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Cu/Zn superoxide dismutase activity and respective gene expression during cold acclimation and freezing stress in barley cultivars

C. KAYIHAN[1], F. EYIDOGAN[2]*, N. AFSAR[1], H.A. OKTEM[1] and M. YUCEL[1]

Middle East Technical University, Department of Biology, 06531 Ankara, Turkey[1]
Baskent University, Faculty of Education, 06810, Ankara, Turkey[2]

Abstract

The transcript levels and activities of the superoxide dismutase isoenzyme (Cu/ZnSOD) were assessed in winter (Tarm-92) and spring (Zafer-160) barley cultivars during cold acclimation, freezing stress and after rewarming. Leaf Cu/ZnSOD activity and *Cu/ZnSOD* expression level were not significantly changed during cold acclimation. The *Cu/ZnSOD* expression increased evidently at mild freezing stress (-3 °C; F1), while Cu/ZnSOD1 activity did not show any response and Cu/ZnSOD2 activity decreased continuously during F1 and F2 (-7 °C) in Tarm-92. On the other hand, root Cu/ZnSOD2 activity was in accordance with *Cu/ZnSOD* expression in Zafer-160 after F2 treatment. Rewarming periods did not cause any significant changes in the Cu/ZnSOD activity and expression of *Cu/ZnSOD* in both cultivars when compared to freezing stresses. These results showed that freezing stress can regulate differently *Cu/ZnSOD* transcription and enzyme activity.

Additional key words: antioxidant enzymes, *Hordeum vulgare*, low temperature, rewarming.

Introduction

Various environmental stresses result in the increased generation of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) (Okuda *et al.* 1991, Alscher *et al.* 1997). Overproduction of ROS can lead to negative effects on cellular structures and metabolism, and even cell death since these ROS are capable of causing oxidative damage to proteins, DNA, and membrane lipids (Kendall and McKersie 1989, Apel and Hirt 2004). On the other hand, plant cells have evolved several enzymatic and non-enzymatic antioxidant systems to keep low concentrations of ROS. Enzymatic antioxidant defenses in plants comprise mainly superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). Superoxide dismutases (SODs, EC 1.15.1.1) are a family of metalloenzymes which catalyse the dismutation of toxic superoxide radicals to O_2 and H_2O_2, and thus have central role in the antioxidant defence network. Based on the metal co-factor used by the enzyme, SODs are classified into three groups: FeSOD, MnSOD and Cu/ZnSOD (Fridovich 1989). These SOD isozymes are located in different cell compartments. FeSOD can be found in the chloroplasts, MnSOD in the mitochondria and the peroxisomes, whereas Cu/ZnSOD in cytosol, chloroplasts, mitochondria, peroxisomes, glyoxisomes and extracellular space (Bannister *et al.* 1987).

It is well known that SOD is important in plant stress tolerance (Gill and Tuteja 2010). Furthermore, Cu/ZnSOD has been postulated as a general stress protein (Kliebenstein *et al.* 1998). In several plant species, it has been observed that Cu/ZnSOD expression can be induced by herbicide paraquat, ozone, NaCl, hormones, high or low temperature and high irradiance (Abercrombie *et al.* 2008, Guan and Scandalios 1998, Kliebenstein *et al.* 1998, Sakamoto *et al.* 1992).

One of the most important agronomic traits in barley (*Hordeum vulgare* L.) is winter-hardiness because barley production has been shifted from spring to autumn sowing due to higher yields of winter cultivars than spring ones. A fundamental component of winter-hardiness is freezing tolerance, which is based on an

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Abbreviations: APX - ascorbate peroxidase; CA - cold-acclimated; CAT - catalase; GR - glutathione reductase; NBT - nitroblue tetrazolium; ROS - reactive oxygen species; RT-PCR - reverse transcription polymerase chain reaction; SOD - superoxide dismutase.
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* Corresponding author: fax: (+90) 3122466628, e-mail: fusunie@baskent.edu.tr

inducible process, known as hardening or cold acclimation, that occurs when plants are exposed to low non-freezing temperatures. Cold acclimation may protect the integrity of cellular membranes, enhance the antioxidative mechanisms and finally improve freezing stress tolerance (Mahajan and Tuteja 2005). The responses to low temperature stress has been widely studied in several crops, including barley. However, the mechanisms of these processes are not satisfactory elucidated. Therefore, the aim of this study is to unravel the changes in the expression and the activity of Cu/ZnSOD under freezing stress, with prior cold acclimation as well as under subsequent rewarming in two barley cultivars. This approach may help to understand the differential cellular responses of winter and spring cultivars to cold stress.

Materials and methods

Seeds of two barley (*Hordeum vulgare* L.) cultivars (winter cv. Tarm-92 and spring cv. Zafer-160) were provided by the Turkish Ministry of Agriculture and Rural Affairs, Ankara. Seeds of both cultivars were sown in plastic pots filled with *Perlite* moistened with half strength Hoagland's solution (Hoagland and Arnon 1950). Plants were grown for 8 d in a growth chamber with supplementary irradiance (300 μmol m^{-2} s^{-1}) providing a 16-h photoperiod with a temperature of 23 °C. After 8 d, plants were cold-acclimated for 3 d at 4 °C (CA). Then, freezing stresses were applied at -3 °C (F1) or -7 °C (F2) for 3 and 1.5 h, respectively in a cooling incubator. Subsequently, rewarming treatments after both freezing temperatures were performed at 4 °C for 4 d (R1 and R2).

SOD activity was measured according to Burke and Oliver (1992). Leaf tissue (1 g) from control and treated plants was homogenized in buffer containing 9 mM Tris-HCl, pH 6.8, and 13.6 % (v/v) glycerol. The homogenate was centrifuged at 18 000 *g* for 5 min and the supernatant was used for enzyme assay. Root tissue (1 g) from control and treated plants was ground in a mortar on ice using a homogenization medium consisting of 0.2 M sodium phosphate buffer (pH 7.8) and EDTA. The suspension was centrifuged at 10 000 *g* for 30 min and the supernatant was used for the enzyme assay. Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard. Equal amounts of proteins were loaded onto non-denaturing (native) polyacrylamide gel electrophoresis. SOD activity was detected by determining its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971). The changes in SOD isozymes were evaluated from band intensities by using *Scion* imaging software program. The unit of SOD activity was determined by running a SOD standard (specific activity = 3350 units mg^{-1}) in the same gel. One unit of standard SOD activity inhibits the rate of reduction of cytochrome *c* by 50 % in a coupled system with xanthine oxidase at pH 7.8 and 25 °C in a 3 cm^3 reaction medium. For determination of SOD isozymes, staining assays were performed in the presence of selective inhibitors. KCN (3 mM) inhibited only Cu/ZnSOD, while H_2O_2 (5 mM) inhibited both Cu/ZnSOD and FeSOD. MnSOD was inhibited neither by KCN nor H_2O_2. On the other hand, 5 mM NaN_3 inhibited only FeSOD.

Total RNA was isolated from leaf and root tissues and stored at -70 °C until used for semi-quantitative RT-PCR. Total RNA extraction and semi-quantitative RT-PCR were replicated four times. RNA was reverse-transcribed into cDNA using *Moloney* murine leukemia virus reverse transcriptase (*Fermentas*, Burlington, Canada) as recommended by the manufacturer. The SOD primers for *HvSOD* were constructed with the *Primer3* program using the barley gene sequences in the *TIGR* database or GenBank. The primer for SOD was designed to produce an amplicon of about 180 nucleotides. Produced cDNA was used for PCR reactions in a 0.05 cm^3 reaction mixture containing: 3 mm^3 of cDNA from control and treated plants, 0.05 U mm^{-3} *Taq* DNA Polymerase (*Applichem*, Ottoweg, Germany), 10× *Taq* DNA polymerase buffer, 2 mM dNTPs, 25 mM $MgCI_2$, 10 μM primers Sod-forward (5'- TCATCAGCATCACCATG GAC-3') and Sod-reverse (5'- ATGTCAACTGGACC GCACTT-3') to amplify *HvSOD*. Elongation factor, Ef (*HvEF-1β*), was used as an internal control. The primers Ef-forward (5'-CCAAGAATCCAGCAGCAACA-3') and Ef-reverse (5'-TAAGCTCATGCCTGTGGGTTA-3') were utilized to amplify *HvEF-1β*. Amplification conditions were as follows: 94 °C for 5 min, 33 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. RT-PCR reaction mixtures were loaded onto 2 % agarose gels in TAE buffer. A 50 bp DNA ladder (*Fermentas*, SM0371) was run on every gel. Quantification of the bands was determined by using *Scion* imaging software program. A ratio of *HvSOD* band intensities to *HvEF-1β* band intensities was calculated.

The experiments were performed in a completely randomized design, and the *SPSS* statistical program was used to determine the differences between the cultivars and treatments. To confirm the variability of data and validity of results, all the data were subjected to an analysis of variance (*ANOVA*) and to determine differences between cultivars and treatments, the least significant difference (LSD) was calculated at 5 % level ($P < 0.05$).

Results

Separating of leaf and root extracts by non-denaturing PAGE and staining for SOD activity revealed up to three isoformic bands (Fig. 1*A*). The band showing resistance to inhibition by KCN, H_2O_2 and NaN_3 was identified as MnSOD (Fig. 1*B*). The other two isozymes were sensitive to both KCN and H_2O_2. Therefore, they were assigned as Cu/ZnSOD. The band with higher molecular mass was named Cu/ZnSOD1 while the one with higher relative mobility was named Cu/ZnSOD2. FeSOD isoform was not observed in the native gels (Fig. 1*B*).

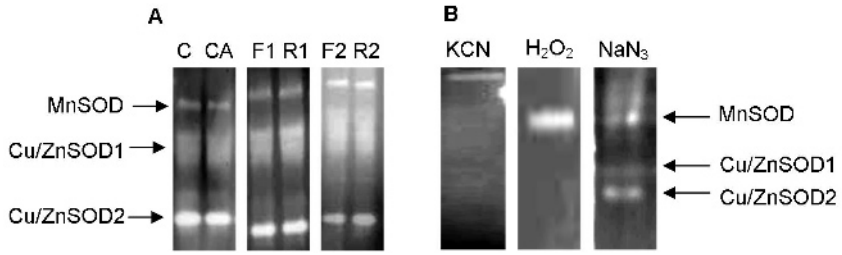

Fig. 1. Native gels stained for the activity of SOD (*A*) and the identification of SOD isoforms in the leaves of barley plants (Tarm-92) (*B*). C - control plants (23 °C), CA - cold-acclimated plants (3 d at 4 °C), F1 - freezing at -3 °C for 3 h after CA, R1 - rewarming at 4 °C for 4 d after F1, F2 - freezing at -7°C for 1.5 h after CA, R2 - rewarming at 4 °C for 4 d after F2. SOD activity was performed without any inhibitor (control) and in the presence of KCN, H_2O_2 and NaN_3.

Table 1. Cu/ZnSOD1 and Cu/ZnSOD2 activities [U μg^{-1}(protein)] in leaves and roots of two barley cultivars. C - control plants (23 °C), CA - cold-acclimated plants (3 d at 4 °C), F1 - freezing at -3 °C for 3 h after CA, R1 - rewarming at 4 °C for 4 d after F1, F2 - freezing at -7°C for 1.5 h after CA, R2 - rewarming at 4 °C for 4 d after F2. Means ± SE, n = 4, the same letters within a column indicate no significant difference at 95 % probability according to LSD test.

Parameters	Cultivars	Tissues	C	CA	F1	R1	F2	R2
Cu/ZnSOD1	Tarm-92	leaf	20.0±1.13^{a}	18.4±1.46^{a}	19.0±0.58^{a}	18.9±1.09^{a}	18.5±0.77^{a}	18.7±0.70^{a}
	Tarm-92	root	24.0±1.26^{a}	26.0±1.37^{a}	17.6±0.92^{b}	19.2±0.62^{bc}	20.3±1.34^{c}	22.8±0.88^{ac}
	Zafer-160	leaf	22.0±1.38^{a}	21.5±0.80^{a}	13.8±0.92^{b}	15.0±0.24^{b}	18.4±1.30^{a}	22.0±1.56^{a}
	Zafer-160	root	9.0±1.46^{a}	13.3±1.37^{a}	12.1±2.27^{a}	11.0±0.75^{a}	22.8±1.74^{b}	27.5±1.96^{b}
Cu/ZnSOD2	Tarm-92	leaf	24.9±1.15^{a}	24.0±1.60^{a}	20.0±1.37^{b}	21.2±0.95^{ab}	14.1±0.82^{c}	14.4±0.70^{c}
	Tarm-92	root	18.4±1.27^{a}	18.6±1.71^{a}	9.8±1.18^{b}	10.6±1.96^{b}	17.8±1.42^{a}	19.0±0.70^{a}
	Zafer-160	leaf	27.3±1.80^{a}	26.0±1.23^{a}	16.3±0.87^{b}	17.6±0.26^{b}	18.5±1.19^{b}	20.1±0.77^{b}
	Zafer-160	root	13.0±1.66^{a}	11.1±1.70^{a}	5.2±0.12^{b}	5.5±0.49^{b}	21.0±2.08^{c}	20.6±1.80^{c}

Cold acclimation (CA), freezing stresses (F1 and F2) and rewarming periods (R1 and R2) did not cause any significant change in the leaf Cu/ZnSOD1 activity in Tarm-92 (Table 1). However, compared to control (C) and CA, the leaf Cu/ZnSOD1 activity significantly decreased during F1 and R1 and increased during F2 and R2 in Zafer-160. In Tarm-92 roots, F1 and F2 treatment caused a significant decrease in Cu/ZnSOD1 activity (Table 1). On the other hand, the root Cu/ZnSOD1 activity in F2 was higher than that in C and CA in Zafer-160.

Cu/ZnSOD2 activity decreased rapidly in the leaves of Tarm-92 after exposure to F1 and maintained a decreased level in F2 as well. Similarly, the leaf Cu/ZnSOD2 activities in F1 and F2 were significantly lower than those in CA and C in Zafer-160. Rewarming periods, R1 and R2, did not lead to any significant changes in the leaf Cu/ZnSOD2 activities in both cultivars when compared to F1 and F2, respectively (Table 1). In roots of both cultivars, CA did not cause any significant change in Cu/ZnSOD2 activities. When both cultivars were exposed to F1, the root Cu/ZnSOD2 activities were significantly decreased and maintained a reduced level in R1 but they increased markedly in response to F2 and maintained an elevated level in R2 (Table 1).

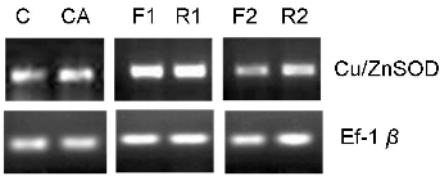

Fig. 2. RT-PCR results of Cu/ZnSOD in the leaves of barley plants (Tarm-92). C - control plants (23 °C), CA - cold-acclimated plants (3 d at 4 °C), F1 - freezing at -3 °C for 3 h after CA, R1 - rewarming at 4 °C for 4 d after F1, F2 - freezing at -7°C for 1.5 h after CA, R2 - rewarming at 4 °C for 4 d after F2. Bands at the bottom indicate expression level of internal control (*HvEF-1β*).

Table 2. Relative RT-PCR analysis of Cu/ZnSOD gene transcripts in the leaves and roots of two barley cultivars. C - control plants (23 °C), CA - cold-acclimated plants (3 d at 4 °C), F1 - freezing at -3 °C for 3 h after CA, R1 - rewarming at 4 °C for 4 d after F1, F2 - freezing at -7 °C for 1.5 h after CA, R2 - rewarming at 4 °C for 4 d after F2. Means ± SE, $n = 4$, the same letters within a column indicate no significant difference at 95 % probability according to LSD test.

Parameters	Cultivars	Tissues	C	CA	F1	R1	F2	R2
Cu/ZnSOD	Tarm-92	leaf	1.49±0.08^{a}	1.01±0.06^{b}	1.33±0.05^{ab}	1.37±0.03^{a}	1.16±0.04^{bc}	1.30±0.00^{ac}
	Tarm-92	root	0.70±0.05^{a}	0.69±0.02^{a}	1.04±0.00^{b}	1.14±0.01^{b}	0.52±0.02^{c}	0.60±0.03^{ac}
	Zafer-160	leaf	0.89±0.09^{a}	0.85±0.05^{a}	0.83±0.02^{a}	0.94±0.00^{ab}	1.09±0.08^{bc}	1.20±0.06^{c}
	Zafer-160	root	1.07±0.17^{ab}	0.94±0.03^{a}	1.17±0.03^{b}	1.11±0.05^{b}	1.87±0.03^{c}	1.53±0.09^{d}

The expression patterns of the *Cu/ZnSOD* gene in barley seedlings under cold acclimation, freezing stresses and rewarming were investigated by RT-PCR (Fig. 2). The leaf *Cu/ZnSOD* expression decreased significantly in CA in Tarm-92 (Table 2). In comparison with CA, F1 and F2 treatments induced a significant increase in the leaf *Cu/ZnSOD* expression in Tarm-92. In leaf tissues of this cultivar, the expression level of *Cu/ZnSOD* after R1 and R2 was not significantly different from that prior to F1 and F2. On the other hand, there was no significant difference of *Cu/Zn-SOD* expression among CA and C in the leaves of Zafer-160. In this cultivar, the leaf *Cu/ZnSOD* transcript level increased significantly in F2 and the maximum expression was detected after R2 (Table 2). The root *Cu/ZnSOD* expression after F1 and R1 was significantly higher than that of the C and CA, but declined sharply in F2 and R2 in Tarm-92 (Table 2). On the other hand, the root *Cu/ZnSOD* expression was greatly induced in F2 and then decreased in R2 in Zafer-160.

Discussion

Exposure to low or freezing temperatures raises ROS content, which results in oxidative stress in plants (Prasad *et al.* 1994). However, exposure of winter cereals to cold acclimation temperatures induces an increased tolerance to oxidative stress (Bridger *et al.* 1994). It has been suggested that an increased cold tolerance can be accompanied by the increase of expression of specific genes encoding antioxidant enzymes (Baek and Skinner 2003). Furthermore, the activities of several antioxidant enzymes increased during cold acclimation in wheat (Scebba *et al.* 1999). In the present work, Cu/ZnSOD1 and Cu/ZnSOD2 activities and MnSOD activity (data not shown) and expression of *Cu/ZnSOD* gene (except for leaf tissues of Tarm-92) were not significantly changed by cold acclimation and there were no significant differences between the winter and spring barley cultivars. These findings suggest that the maintenance of constant SOD amount and activity might be sufficient to ensure protection against the superoxide radicals produced during cold acclimation. An efficient system to protect the plant from ROS could maintain the constant activity of Cu/ZnSOD but increase the activities of enzymes that scavenge the H_2O_2 produced by Cu/ZnSOD during cold acclimation since Cu/ZnSOD is very sensitive to accumulation of H_2O_2 (Bowler *et al.* 1992). Likewise, Afsar (2007) showed that enhanced APX and glutathione reductase activities prevented accumulation of H_2O_2 in leaf tissue during cold acclimation in winter and spring barley cultivars.

Freeze-induced cellular dehydration as well as production of ROS contribute to membrane damage (McKersie and Bowley 1998). To date, the protective role of SOD in plants has been explored by transgenic approaches or by correlation of SOD expression to the degree of oxidative stress resistance (Gupta *et al.* 1993, McKersie *et al.* 1993, Hernández-Nistal *et al.* 2002). In this study, F2 induced the activity and expression level of Cu/ZnSOD in the root of Zafer-160. The root Cu/ZnSOD2 activity was in accordance with respective gene expression after F2. On the other hand, the leaf *Cu/ZnSOD* expression increased evidently in F1, while Cu/ZnSOD1 activity did not show any response to enhanced freezing tolerance and Cu/ZnSOD2 activity decreased continuously in F1 and F2, especially in F2, in Tarm-92. Furthermore, in both cultivars, the root *Cu/ZnSOD* expression increased significantly in F1, while Cu/ZnSOD2 activity decreased sharply. This implied that the changes of Cu/ZnSOD1 and Cu/ZnSOD2 activities did not absolutely correspond to the changes of their gene expression in roots of both cultivars in F1. Likewise, Cu/ZnSOD activity and expression level were not coordinately regulated in the leaves of Zafer-160 after F1. It is possible that post-transcriptional regulations of *Cu/ZnSOD* gene may have a key role for freezing stress tolerance. It was indicated that *Cu/ZnSOD* expression in *Arabidopsis* is regulated by microRNA (Sunkar *et al.* 2006). Although Perl *et al.* (1993) proposed that the pathways controlling two different Cu/ZnSODs may be coordinately regulated, we detected different responses of Cu/ZnSOD1 and Cu/ZnSOD2 under the same conditions in the roots and leaves of both cultivars. These findings are in agreement with previous reports on *Nicotiana*

plumbaginifolia (Tsang *et al.* 1991) and cucumber (Lee and Lee 2000). Rewarming period after freezing stress is especially important to understand the changes in antioxidants and antioxidant enzymes. Fu *et al.* (2011) suggests that SOD activity is partially restored after rewarming. In this study, in rewarmed plants, the SOD activity and expression of *Cu/ZnSOD* were on the same level as during freezing in both cultivars.

In conclusion, there were not dramatic differences between the two barley cultivars at early cold acclimation, but significant differences occurred during freezing after cold acclimation with respect to changes in Cu/ZnSOD activity and expression of *Cu/ZnSOD*. It was observed previously, that the increase in electrolyte leakage and H_2O_2 content after freezing treatment were higher in Zafer-160 than that in Tarm-92 and Tarm-92 showed a lower cellular damage and a higher cold tolerance (Afsar 2007). However, the responses of *Cu/ZnSOD* expression to freezing stresses did not go along with the changes in Cu/ZnSOD activity because SOD might have a very fast catalytic rate of converting superoxide into H_2O_2 or/and possible post-transcriptional regulation. Therefore, the activities of enzymes that scavenge the H_2O_2 produced by SOD and other factors after transcription are critical to better understand the role of SOD in freezing tolerance. Determining the nature of the genes and mechanisms responsible for freezing tolerance would be highly necessary as traditional plant breeding approaches have had limited success in improving freezing tolerance.

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