

This is an author-generated copy of the paper published in: *Plant Science* 165 (2003) 1221–1227. The published version is available online at:
<http://www.sciencedirect.com/science/journal/01689452>

Alteration of antioxidant enzyme gene expression during cold acclimation of near isogenic wheat lines

Kwang-Hyun Baek^a, Daniel Z. Skinner^{a,b,*}

^a *Department of Crop and Soil Sciences, 210 Johnson Hall, Washington State University, Pullman, WA 99164-6420, USA*

^b *USDA/ARS, Department of Crop and Soil Sciences, 209 Johnson Hall, Washington State University, Pullman, WA 99164-6420. USA*

*Corresponding author. Tel.: +2-509-335-3475; fax: +2-509-335-2553

E-mail address: dzs@wsu.edu (D.Z. Skinner)

Abstract

Reactive oxygen species (ROS) are harmful to living organisms due to the potential oxidation of membranes, DNA, proteins, and carbohydrates. Freezing injury has been shown to involve the attack of ROS. Antioxidant enzymes can protect plant cells from oxidative stress imposed by freezing injury, therefore, cold acclimation may involve an increase in the expression of antioxidant enzymes. In this study, quantitative RT-PCR was used to measure the expression levels of antioxidant enzymes during cold acclimation in near isogenic lines (NILs) of wheat, differing in the Vrn1-Fr1 chromosome region that conditions winter versus spring wheat growth habit. The antioxidant genes monitored were mitochondrial manganese-superoxide dismutase (MnSOD), chloroplastic Cu,Zn-superoxide dismutase (Cu,ZnSOD), iron-superoxide dismutase (FeSOD), catalase (CAT), thylakoid-bound ascorbate peroxidase (t-APX), cytosolic glutathione reductase (GR), glutathione peroxidase (GPX), cytosolic mono-dehydroascorbate reductase (MDAR), chloroplastic dehydroascorbate reductase (DHAR). The expression levels were up-regulated (MnSOD, MDAR, t-APX, DHAR, GPX, and GR), down-regulated (CAT), or relatively constant (FeSOD and Cu,ZnSOD). The Vrn1-Fr1 region seemed to have a role in regulating the expression level of some of the antioxidant enzyme genes because t-APX, CAT and MnSOD expressed to significantly higher levels in the winter wheat NIL than the spring wheat NIL after 4 weeks cold acclimation.

Keywords Antioxidant enzymes, quantitative real-time PCR, cold acclimation, wheat, near-isogenic lines.

Abbreviations: t-APX, thylakoid-bound ascorbate peroxidase; CAT, catalase; Cu,ZnSOD chloroplast Cu, Zn-superoxide dismutase; DHAR, chloroplastic dehydroascorbate peroxidase; FeSOD, iron-superoxide dismutase; GPX, glutathione peroxidase; GR, cytosolic glutathione reductase; MDAR, cytosolic mono-dehydroascorbate reductase; MnSOD, mitochondrial manganese-superoxide dismutase; NIL, near isogenic line.

1. Introduction

Cold temperature is one of the most severe environmental stresses limiting the distribution of native flora and reducing agricultural productivity [1, 2]. Many crop plants can significantly lose productivity due to an untimely frost or extremely cold winter temperatures [2, 3]. The mechanisms of cold temperature injury are exceedingly complex and the causes of injury are not clearly understood [2, 4]. The mechanisms involved differ depending on species, degree of hardiness, and temperature conditions, including cooling rates and the time of initiation of ice formation [2, 5]. The condition of the plasma membrane has been shown to be critical to cellular behavior during cold temperature exposure [6]. Several mechanisms have been suggested to be involved in membrane damage at cold temperatures including structural transitions [7], injury to membrane-bound ATPase [5], membrane phase transitions [8], loss of bound water [9], and phospholipid degradation by phospholipase D [10].

Oxidative stress, the harmful condition that occurs when there is an excess of free radicals within the cells, also has been proposed as one of the causes of cold temperature damage [11, 12]. Hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and the hydroxyl radical (OH^\cdot), called reactive oxygen species (ROS) because of their high oxidizing potentials, are responsible for oxidative stress. While ROS are generated in organisms on a regular basis and play a role in signal transduction [13, 14], excessive production or inefficient deactivation of ROS can cause plant injury and eventually cell death [15]. The roles ROS play in this process have been shown to include peroxidation of cell membrane components, degradation of polysaccharides, denaturation of enzymes, and the nicking, cross linking and scission of DNA strands [12, 15].

The accumulation of ROS has been shown to occur in bacterial cells as a result of exposure to cold temperature [15]. Similarities between freezing injury and oxidative stress were reported by simulating oxidative stress on isolated wheat microsomal membranes [16]. The emission of chemiluminescence following freezing is considered to be due to the evolution of ROS [17], which may occur partially by cavitation of the cytoplasm during freezing [18]. These data suggest that ROS are produced during exposure to cold temperature and that oxidative stress contributes to cold temperature-induced damage of plant cells.

To protect from oxidative stress, plants have evolved very efficient antioxidant systems to scavenge ROS [19]. The antioxidant systems can be divided into three groups: (1) antioxidant enzymes, (2) lipid soluble, membrane associated antioxidants (e.g. α -tocopherol, β -carotene, and ubiquinone), and (3) water soluble antioxidants (e.g., glutathione and ascorbate) [12]. Among these systems, antioxidant enzymes are the most active and efficient protective mechanism against oxidative stress [12]. The antioxidant enzymes include catalase (CAT), superoxide dismutase (SOD), peroxidase, and enzymes in the water-water cycle [19]. CAT (EC 1.11.1.6) degrades H_2O_2 into H_2O and O_2 . At least 3 different wheat CAT genes have been reported in GenBank. SOD (EC. 1.15.1.1) catalyzes the dismutation of two superoxide radicals (O_2^-), resulting in the production of H_2O_2 and O_2 . SOD has several isozymes, which can be classified by the location and catalytic metals; MnSOD in mitochondria, FeSOD in chloroplast, Cu,ZnSOD in chloroplast and cytosol [19]. Peroxidase oxidizes an organic substrate (RH_2) with H_2O_2 producing oxidized substrate and water. The water-water cycle, also known as Halliwell-Asada or ascorbate-glutathione cycle, includes the activity of APX, DHAR, GR and MDAR, and is a pathway involved in scavenging superoxide radicals and H_2O_2 [20].

Antioxidant enzymes have been shown to condition tolerance to low temperature stress [21, 22, 23]. CAT was induced by low temperature treatment and is an essential enzyme to protect mitochondria against chilling stress (non-freezing cold temperatures) in maize [23]. A high amount of MnSOD in *Chlorella* was related to increased chilling tolerance [21]. Freezing tolerance was increased by transforming alfalfa to overexpress a tobacco mitochondrial MnSOD gene in chloroplasts or mitochondria [22]. Overexpression of a chloroplast Cu,ZnSOD gene increased resistance to oxidative stress in tobacco [24]. In stressful conditions, plants generally increase the activity of one or more antioxidant enzymes; the elevated activity level usually correlates with increased stress tolerance [19]. Therefore, increased expression of antioxidant systems in plants might afford protection from ROS generated as a result of cold stress.

Many plants can become more tolerant of cold temperatures through a process known as cold acclimation, which occurs as a result of exposure to cold temperatures. During cold acclimation, many physiological changes occur that result in increased freezing tolerance [6]. One of these changes may be increased expression of antioxidant enzyme genes to protect from oxidative

stress. The objective of this study was to measure the expression levels of antioxidant enzyme genes in wheat plants undergoing cold acclimation.

2. Materials and Methods

2.1 Wheat Growth and Sample Collection

The plant lines used were NILs 442 (winter wheat) and 443 (spring wheat), which should differ only in the *Vrn1-Fr1* region of chromosome 5A. NIL 442 (*vrn1vrn1Fr1Fr1*) displays a winter wheat habit and NIL 443 (*Vrn1Vrn1fr1fr1*) displays a spring wheat growth habit [25]. NIL 442 and NIL 443 wheat seeds were planted and grown for 14 days in a growth chamber maintained at 20°C under 200 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$, 16 hr photoperiod. The temperature then was changed to 2°C to induce cold acclimation. Leaves were collected approximately 4 cm above the crowns on 0, 7, 14, and 28 days of cold acclimation and used for RNA extraction.

2.2 RNA Extraction and Quantitative RT-PCR (qRT-PCR)

RNA was extracted using Trizol (Invitrogen, San Diego, CA) according to the manufacturer's instructions and stored at -70°C before being used for qRT-PCR. Each extraction and qRT-PCR was replicated three times. The primers for genes encoding antioxidant enzymes were constructed by using the Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), using the wheat gene sequences in the TIGR database (<http://www.tigr.org/tdb/tgi/tagi/>) or GenBank (<http://www.ncbi.nlm.nih.gov>) (Table 1). Each primer was composed of about 20 nucleotides with melting temperatures around 60 °C. The primer set for each antioxidant enzyme was designed to produce an amplicon ranging from about 90 to 110 nucleotides. A total of 125 ng RNA were used for the template for SuperScript One-step RT-PCR with platinum Tag kit (Invitrogen, San Diego, CA) and a Rotor-Gene2000 Real-Time PCR machine (Corbett Research, Sydney, Australia). The reverse transcription and PCR steps were carried out as: cDNA construction for 15 min at 50 °C, followed by qRT-PCR with an amplification profile of 3 min at 95 °C, then 32 cycles of 95 °C for 15 sec, 54 °C for 20 sec, and 72 °C for 17 sec. SuperScript One-Step RT-PCR solution had 2 mM MgCl_2 , 100 nM primers, 1:40,000 Sybr Green (Molecular Probe, Eugene, OR), in 20 μL solution. 8 μl mineral oil (Sigma, St. Louis, MO) was loaded on the top of the qRT-PCR solution to avoid evaporation. We used known concentrations of cloned MnSOD double-stranded DNA to generate data for a standard

curve. The cloned MnSOD DNA consisted of about 500 bp from the middle of the MnSOD gene to the 3' UTR. The gene fragment was cloned using Topo-XL PCR cloning kit (Invitrogen, San Diego, CA) and sequenced to confirm the PCR product was indeed MnSOD. Sequencing was carried out with T7 and M13 reverse primers at the DNA sequencing facility, Washington State University, Pullman. The expression calculation program included with the Rotor-Gene2000 apparatus calculated the transcript copy numbers of the antioxidant enzyme genes. Melting curves of the final RT-PCR products were generated to confirm a single PCR product had been generated. We also analyzed the qRT-PCR products on 1.2 % agarose gels to confirm that a single product of about 100 bp had been generated. We independently repeated each determination three times with one pair of primers for each antioxidant enzyme gene, and also confirmed the data with a second pair of primers for each gene, with agarose gels of RT-PCR, and Northern blot analysis of selected enzyme genes and time points. The expression data for the transcript copy numbers were analyzed by Duncans means separation available in PROC ANOVA of the software SAS (SAS Inc., Cary, NC).

3. Results

3.1 SOD Genes

Expression levels of the SOD genes were not significantly different in NILs 442 and 443 prior to exposure to cold temperatures (Figs. 1A-1C). The Cu,ZnSOD and FeSOD gene expression levels remained nearly constant throughout the 4-week acclimation period (Fig. 1A and 1B), except FeSOD expression increased significantly in the winter wheat NIL (442) after one week of cold acclimation and maintained the elevated level of expression through the second week (Fig. 1B). The expression levels of both of these genes in both NILs had returned to a level not significantly different from the pre-cold level by the end of the fourth week (Figs. 1A and 1B).

Unlike the other SOD genes, MnSOD gene expression increased significantly each week over two weeks of cold acclimation in both NIL 442 and 443 (Fig. 1C). The elevated expression level was maintained through the fourth week in the winter wheat NIL, but declined significantly in the spring wheat between the end of the second week and the end of the fourth week of cold acclimation (Fig. 1C).

3.2 *CAT Gene*

The expression level of the CAT gene was significantly higher in the winter wheat NIL (442) than the spring wheat NIL (about 2 times greater, Fig. 1D) prior to cold acclimation. The level of expression decreased rapidly in the winter wheat NIL after exposure to cold temperature and maintained the decreased level of expression through the end of the fourth week (Fig. 1D). The expression level of the CAT gene remained essentially constant in the spring wheat NIL (Fig. 1D). After four weeks of cold acclimation, the expression level of the CAT gene in the winter wheat NIL was 1.8 times that of the spring wheat NIL (Fig. 1D).

3.3 *Genes encoding t-APX, MDAR, DHAR, GR, and GPX*

The expression level of the t-APX gene was increased 3.7 or 4.9 times after one week of cold acclimation in the winter and spring wheat NILs, respectively (Fig. 1E). The expression level was maintained or slightly increased through the end of the second week of cold acclimation in both NILs, then decreased significantly in the spring wheat NIL, but not the winter wheat NIL, by the end of the fourth week (Fig. 1E).

The MDAR, GR and GPX genes showed similar expression patterns throughout the four-week cold acclimation (Figs. 1F, 1H and 1I, respectively). The MDAR gene increased expression levels significantly (about 10-fold) in both the winter and spring wheat NILs after one week of cold acclimation (Fig. 1F). The expression level was highest after the first week of cold acclimation, then decreased continuously through the fourth week of cold acclimation in both NILs (Fig. 1F). Of the enzyme genes investigated in this study, the MDAR gene showed the largest response in expression after cold exposure in both NILs.

Like MDAR, the expression levels of GR and GPX genes were expressed to the highest level after one week of cold acclimation in both NILs, then decreased continuously through the end of the fourth week (Figs. 1H and 1I).

The DHAR gene slowly increased expression levels in both NILs in response to low temperature (Fig. 1G). In the winter wheat NIL, the expression level of DHAR after one week of cold acclimation was not significantly different from that prior to exposure to cold, but the expression

level increased continuously through the end of the fourth week (Fig. 1G). In the spring wheat NIL, the expression level increased after the first week of cold exposure, then gradually decreased expression through the end of the fourth week (Fig. 1G).

3.4 Replication of measurements with a second primer set

Each determination for each enzyme on RNA extracted from plants with 0 and 7 days cold exposure as reported above was confirmed with a second, independent set of PCR primers (Table 2) and RNA from two additional plants, independently grown from the plants used with the first primer sets. The result from CAT was especially unexpected, but the second set of primers confirmed the results from the first primer pair, *i.e.* a significantly higher level of expression in the winter wheat NIL prior to cold exposure, and a decline to a significantly lower level, equivalent to the spring wheat NIL, after 7 days cold exposure. The determinations of expression levels of the other enzyme genes with the second primer pairs (Table 2), confirmed the results of the first primer pair in each case, with one exception. That exception was that the second primer pair from Cu,ZnSOD indicated a statistically significant, 24% reduction in transcript copy number after 7 days cold exposure of the winter wheat NIL, while the first primer pair indicated no significant change (Fig. 1A).

4. Discussion

Exposure to low temperature may result in the increased generation of ROS in plants [26, 23]. The ROS may attack plant cellular components or may deliver signals for detecting the changed environment, or both [13, 23]. Exposure of winter cereals to cold acclimation temperatures induced an increased tolerance to oxidative stress [27]. Furthermore, the activities of several antioxidant enzymes increased during cold acclimation in wheat [28, 29]. These data suggest that increased cold tolerance can be accompanied by increased expression of specific genes encoding antioxidant enzymes.

In contrast to Cu,ZnSOD and FeSOD, the expression of the MnSOD gene was significantly increased in response to cold temperature in both the winter and spring wheat NILs (Fig. 1B). The MnSOD genes are nuclear genes whose protein products are targeted to the mitochondria. The increased expression observed in this study suggests a protective mechanism targeted to the

mitochondria. The electron transport system of mitochondria is a well-documented source of ROS [30]. Because the electron transport chain in mitochondria has been shown to be very labile at low temperatures [31], the electrons that are not transported to the final electron acceptor O_2 to produce H_2O , might be used to form superoxide (O_2^-). The operation of the mitochondrial alternative pathway at low temperature may produce additional superoxide radicals. Possibly in response, the expression level of the MnSOD gene increased quickly (Fig. 1B) and appeared to show increased expression in Northern blot and RT-PCR data after only one day of cold exposure (data not shown). The expression of MnSOD decreased significantly after four weeks cold exposure in the spring wheat NIL, but not the winter wheat (Fig. 1B). This result may have indicated that mitochondrial activity decreased more rapidly in the spring wheat NIL than the winter wheat NIL, at cold temperature.

Cu,ZnSOD and FeSOD genes maintained relatively constant expression levels during four weeks of cold acclimation in both the winter and spring wheat NILs. The Cu,ZnSOD and FeSOD genes we measured were located in the chloroplast, suggesting the constant expression of Cu,ZnSOD and FeSOD genes may be enough to protect chloroplasts from damage by superoxide during cold acclimation in winter and spring wheats. If we consider that SOD has a very fast catalytic rate of converting superoxide into H_2O_2 but the chloroplastic Cu,ZnSOD and FeSOD are very sensitive to H_2O_2 [32], it seems reasonable that an efficient system to protect chloroplasts from ROS could maintain the constant expression of Cu,ZnSOD and FeSOD genes but increase the expression levels of genes encoding enzymes that scavenge the H_2O_2 produced by these SODs in chloroplasts during cold acclimation. This possibility is supported by the observed increased levels of expression of genes for t-APX and GSH-dependent DHAR. T-APX quickly scavenges H_2O_2 at the thylakoid membrane and GSH-dependent DHAR is located in the stroma of chloroplasts; both enzymes operate in the water-water cycle that removes H_2O_2 from the cell [20]. The transcription levels of Cu,ZnSOD and FeSOD were indistinguishable in winter and spring wheat (Fig. 1A and 1C), suggesting that chloroplast activity is impacted by exposure to cold temperature equally in winter and spring wheat.

The enzymes MDAR, GPX, and GR catalyze reactions, such as the reduction of oxidized ascorbate, that ultimately oxidize NAD(P)H to NAD(P)⁺ [20]. Also, MDAR, GR, and GPX

genes exhibited similar patterns of expression following exposure to cold: rapid increase in the first week of exposure, then a gradual decline in expression through the end of the fourth week (Fig. 1F, 1H, and 1I). These observations suggest that MDAR, GPX, and GR are involved in production of NAD(P)⁺, which may then be incorporated into light energy capture [33], the oxidative portion of the pentose phosphate pathway, or glycolysis. These processes contribute to the net carbon assimilation and relative growth rates, which, similar to the expression of MDAR, GPX, and GR, undergo a rapid, then gradual reduction as nonhardened wheat plants grow at cold temperature [34].

Expression of the gene encoding CAT was observed to decrease or remain constant during four weeks of cold acclimation. This CAT gene expression profile is very well matched with the data for CAT enzyme activity in cold-acclimated wheat leaves presented by Janda et al. [29], in which CAT activity in leaves was significantly reduced after 7 weeks of cold acclimation of six of seven wheat lines.

The Vrn1-Fr1 region of chromosome 5A confers winter versus spring wheat growth habit and also seems to have a significant role of regulating the expression levels of some antioxidant enzymes. With genes encoding t-APX, CAT and MnSOD, expression levels were significantly less in the spring wheat NIL than the winter wheat NIL after four weeks of acclimation. A significantly higher level of CAT transcript was observed in the winter wheat NIL, than the spring wheat NIL, prior to the start of cold acclimation. The meaning of this observed difference in NILs differing at the Vrn1-Fr1 region is unknown but suggests a difference in the antioxidant capacity of wheat plants grown at 20°C may be conditioned by gene(s) in the Vrn1-Fr1 region.

The results presented here suggest that genes encoding antioxidant enzymes may play a pivotal role in cold response either through increased expression, or through the decrease in function of some enzyme forms. All genes for antioxidant enzymes, except CAT, increased or maintained expression levels during cold acclimation of wheat, and the over-expressed antioxidant enzymes

may be used to protect plant cells from oxidative stress resulting from exposure to cold temperature. Based on the magnitude of upregulation, it appeared that MnSOD in mitochondria and enzymes active in the water-water cycle in chloroplasts were the primary antioxidant enzymes protecting the organelles. The Vrn1-Fr1 chromosomal region appeared to play a role in the regulation of expression of some of the antioxidant genes, primarily by influencing the duration of increased expression. This possibility could be investigated further by examining antioxidant gene expression in a series of independent Vrn1-vrn1 and Fr1-fr1 near isogenic line pairs. Linkage of Vrn1 and Fr1 has been broken [35], but NILs for each locus do not yet exist, insofar as we are aware. Development of such NILs would enable the elucidation of the role each locus plays on the control of antioxidant gene expression during cold acclimation.

Acknowledgements

The authors thank Dr. Gerald Edwards, Washington State University, for critically reviewing the manuscript and providing helpful suggestions.

References

- [1] M.J. Burke, L.V. Gusta, H.A. Quamme, C. J Weiser, P. H. Li, Freezing and injury in plants. *Annu. Rev. Plant. Physiol.* 27 (1976) 507-528.
- [2] A. Sakai, W. Larcher, *Frost Survival of Plants*, Springer-Verlag, Berlin Heidelberg, New York, 1987.
- [3] S. Yoshida, M. Uemura, Responses of the plasma membrane to cold acclimation and freezing stress, in: C. Larsson, I. M. Møller, (Eds.), *The Plant Plasma Membrane: Structure, Function, and Molecular biology*. Springer-Verlag, Berlin Heidelberg, 1990, pp. 293-319.
- [4] C. L. Guy, Cold acclimation and freezing stress tolerance: Role of protein metabolism, *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* 41(1990) 187-223.
- [5] S. Iswari, J. P. Palta, Plasma membrane ATPase activity following reversible and irreversible freezing injury, *Plant Physiol.* 90 (1989) 1088-1095.
- [6] M. F. Thomashow, Plant cold acclimation: Freezing tolerance genes and regulatory mechanism, *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 50 (1999) 571-599.
- [7] C. B. Rajashekar, L. V. Gusta, M. J. Burke, Membrane structure transition: probable relation to frost damage in hardy herbaceous species, in: J. M. Lyon, D. Graham, J. K. Raison (Eds.) *Low*

Temperature Stress in Crop Plants: The Role of Membrane, Academic Press, London New York, 1979, pp. 255-274.

[8] R. S. Pearce, J. H. M. William, Wheat tissues freeze-etched during exposure to extracellular freezing: distribution of ice. *Planta* 163 (1985) 295-303.

[9] C. J. Weiser, Cold resistance and injury in woody plants, *Science* 169 (1970) 1269-1278.

[10] S. Yoshida, A. Sakai, Phospholipid degradation in frozen plant cells associated with freezing injury, *Plant Physiol.* 53 (1974) 509-511.

[11] B. D. McKersie, S. R. Bowley, Active oxygen and freezing tolerance in transgenic plants, in: P. H. Li, T. H. H. Chen (Eds.), *Plant Cold Hardiness*, Plenum Press, New York, 1997, pp. 203-214.

[12] B. Halliwell, J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, 3 Ed., Oxford University Press, New York, 1999.

[13] I. Fridovich, Molecular oxygen; Friend and foe, in: E. J. Pell, K. L. Steffen, (Eds.), *Active oxygen/oxidative stress and plant metabolism*, American Society of Plant Physiologists, Rockville, 1991, pp. 1-5.

[14] K. Shinozaki, K. Yamaguchi-Shinozaki, Gene expression and signal transduction in water-stress response, *Plant Physiol.* 115 (1997) 327-334.

[15] L.S. Monk, K. V. Fagerstedt, R. M. M. Crawford, Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress, *Physiol. Plant.* 76 (1989) 456-459.

[16] E. J. Kendall, B. B. McKersie, Free radical and freezing injury to cell membranes of winter wheat, *Physiol. Plant* 76 (1989) 86-94.

[17] E. E. Benson, A. A. Noronha-Dutra, Chemiluminescence in cryopreserved plant tissue cultures: the possible involvement of singlet oxygen in cryoinjury, *Cryo-Letters* 9 (1988) 120-131.

[18] C. B. Rajashekar, Cell tension and cavitation in plants during freezing: Their role in injury, in: P. H. Li, T. H. H. Chen, (Eds.), *Plant Cold Hardiness*, Plenum Press, New York, 1997, pp. 203-214.

[19] R. D. Allen, Dissection of oxidative stress tolerance using transgenic plants, *Plant Physiol.* 107 (1995) 1049-1054.

[20] K. Asada, The water-water cycle in chloroplast: Scavenging of active oxygens and dissipation of excess photons, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50 (1999) 601-639.

- [21] D. A. Clare, H. D. Rabinowitch, I. Fridovich, Superoxide dismutase and chilling injury in *Chlorella ellipsoidea*, Arch. Biochem. Biophys. 231 (1984) 158-163.
- [22] B. D. McKersie, Y. R. Chen, M. De Beus, S. R. Bowler, D. Inzé, K. D'Halluin, J. Botterman, Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.), Plant Physiol. 103 (1993) 1155-1163.
- [23] T. K. Prasad, M. D. Anderson, B. A. Martin, C. R. Stewart, Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide, Plant Cell 6 (1994) 65-74.
- [24] A. S. Gupta, J. L. Heinen, A. S. Holaday, J. J. Burke, R. D. Allen, Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase, Proc. Natl. Acad. Sci. USA 90 (1993) 1629-1633.
- [25] E. W. Storlie, R. E. Allan, M. K. Walker-Simmons, Effect of the Vrn1-Fr1 interval on cold hardiness levels in near isogenic wheat lines, Crop Sci. 38 (1998) 483-488.
- [26] T. Okuda, Y. Matsuda, A. Yamanaka, S. Sagisaka, Abrupt increase in the level of hydrogen-peroxide in leaves of winter-wheat is caused by cold treatment, Plant Physiol. 97 (1991) 1265-1267.
- [27] G. M. Bridger, W. Yang, D. E. Falk, B. D. McKersie, Cold acclimation increases tolerance of activated oxygen in winter cereals, J. Plant Physiol. 144 (1994) 235-240.
- [28] F. Scebba, L. Sebastiani, C. Vitagliano, Protective enzymes against activated oxygen species in wheat (*Triticum aestivum* L.) seedlings: Responses to cold acclimation, J. Plant Physiol. 155 (1999) 762-768.
- [29] T. Janda, G. Szalai, R. Rios-Gonzalez, O. Veisz, E. Paldi, Comparative study of frost tolerance and antioxidant activity in cereals, Plant Sci. 164 (2003) 301-306.
- [30] E. F. Elstner, Mechanisms of oxygen activation in different compartments of plant cells, in: E. J. Pell, K. L. Steffen, (Eds.), Active oxygen/oxidative stress and plant metabolism, American Society of Plant Physiologists, Rockville, 1991, pp.13-25.
- [31] G. C. Vanlerberghe, L. McIntosh, Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco, Plant Physiol. 100 (1992) 115-119.
- [32] C. Bowler, M. Van Montagu, D. Inzé, Superoxide dismutase and stress tolerance, Annu. Rev. Plant Physiol. Plant Mol. Biol 43 (1992) 83-116.

- [33] K. L. Steffen, J. P. Palta, Photosynthesis as a key process in plant response to low temperature: Alteration during low temperature acclimation and impairment during incipient freeze-thaw injury, in: P. H. Li, (Eds.), Plant cold hardiness, Plant Biology Vol. 5, Alan R. Liss, Inc., New York, NY, 1987, pp. 67-99.
- [34] V. M. Hurry, A. Strand, M. Tobiason, P. Gardestrom, G. Oquist, Cold Hardening of Spring and Winter Wheat and Rape Results in Differential Effects on Growth, Carbon Metabolism, and Carbohydrate Content, Plant Physiol. 109 (1995) 697-706.
- [35] G. Galiba, S.A. Quarrie, J. Sutka, A. Morounov, J.W. Snape, RFLP mapping of the vernalization (Vrn1) and frost resistance (Fr1) genes on chromosome 5A of wheat, Theor. Appl. Genet. 90, (1995) 1174-1179.

Table 1. DNA sequences of PCR primers used in quantitative real-time PCR determination of antioxidant enzyme gene copy number in wheat lines undergoing cold acclimation

Antioxidant		Primer pair sequences	Expected Amplicon Size (bp)
Enzyme	Origin ^a		
CAT	GI5711144	CCATGAGATCAAGGCCATCT ATCTTACATGCTCGGCTTGG	103
Cu,ZnSOD	GI1572626	CGCTCAGAGCCTCCTCTTT CTCCTGGGGTGGAGACAAT	98
FeSOD	TC38624 (FeSOD in rice)	GTCCTACTACGGCCTCACCA ACGTAGTCCTGCTGGTGCTT	108
MnSOD	GI1622928	CAGAGGGTGCTGCTTTACAA GGTCACAAGAGGGTCCTGAT	107
APX	TC22268 (thylakoid-bound APX in tobacco)	GCAGCTGCTGAAGGAGAAGT CACTGGGGCCACTACTAAT	99
GPX	TC22467 (GP-like protein in barley)	CCCCCTGTACAAGTTCCTGA GTCAACAACGTGACCCTCCT	106
GR	TC84151 (cytosolic GR in rice)	TGCGTCCCGAAGAAGATACT GTTGATGTCCCCGTTGATCT	96
DHAR	TC26944 (GSH-dependent DHAR in rice)	GACCAAGGAGAACCTGATCG CGTCGCTACTCTCACACGAC	103
MDAR	TC27229 (cytosolic MDAR in rice)	GCTCCTCGACCATAAAGCTC CATAGCTGCGACCAACTTGT	107

^aSequence identifiers are indicated from GenBank (identifier starts with GI) or the TIGR (<http://www.tigr.org>) database (identifier starts with TC).

Table 2. DNA sequences of PCR primers used in quantitative real-time PCR determination of antioxidant enzyme gene copy number in wheat lines undergoing cold acclimation

Antioxidant Enzyme	Origin ^a	Primer pair sequences	Expected Amplicon Size (bp)
CAT	GI5711144	GAGTCGCTCCACATGTTTCAC TTCACCAGCGTGTAGGTGTT	101
Cu,ZnSOD	GI1572626	GTTGTTGGGAGAGCGTTTGT GCAAGTCTTCCACCAGCATT	101
FeSOD	TC38624 (FeSOD in rice)	GAAGCTTGAGGTGGTTCACA TAAGCATGCTCCCACAAGTC	93
APX	TC22268 (thylakoid-bound APX in tobacco)	GGCTAATGCTGGTCTTGTGA GCACTGGCAAGTTGAAACAG	99
GPX	TC22467 (GP-like protein in barley)	GTCGACCTCAGCGTCTACAA CTCAGCTCGGTGTAGTTGGA	92
GR	TC84151 (cytosolic GR in rice)	TCAACCACCAGTTTCATGGT GCGGAAACCAAGAATGATCT	104
DHAR	TC26944 (GSH-dependent DHAR in rice)	AGATCGACAGCGGAGATGTT GTTTCGGAGAAGGCACTTGTT	100
MDAR	TC27229 (cytosolic MDHAR in rice)	TGTGGTGAAGCTGAAGGATG CCTCTTCAACTTGGCCTTTG	104

^aSequence identifiers are indicated from GenBank (identifier starts with GI) or the TIGR (<http://www.tigr.org>) database (identifier starts with TC).

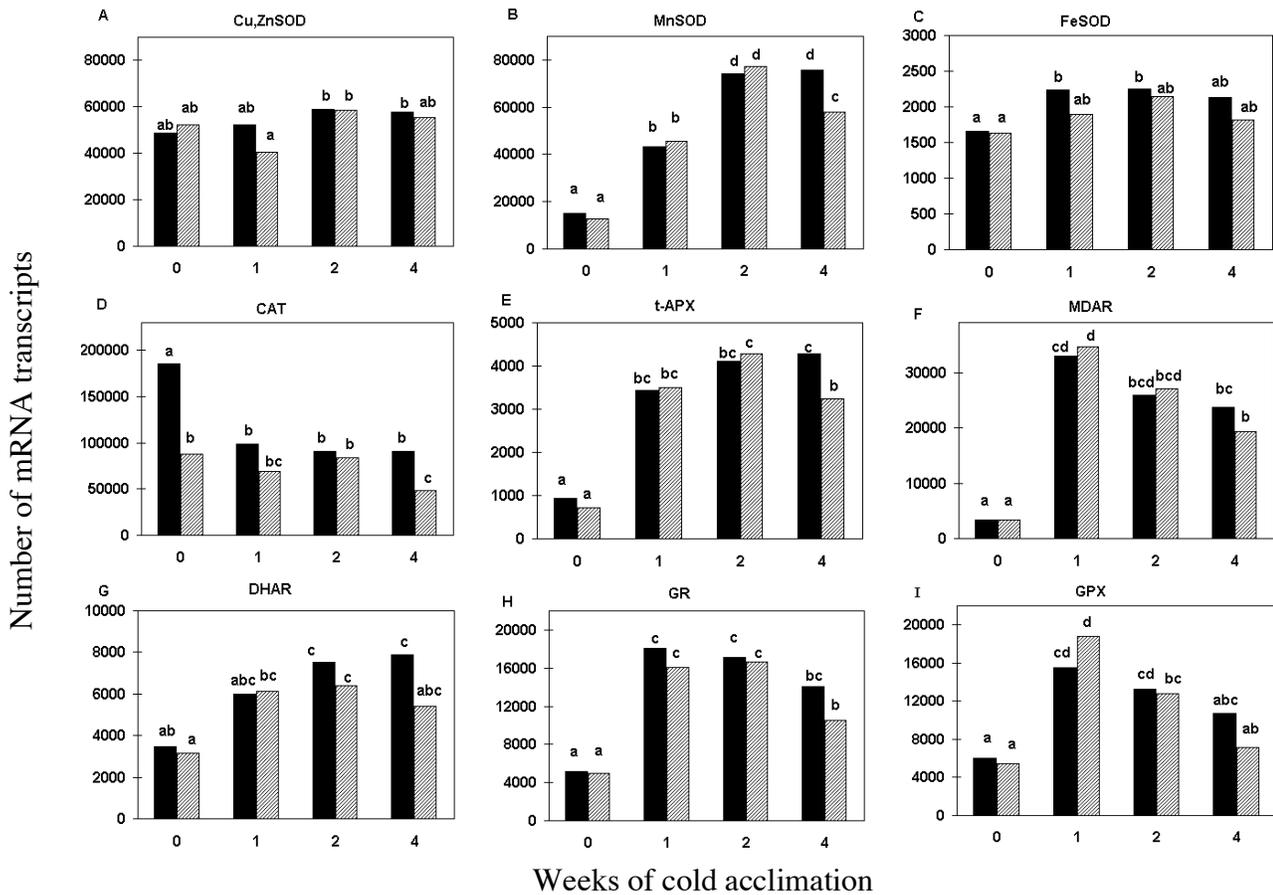


Figure 1. Quantitative real-time PCR determination of the number of copies of mRNA transcript of the indicated antioxidant enzyme gene in 125ng RNA after 0, 1, 2 or 4 weeks of cold acclimation of wheat near-isogenic lines. Solid bars represent NIL 442 (winter wheat) and cross-hatched bars represent NIL 443 (spring wheat).