of these enzymes was also observed during seed development: with α -amylase, the effect was transitory (Figure 5B) whereas, with pullulanase, a 30% lowering of activity persisted from midway to the end of the monitoring period (Figure 5C). With respect to germination, it is noted that Trx *h*, assessed by western blots, decreased rapidly during germination in control barley seeds, whereas, as noted above, the activity of Trx in the null segregant wheat line remained relatively constant (Lozano et al., 1996).

Effect on Preharvest Sprouting

The retardation of germination with homozygous lines underexpressing Trx *h9* suggests that they might show protection

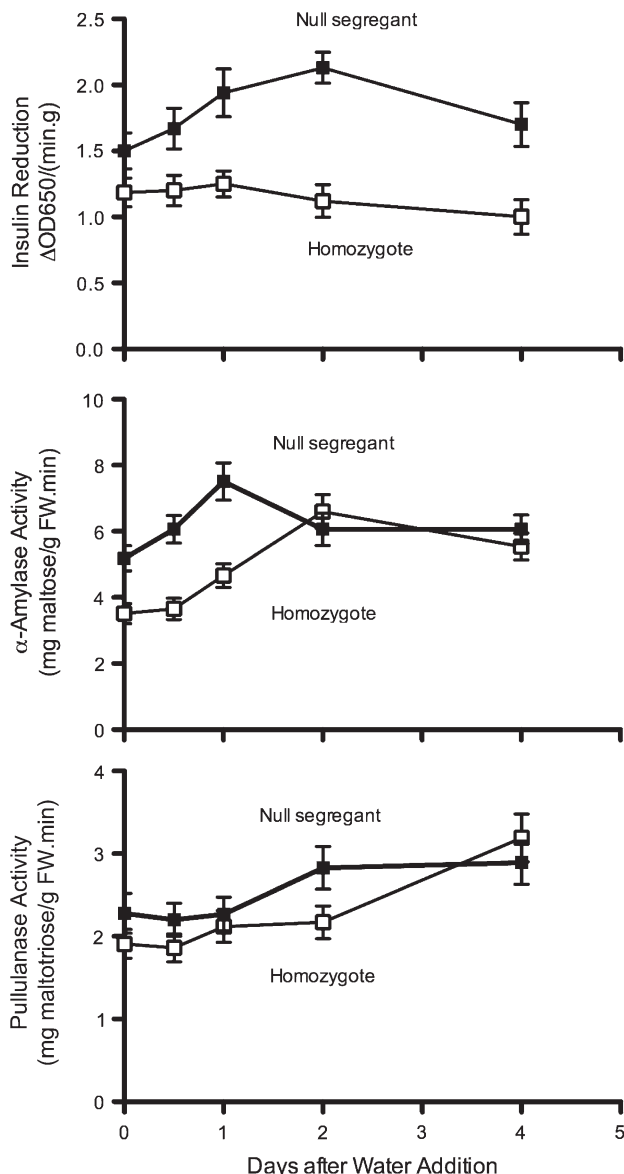


Figure 7. Activity changes of Trx and Associated Enzymes in Germinating Wheat Seeds Underexpressing Trx *h9*.

(A) Trx *h* activity in germinating wheat seeds underexpressing Trx *h9*. (B) α -Amylase activity in germinating wheat seeds underexpressing Trx *h9*. (C) Pullulanase activity in germinating wheat seeds underexpressing Trx *h9*.

against preharvest sprouting—a problem that affects yield as well as the nutritional and processing quality of wheat worldwide (Imtiaz et al., 2008). Experiments conducted in the present study showed this to be the case. The grain in the homozygous line underexpressing Trx failed to germinate in the head 7 d after being subjected to sprouting conditions, whereas the null segregant showed copious new seedlings (Figure 8). Similar results have been obtained with multiple lines of both greenhouse and field-grown Shengkang No. 1 wheat plants. Moreover, the seed has been advanced to the

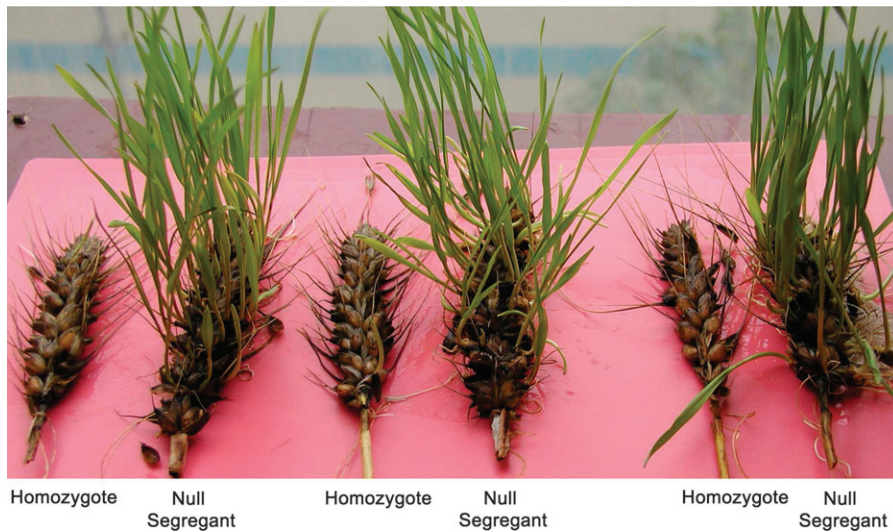


Figure 8. Suppression of Preharvest Sprouting in Transgenic Wheat Spikes Underexpressing Trx *h9*.

Plants were grown in the field and subjected to sprouting conditions in the environmentally controlled chamber.

fourth generation with no diminution in protection against sprouting under wet growth conditions. Finally, tests conducted so far indicate that underexpression of Trx *h9* has no adverse effect on flour quality based on rapid visco analysis (RVA) parameters or, as noted above, on crop yield (data not shown).

Concluding Remarks

The present study represents a milestone in experiments that launched the field of redox biology four decades ago (Buchanan et al., 1967; Buchanan, 1980; Buchanan et al., 2002; Buchanan and Balmer, 2005). Early efforts uncovered a regulatory role of Trx in chloroplasts—a role that now embraces multiple processes fundamental to the plant. As the field developed, an extraplastidic Trx (*h*-type) was identified in several compartments, including the cytosol, and found to function in seed germination, initially in cereals and more recently in legumes. The *in vitro* data obtained in a number of studies showed that Trx *h* acts at multiple levels to foster germination and development of the new seedling. Following water addition, Trx *h* reduces redox-active disulfide groups of storage proteins and enzymes of the dry seed to the sulhydryl state, thereby enhancing the supply of nutrients through solubility and activity changes. More specifically, Trx acts by increasing both the solubility of storage proteins and their susceptibility to proteolysis. These changes are accompanied by activation of enzymes functional in starch and protein utilization, both directly via reduction and indirectly via reductive inactivation of specific disulfide inhibitor proteins. Collectively, these changes facilitate the emergence and growth of the new seedling.

The *in vitro* experiments with seeds were initially supported by genetic engineering studies with barley. Overexpression of Trx *h5* in the protein body of the starchy endosperm showed striking effects: accelerated germination and α -amylase release in concert with an increase in pullulanase activity. The present experiments with wheat take that study in a new di-

rection. Building on the barley work, overexpression of Trx *h5* in the wheat endosperm was found to increase the solubility and decrease the allergenicity of a major group of storage proteins (gliadins) (Figure 9). Underexpression of Trx *h9*, on the other hand, promoted changes opposite to those observed with barley—namely retardation of germination and delayed or decreased expression of the associated enzymes, leading to suppression of preharvest sprouting.

In extending earlier findings, the current results show that wheat Trx *h9* acts jointly with Trx *h5* in regulating germination. To our knowledge, this represents the first instance in which more than one Trx has been linked to the regulation of a physiological process. The mechanism by which these Trxs interact thus emerges as an interesting question for the future. A related question concerns the means by which the *PTrx* antisense gene suppresses germination and protects against preharvest sprouting.

The underexpression experiments have taken Trx research to a new level in moving plants from the laboratory to the field. Multiple field-grown lines underexpressing Trx *h9* showed significant protection against preharvest sprouting—a major problem in China (Xiao et al., 2004) and throughout the world (Imtiaz et al., 2008). In China, 83% of the wheat planting region is subject to sprouting damage and, in one of the major cultivation areas for white-grained varieties, for example, sprouting regularly occurs in more than 20% of the crop (Xiao et al., 2002). In short, if expectations are fulfilled (Buchanan et al., 1994), Trx will have been instrumental in enhancing the quality and supply of a major food crop.

METHODS

Plant Material

For the Trx *h5* (AAF88067) overexpression experiments, the wheat storage proteins were prepared from transgenic white wheat (*Triticum aestivum* L. cv. Yecoro Rojo, and Anza) grains.

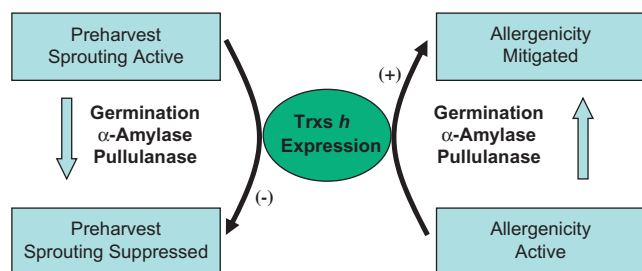


Figure 9. Expression of *h*-type Trx Determines Properties Linked to Applications of Cereal Seeds.

The effects of overexpressing Trx *h5* in both barley and wheat are shown on the right. A summary of the effects of underexpressing Trx *h9* in wheat is shown on the left.

For the Trx underexpression experiments, the seed and other materials were prepared from transgenic white wheat lines (*Triticum aestivum* L. cv. Sheng kang No. 1) harboring an expression cassette consisting of a wheat α -gliadin promoter (GB: TAU51305), antisense *PTrx* sequence (*Phalaris coarulescens*, mRNA no. GB:AF159388.1), and a 3' nos terminator (Figure 3).

Animals

A colony of inbred, high IgE-producing, atopic dogs was sensitized and maintained at the Animal Resources Service, School of Veterinary Medicine, University of California, Davis (Frick and Brooks, 1983; Ermel et al., 1997). The dogs were selected for a genetic predisposition to allergy and have a 15-year history of food and pollen hypersensitivity. As described elsewhere (Buchanan et al., 1997), they were regularly immunized with specific food extracts and periodically characterized for food hypersensitivity following birth.

Chemicals and Enzymes

Reagents for sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad. DTT was purchased from Boehringer Mannheim. Other biochemicals, reagents, and enzymes were purchased from Sigma Chemical Co. (St Louis, MO). Anti-gliadin (wheat) G-9144 was also purchased from Sigma.

Stable Wheat Transformation for Trx *h* Overexpression

The Trx *h5* overexpression experiments were carried out in Berkeley. Two spring cultivars of wheat (*Triticum aestivum* L.), Anza and Yecora Rojo, were grown in a greenhouse as previously described for barley (Lemaux et al., 1996). Whole immature embryos (IEs) of about 1.0–2.0 mm were isolated from these two cultivars from spikes surface-sterilized for 10 min in 20% (v/v) bleach (5.25% sodium hypochlorite) followed by three washes in sterile water. Green regenerative tissues were induced and proliferated by culturing IEs on DBC3 medium containing 1.0 mg L⁻¹ 2,4-D, 0.5 mg L⁻¹ 6-Benzylaminopurine (BAP), and 5.0 μ M CuSO₄ (Cho et al., 1998) and used for

bombardment. Highly regenerative tissues, approximately 2–5 months old, were used as targets for bombardment. Tissues (4–6 mm) were transferred for osmotic pretreatment to DBC3 medium containing equimolar amounts of mannitol and sorbitol to give a final concentration of 0.4 M (Cho et al., 1998). After 4 h, tissues were bombarded as previously described (Lemaux et al., 1996) with modifications. In summary, gold particles (1.0 μ m) were coated with 25 μ g of a 1:1 molar ratio of a mixture of pAct1IHPT-4 and pdBhssWTRN3-8 (Figure 1) followed by bombardment using a PDS-1000 He biolistic device (Bio-Rad, Inc., Hercules, CA) at 900 psi. The plasmid pAct1IHPT-4 contains the hygromycin phosphotransferase (*hpt*) coding sequence under control of the rice actin1 promoter (*Act1*), its intron, and the *nos* 3' terminator (Cho et al., 1998). pdBhssWTRN3-8 contains the wheat Trx *h* gene pTaM13.38 (Gautier et al., 1998), here designated wheat Trx *h5*, under control of the barley endosperm-specific B₁-hordein promoter with its signal peptide sequence and the *nos* 3' terminator (Cho et al., 1999; Figure 1). Sixteen to 18 h after bombardment, the bombarded tissues were placed on DBC3 medium without osmoticum and grown at 24 $\pm$ 1°C under dim light with 10–30 μ mol m⁻² s⁻¹.

Following the initial 10–14-d culturing period, each regenerative tissue was broken into one to three pieces, depending on tissue size, and transferred to DBC2 medium containing 2.0 mg L⁻¹ 2,4-D, 0.1 mg L⁻¹ 6-Benzylaminopurine (BAP), and 5.0 μ M CuSO₄ (Cho et al., 1998) or DBC3 medium supplemented with 25 mg L⁻¹ hygromycin B (Boehringer Mannheim, Mannheim, Germany). Three weeks after the first round of selection, cultures were transferred to fresh DBC2 or DBC3 medium containing 30 mg L⁻¹ hygromycin B. From the third-round selection, the tissues were sub-cultured and maintained on DBC3 medium containing 30 mg L⁻¹ hygromycin B at 3–4-week intervals. After the fourth round of selection, surviving tissues were transferred to DBC3 medium without selective agent. Following identification of sufficient-sized green, regenerative structures on DBC3, tissues were plated on solid regeneration medium without selective agent and exposed to higher intensity light (approximately 45–55 μ mol m⁻² s⁻¹). After 4 weeks on regeneration medium (callus-induction medium without phytohormones), regenerated shoots were transferred to Magenta boxes containing the same medium without selective agent. When shoots reached the top of the box, plantlets were transferred to soil.

PCR Screening of Transgenic Lines

Total genomic DNA from leaf tissue was purified as described (Dellaporta, 1994). To test for presence of *wtrxh* in genomic DNA of putatively transformed lines, 250 ng of genomic DNA was used for amplification by PCR using one of two primer sets unique to *wtrxh*, Wtrxh1 plus Wtrxh2R (Cho et al., 1999) or Wtrxh4 (5'-CCAAGAAGTCCCAGCTGC-3') plus Wtrxh5R (5'-ATAGCTGCGACAACCTGTCCTT-3'). The presence of *hpt* was determined using the primer set, HP-T6F (5'-AAGCCTGAACCTACCGCGACG-3') plus HPT5R

(5'-AAGACCAATGCGGAGCATATAC-3') (Cho et al., 1998). Amplifications were performed with *Taq* DNA polymerase in a 25- μ l reaction (Cho et al., 1998) with modifications, namely *Taq* DNA polymerase (Qiagen, Valencia, CA) with Q-solution was used. Twenty-five microliters of the PCR product with loading dye was subjected to electrophoresis in a 1.0% agarose gel with ethidium bromide and photographed using exposure to UV light. Presence of 0.4-kb (*Wtrxh1*, *Wtrxh2R*) and 0.14-kb (*Wtrxh4*, *Wtrxh5R*) fragments was consistent with intact and partial *wtrxh* fragments, respectively; an internal 0.81-kb fragment was produced with *hpt* primers. Putative homozygous *wtrxh* and null segregant lines for *wtrxh* were identified by segregation analysis using PCR in the T_1 , T_2 , or T_3 generations.

Protein Blot Analysis of Overexpression of Trx *h5*

Soluble proteins were extracted from T_1 seeds to determine levels of Trx *h5*. Ten mature seeds were ground into fine powder using a mortar and pestle and mixed with 1 ml extraction buffer containing 50 mM Tris/HCl, pH 7.9, 1 mM EDTA and 0.5 mM phenylmethanesulphonyl fluoride (PMSF). After 10 min centrifugation at 14 000 *g*, the supernate was collected and analyzed for protein with the dye-binding procedure using γ -globulin as a standard (Bradford, 1976). 40–50 μ g of protein were subjected to SDS–PAGE on 10–20% polyacrylamide gradient gels at pH 8.5 at 150 mA (Laemmli, 1970). Proteins were transferred onto 0.1 μ m nitrocellulose membranes at 90 V for 1.5 h using a TE 42 Transphor Electro-Transfer Unit (Hoeffer Scientific Instruments, San Francisco, CA). The blot was blocked in TBS supplemented with 5% nonfat dry milk for 30 min, and incubated overnight at 4°C in antiserum against wheat Trx *h5* diluted 1:500 with 5% nonfat dry milk in TBS. The blot was washed three times for 20 min each in a solution of TBS containing 0.05% Tween 20 at room temperature, and then incubated for 1 h with the secondary antibody, goat anti-rabbit IgG-HRP conjugate, diluted 1:2500 with 5% milk in TBS (Bio-Rad, Hercules, CA). The blot was again washed twice with TBS/Tween 20, then HRP activity was visualized using a TMB detection kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA).

Quantification of Immunoblot

To determine the relative amount of overexpression of Trx *h5* in various wheat lines, an image of the immunoblot was scanned with a UMAX PowerLook 1100 scanner using UMAX MagicScan version 4.4 within Adobe Photoshop, v.6 (Adobe Systems, San Jose, CA). Levels of expression were quantified as volume (intensity $\times$ area) of immuno-reactive bands using Quantity One software (Bio-Rad, Hercules, CA). Level of overexpression was calculated as the ratio of volume of heterozygote vs. null segregant (heterozygote vs. nontransgenic for the Anza cultivar).

Solubility of Gliadins

200 mg of flour from homozygous T_2 transgenic wheat grain overexpressing Trx *5h* and the companion null segregant were analyzed. Samples were mixed and then extracted in 1 ml

double-distilled H_2O in microfuge tubes on a rotating wheel (Glas-Col, Terre Haute, IN, USA) at a speed setting of 40% for 60 min at room temperature. Samples were centrifuged at 14 000 rpm for 10 min. Clear supernates were transferred to another set of microfuge tubes. Protein was determined by the dye-binding method as above. Aliquots of extracts giving 50 μ g of protein were precipitated by cold acetone overnight. Protein pellets recovered after centrifugation were air-dried and then re-dissolved in (1) 30 μ l 1 $\times$ Laemmli Sample Buffer without 2-ME (non-reducing), and (2) 30 μ l of the same buffer plus 2.5% (v/v) 2-ME (reducing) and vortexed for 20 min. Dissolved samples were boiled for 5 min and clarified by centrifugation for 30 s. Aliquots (15 μ l) of each sample were loaded onto Criterion Tris-HCl pre-cast 10–20% gradient gel (Bio-Rad, Hercules, CA). Separation and transfer of protein to NC membrane were as described above. The immunoblotting was also similar, except the first antibody was rabbit-anti-gliadin (1:1250 dilution) (Sigma, St Louis, MO). The quantification of volume of immunoblot of gliadin was as described above.

Allergenicity of Transgenic Wheat

Allergenicity of the transgenic wheat grains was tested using the atopic dog colony. Dogs were maintained, sensitized, and skin tested as previously described (Buchanan et al., 1997; Ermel et al., 1997). Six wheat-sensitive dogs were used in this study. The wheat allergen preparation used for sensitization was from Bayer (Tarrytown, NY). Earlier protocols used for the isolation of individual protein fractions from wheat based on solubility (Buchanan et al., 1997) were applied to transgenic wheat overexpressing Trx *h5* (Cho et al., 1999) and a companion null segregant. Protein concentration of each fraction was determined as above. After estimation of protein concentration, each fraction (10^{-2} to 10^{-8} mg protein) was serially diluted in physiological buffered saline (PBS) and then used for the skin tests. The following numbers of tests were conducted for the wheat protein fractions: albumins/globulins, 33; gliadins, 29; glutenins, 28. An appropriate control (minus protein) was included for each animal tested.

Skin Tests

About 3 min prior to skin testing, each dog received 4–5 ml of filtered 0.5% Evans blue dye solution (equivalent to 0.2 ml of 0.5% Evans blue dye per kg of weight) through a cephalic vein to enhance assessment of the cutaneous IgE antibodies (Buchanan et al., 1997; Ermel et al., 1997). Serial dilutions of 100 μ l of each sample were injected intradermally on the abdominal skin to establish the titer. After 15–20 min, the allergic response was determined by measuring the size of the blue wheal reaction (maximum length and width). An appropriate negative control (solution of glycerol, or ethanol diluted in physiological saline buffer) was included for each animal tested.

Data Analysis

The statistical significance of the overexpressed Trx-linked mitigation of the canine cutaneous response was determined by

paired one-tailed *t*-tests. The null hypothesis assuming no difference in wheal area induced by null segregant vs. homozygote overexpressing Trx *h5* extracts was tested against the alternative hypothesis that overexpressing Trx resulted in mitigation of allergic response. The paired one-tailed *t*-tests were completed for each dilution series and shown to decrease the gliadin response at 0.05 level of significance with all sensitive dogs.

Stable Wheat Transformation for Trx Underexpression

The Trx *h9* underexpression experiments were carried out in Zhengzhou. A winter variety of white wheat (*Triticum aestivum* L.), Shengkang No. 1, was grown in the field and the immature embryos (13 d after pollination) were prepared for genetic transformation based on particle bombardment of scutellar tissues. The wheat transformation was performed according to Becker et al. (1994), with the following minor modifications. Two expression vectors, pBS-*bar* and pBS-Gli-AntiTrx (Figure 3), were used for co-transformation. The plasmid pBS-*bar* contained the *bar* gene sequence under control of the CaMV 35S promoter and the nos 3' terminator. The vector pBS-Gli-AntiTrx includes the transcription elements of the antisense *PTrx* sequence (AF159388) (Figure 3). Gold particles (42 µg, 1.0 µm) coated with 0.21 µg of a 1:1 molar ratio of a mixture of these two plasmids was used for each bombardment with a PDS-1000/He biolistic device (Bio-Rad) at 900 psi. The culture and selection conditions were mainly according to Becker et al. (1994). Induction of somatic embryogenesis was carried out at 26°C in the dark for 14 d without a selective agent, and calli were then transferred for an additional 14 d to induction media containing 5 mg l⁻¹ PPT (phosphinothricin). The same amount of selective agent used during the callus induction phase was also included in the plant regeneration medium. Plant regeneration was performed at 26°C under fluorescent light (irradiance of 240 µmol m⁻² s⁻¹ or PAR for 16 h) and other culture conditions were the same as described (Becker et al., 1994). The T₀ transgenic plantlets were pot planted in the temperature-controlled greenhouse at 25–27°C. As seen in Figure 4, nine transgenic lines were isolated from independent transformation events (Supplemental Figure 2).

PCR Screening of Transgenic Lines

Genomic DNA was extracted from the wheat leaves according to the CTAB method (Saghai-Maroo et al., 1984), with minor modification of sample preparation. A Retsch Mixer Mill MM301 was used to grind samples. Briefly, 50 mg of fresh leaf tissue together with two washed, autoclaved stainless steel ball bearings (4 mm diameter) were placed in microfuge tubes. Tubes were loaded into the Adapter Rack, which was immersed in liquid nitrogen to freeze the plant tissue and cool the ball bearings. The mill was assembled and the samples were then ground. Specific primer sets were designed based on the transgene element sequence, such as the primer Phr-7 (5'-TCTGTGCCAGCCATGCTTAT-3') to target the wheat

α-gliadin promoter region and the primer Phr-8 (5'-TTTCAAAGGTGGGAATGTGC-3') to target the antisense *PTrx* sequence, while the primer bar-3 (5'-CCTTCGCAAGACCCTTCCTC-3') targeted the CaMV 35S promoter region and bar-4 (5'-ACCCACGTCATGCCAGTTCC-3') targeted the *bar* gene region. The presence of the transgene in the genome of putatively transformed lines was identified by performing PCR using one of these primer sets. PCR was carried out using about 200 ng of genomic DNA as template and 125 pmol of each primer in a 20-µl reaction system containing 0.2 mM of each dNTPs, 1.5 mM MgCl₂, and 1 U Taq polymerase. PCR program was as follows: 5 min at 94°C; followed by 35 cycles of 1 min at 95°C, 40 s at 58°C, and 1 min at 72°C; then a final extension of 10 min at 72°C. PCR products were separated on a 1% agarose gel (with ethidium bromide) by electrophoresis and photographed using exposure to UV light. Presence of 0.76-kb (Phr-7, Phr-8) or 0.56-kb (bar-3, bar-4) fragment was consistent with partial gliadin/anti-*PTrx* or CaMV 35S/*bar* fragment, respectively. Putative homozygous and null segregant lines for gliadin/anti-*PTrx* were identified by segregation analysis using PCR in the T₁, T₂, or T₃ generations.

Semi-Quantitative RT-PCR Analysis

Seed samples of the homozygote (T₃ generation) and null segregant Shengkang No. 1 lines were prepared in triplicate. Total RNA was isolated from the samples using the RNeasy kit (Sangon, Shanghai) according to the manufacturer's instructions and subjected to DNase digestion in the presence of ribonuclease inhibitor. RNA was then extracted sequentially with phenol: chloroform (1:1) and chloroform, precipitated with ethanol and, finally, dissolved in double-distilled H₂O treated with diethylpyrocarbonate (DEPC). Equal amounts (2 µg) of total RNA were reverse-transcribed in a 20-µl reaction system containing 50 mM Tris-HCl, pH 8.3, 75 mM MgCl₂, 10 mM DTT, 50 µM dNTP, 200 U MMV reverse transcriptase (Promega, Beijing) and 50 pmol Olig-dT (15) nucleotides, (42°C, for 60 min) and finally denatured at 95°C for 5 min. Primer sets specific to the Trx *h9* sequence (AF438359), P-4 (5'-GCAGAAGCAAACAAGGATGGG-3') and P-6 (5'-ACTGCCATCGCCAAGAGC-3'), were used in the RT-PCR analysis. TaAc-1 (5'-GTTCCAATCTATGAGGGATACACGC-3') and TaAc-2 (5'-GAACCTCCACTGAACAACATTACC-3') specific to the actin gene (AB181991) were included as the control. PCR was carried out using 2 µl of the cDNA as template and 125 pmol of each primer in a 20-µl reaction system containing 0.2 mM of each dNTPs, 1.5 mM MgCl₂, and 1 U Taq polymerase. The PCR program was as follows: 5 min at 94°C, followed by 27 cycles of 30 s at 95°C, 30 s at 58°C, and 40 s at 72°C. A final extension of 5 min at 72°C was then added. PCR products were separated by 1% agarose gel electrophoresis and scanned using a gel scanner JEDA801E (Jieda Science and Technology, Jiangsu, China). Two bands, 422 and 285 bp, were detected for the actin and Trx *h9* genes, respectively, and their Integrated Optical Density (IOD) was determined using JD801 gel analysis software. The average IOD value ratios of Trx *h9* to actin were

calculated and shown as the relative abundance of Trx *h9* mRNA in the samples. Statistical analysis was performed using SPSS 10.0 software. Transgenic line 5 of the Shengkang No. 1 variety was used in the subsequent experiments (see below).

Assays for Trx and Associated Enzymes during Grain Development and Germination

For the development study, assays were conducted with seeds harvested and sampled from both transgenic and null segregant plants 20, 30, 40, 50, 60, and 70 d post anthesis (heads are harvested 40 d post anthesis and then stored at room temperature for after-ripening). For the germination analysis, mature seeds were sterilized in 10% Chlorox, washed three times in sterilized water and placed on filter paper soaked with 10 ml of double-distilled H₂O at 26°C in a set of 9-cm Petri dishes that were subjected to 12 h light with an irradiance of 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) and 12 h dark periods. The imbibing grains were sampled 0, 0.5, 1, 2, and 4 d after water addition. A suitable number of grains were ground to fine powder in liquid nitrogen with a mortar and pestle. Seed extracts used for enzyme assays were prepared according to Cho et al. (1999) with minor modifications. Briefly, protein was extracted with 1.4 ml 30 mM Tris-HCl buffer (pH 7.9) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA for 30 min at 4°C. Extracts were centrifuged for 10 min at 25 000 *g* and the supernate (containing Trx *h* and enzymes) was collected and stored at -80°C. Trx *h* was assayed according to Holmgren (1979) and pullulanase according to Yamada and Izawa (1979). α -Amylase activity was measured by the 3,5-dinitrosalicylic (DNS) procedure (Miller, 1959).

Assays for Germination and Preharvest Sprouting Resistance

For germination tests, heads from both the transgenic and null segregant plants (40 d post anthesis) were harvested and grains from the middle section of ears were dissected and collected. The grains were surface sterilized in 5% sodium hypochloride for 12 min and washed four times (2 min each) with sterilized water. Three groups (100 grains/group) were distributed on two layers of filter paper in a 9-cm Petri dish containing 10 ml of sterilized water, and germinated at 26°C PAR. Germination refers to emergence of radicle through the seed coat. Preharvest Sprouting Resistance tests were performed with intact spikes (40 d post anthesis) rolled in paper towels (Hagemann and Ciha, 1984) at 26°C (12-h photoperiod at a light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). All tests were performed in an environmental-controlled weather chamber.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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