EFFECT OF COLD STRESS ON BARLEY (*Hordeum vulgare* L.) SUPEROXIDE DISMUTASE ISOZYME ACTIVITIES AND EXPRESSION LEVELS OF Cu/ZnSOD GENE

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ABSTRACT

EFFECT OF COLD STRESS ON BARLEY (*Hordeum vulgare* L.) SUPEROXIDE DISMUTASE ISOZYME ACTIVITIES AND EXPRESSION LEVELS OF Cu/ZnSOD GENE

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In this study, effect of cold stress and recovery on the superoxide dismutase (SOD) activities and the expression levels of Cu/ZnSOD gene were investigated in two barley cultivars (Tarm-92-winter type, Zafer-160-spring type). Eight days old barley seedlings were subjected to two different cold stresses; chilling stress at 4°C for 1, 3, 7 days and freezing stress at -3°C and -7°C. Analyses were performed both on leaf and root tissues. The SOD activities and isozyme patterns were determined by Native PAGE activity staining technique. Relative RT-PCR was used for the transcript levels of Cu/ZnSOD gene. The SOD activities and expression levels of control and cold stressed plants were compared by densitometric analysis. Under chilling stress, the activities of Cu/ZnSODx and Cu/ZnSOD2 did not have any significant change in leaf and root tissues of both cultivars. However, the expression levels of Cu/ZnSOD gene were more variable than activity results. After -3°C freezing stress,

the activities of SOD enzyme in leaf tissues of Tarm-92 increased significantly, however, these activities significantly decreased in leaves of Zafer-160. These results suggested that both cultivars were not affected by chilling stress in terms of SOD enzyme activities and expression levels. Furthermore, under freezing stress conditions, the increment of SOD activities and expression levels in Zafer-160 was higher than Tarm-92. In conclusion, the changes in SOD isozyme activities and expression levels may not be enough for understanding of the cold stress mechanism. Therefore, further studies have to be carried on other antioxidant enzyme systems.

Key words: Superoxide dismutase, barley, cold stress, relative RT-PCR.

ÖZ

SOĞUK STRESİNİN ARPA (*Hordeum vulgare* L.) SÜPEROKSİT DİSMUTAZ İZOENZİM AKTİVİTELERİ VE Cu/ZnSOD GENİNİN EKSPRESYON SEVİYESİ ÜZERİNE ETKİSİ

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Bu çalışmada, soğuk stresinin SOD izozim aktivitelerine ve Cu/ZnSOD izoziminin ekspresyon seviyesi üzerine etkisi soğuğa hassaslıkları iki arpa türünde araştırılmıştır. (Tarm-92- kışlık tip, Zafer-160- yazlık tip). Sekiz gün büyütülen arpalar, soğuk streslerinden biri olan üşüme stresine +4°C'de 1, 3 ve 7 gün şeklinde maruz bırakılmış, donma stresi ise -3°C ve -7°C'de maruz bırakılarak uygulanmıştır. Bu çalışmada analizler, hem kök hemde yaprak kısımları kullanılarak yapılmıştır. Bu analizlerden biri olan SOD aktivite değişimi ve izozim belirleme çalışması 1-D Native PAGE tekniği kullanılarak yapılmıştır. Cu/ZnSOD geninin transkripsiyon analizi için ise relative RT-PCR tekniği kullanılmıştır. Ayrıca kontrol ve soğuk stresine maruz kalmış bitkide SOD izozim aktivite ve ekspresyon değişimi densiyometrik analiz yöntemi kullanılarak karşılaştırılmıştır. Üşüme stresi altında, her iki türün yaprak ve kök dokularının Cu/ZnSODx ve

Cu/ZnSOD2 izozimleri önemli bir aktivite değişimine sahip değildir. Buna karşın, Cu/ZnSOD geninin ekspresyon değişimi aktivite datalarına gore daha değişkendir.

-3°C stresine maruz kaldıktan sonra Tarm-92 türünün yaprağındaki SOD aktivite değişimi önemli ölçüde artmıştır, buna karşın, Zafer-160 türünün yaprak kısmında düşüş gözlenmiştir. Bu sonuçlar, SOD izozimlerinin aktivite ve ekspresyon değişim datalarına göre her iki türünde üşüme stresinden etkilenmediğini göstermiştir. Ek olarak, donma stresleri altında, Zafer-160 türündeki SOD izozimlerinin aktivite ve ekspresyon artışı Tarm-92'inden daha yüksektir. Sonuç olarak, SOD izozimlerinin aktivite ve ekspresyon değişimleri soğuk stress mekanizmasını anlamak için yeterli olmayabilir. Bundan dolayı, diğer antioksidan enzim sistemleriyle ilgili ilave çalışmalar yapılmak zorundadır.

Key words: Süperoksit dismutaz, arpa, soğuk stresi, göreli Ters transkriptaz Polimeraz Zincir Reaksiyonu.

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LIST OF ABBREVIATIONS

ABA	Absisic acid
AOS	Activated oxygen species
APX	Ascorbate peroxidase
CAT	Catalase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
KCN	Potassium ciyanide
MDA	Malondialdehyde
O_2^1	Singlet oxygen
$O_2^{\cdot -}$	Superoxide radical
$OH^{\cdot}$	Hyroxyl radical
PAGE	Polyacrilamide gel electrophoresis
ROS	Reactive oxygen species
SEM	Standart error of mean
SOD	Superoxide dismutase

CHAPTER 1

INTRODUCTION

1.1. Barley

Barley (*Hordeum vulgare* L.) ranks fourth among the cereals in worldwide production after wheat, maize, and rice. It is an important crop for direct human consumption and for animal feed. It is unique as a source of malt for beer and other products and its annual world production is 150 million metric tons (FAOSTAT, 2005). Barley is one of the most widely adapted cereal crops. It is grown in a range of extreme environments that vary from northern Scandinavia to the Himalayan mountains to monsoon paddies (Hayes *et al*., 2003). Russia is the top barley producing country in the world. France and Germany are the leading European producers and Turkey is the sixth country in the world with 9 million metric tons. (FAOSTAT, 2005)

Barley is grown for many purposes, but the majority of all barley is used for animal feed, human consumption, or malting. High protein barleys are generally valued for food and feeding, and starchy barley for malting (Duke, 1983). It is basically a livestock feed that can be rolled, ground, flaked or pelleted (Kling, 2004). Barley is also used for pasture, greenfeed, hay, roughage and bedding (Young, 2001). The second most important use of barley is for malt which is used to produce beer, distilled alcohol, malt syrup, malted milk, flavorings and breakfast foods (Small, 1999).

Hordeum vulgare L. belongs to the division *Magnoliophyta* (flowering plants), class *Liliopsida* (monocotyledons), subclass *Commelinidae,* order *Cyperales*, and family *Poaceae* (Graminae-grasses).

It is an annual diploid species with 2n=14 chromosomes and the wild ancestor of barley (*H. vulgare* subsp. *spontaneum*) is thought to be a subspecies of cultivated barley which crosses readily with them and is cytologically indistinguishable (Janick *et al*., 1981).

The exact origin of barley is debatable, possibly originating in Egypt, Ethiopia, the Near East or Tibet (Duke,1983). However, it is thought that barley was among the earliest cultivated grains, around the same time as domestication of wheat. Barley was grown in the Middle East prior to 10,000 BC, but barley's cultivation in China and India probably occurred later (Kling, 2004).

Depending on degree of winter hardiness, barley varieties are divided into two types, namely spring and winter barley which has a wider ecological range than any other cereal because it is more adaptable than other cereals, tolerating many diverse environments except for acidic and wet soils. (Duke, 1983).

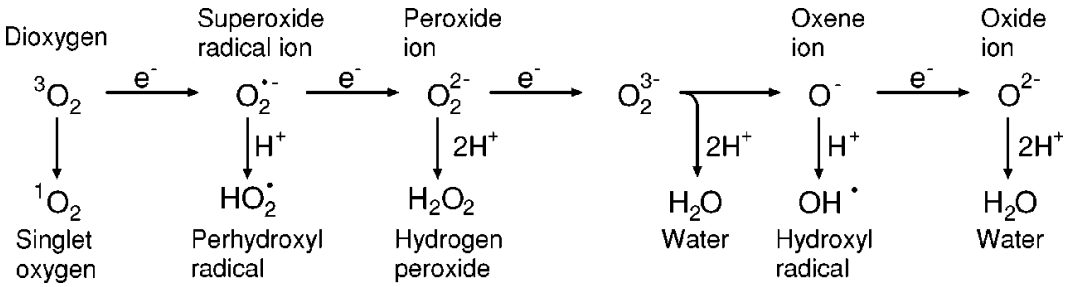
1.2. Environmental Stresses

Reactive oxygen species (ROS) are continuously produced in chloroplasts, mitochondria, and peroxisomes. Production and removal of ROS must be controlled, however, extreme environmental stress conditions such as high light, drought, low temperature, high temperature and mechanical stress can disturb the balance between production and scavenging of AOS (Tsugane *et al., 1999)*.

1.2.1. Generation of Reactive Oxygen Species

An atom is considered to be at ground state when every electron in the outermost shell has a complimentary electron that spins in the opposite direction. By definition a free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell, and is capable

of independent existence. A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom.(Karlsson, 1997). There are lots of types of free radicals that can be formed within the organism. Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). These are the superoxide anion (O_2^-), the hydroxyl radical ($OH^\cdot$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2).



Superoxide anions are formed when oxygen (O_2) acquires an additional electron, leaving the molecule with only one unpaired electron. Hydroxyl radicals are short-lived, but the most damaging radicals within the body. This type of free radical can be formed from O_2^- and H_2O_2 via the Harber-Weiss reaction and the interaction of copper or iron and H_2O_2 also produce $OH\cdot$ as first observed by fenton in the late nineteenth century.

$H_2O_2 + O_{2.}^- \text{----------}\rightarrow OH^- + O_2 + OH^\cdot$ (Haber-Weiss reaction).

$Fe^{2}+ + H_2O_2 \text{------}\rightarrow OH^\cdot + OH^-$ (Fenton reaction).

Hydrogen peroxide is unique in that it can be converted to the highly damaging hydroxyl radical or be catalyzed and excreted harmlessly as water. If H_2O_2 is not converted into water, $^1O_2$ is formed. Singlet oxygen is not a free radical, but can be formed during radical reactions and also cause further reactions.

1.2.1.1. Sources of Reactive Oxygen Species

There are many potential sources of ROS in plants. Some are reactions involved in normal metabolism, such as photosynthesis and respiration. These are in line with the traditional concept, considering ROS as unavoidable byproducts of aerobic metabolism (Asada *et.al.,* 1987). Other sources of ROS belong to pathways enhanced during abiotic stresses, such as glycolate oxidase in peroxisomes during photorespiration.

There are at least four sites within the chloroplast that can activate oxygen. Firstly, the reducing side of PSI is thought to contribute significantly to the monovalent reduction of oxygen under conditions where NADP is limiting. This would occur, for example, if the Calvin cycle did not oxidise NADPH as rapidly as PSI supplied electrons. Secondly, photoactivated chlorophyll normally transfers its excitation energy to the PS reaction centres, but under conditions that prevent the captured light energy from being utilized in the electron transport systems, this energy can excite oxygen from the triplet to singlet form. Thirdly, the oxidizing side of PSII facilitates four single electron transfers from water to the PSII reaction centre releasing triplet or ground-state oxygen. Leaks of electrons from this site to molecular oxygen, or release of partially reduced oxygen products are thought to make a relatively minor contribution to activated oxygen production. (Elstner, 1991)

The mitochondrial electron transport chain is a major site of 0_2 generation in cells. 0_2 is generated towards the matrix surface of the inner mitochondrial membrane and being a charged molecule it tends to stay within the inner mitochondrial compartment where it is converted into H_20_2 (Rajindar, 1997). The various Fe-S proteins and NADH dehydrogenase have also been implicated as possible sites of superoxide and hydrogen peroxide formation (Turrens *et al*., 1982).

Peroxisomes are organelles with a single membrane that compartmentalise enzymes involved in the ß-oxidation of fatty acids, and the glyoxylic acid cycle including glycolate oxidase, catalase and various peroxidases. Glycolate oxidase produces H_2O_2 in a two electron transfer from glycolate to oxygen (Lindqvist *et al*., 1991). Xanthine oxidase, urate oxidase and NADH oxidase generate superoxide as a consequence of the oxidation of their substrates (Fridovich, 1970).

1.2.1.2. Free Radical Dependent Damage on Plant Growth

The reactions of activated oxygen with organic substrates are complex, but in biological systems there are even more complications due to the surface properties of membranes, electrical charges, binding properties of macromolecules, and compartmentalization of enzymes, substrates and catalysts.

In plants, ROS are continuously produced as byproducts of various metabolic pathways localized in different cellular compartments (Foyer *et.al.,* 1994). A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids.

The peroxidation of lipids involves three distinct steps: initiation, propagation and termination. The initiation reaction between an unsaturated fatty acid (e.g. linoleate) and the hydroxyl radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid. In the propagation reactions, this resonance structure reacts with triplet oxygen, which is a biradical having two unpaired electrons and therefore reacts readily with other radicals. This reaction forms a peroxy radical. The peroxy radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another carbon centred free radical that can participate in a second H abstraction. Therefore, once one hydroxyl radical initiates the peroxidation reaction by abstracting a single H atom, it creates a carbon radical product that is capable of reacting with ground

state oxygen in a chain reaction. The peroxidation reactions in membrane lipids are terminated when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals (McKersie, 1996).

The lipid hydroperoxide is unstable in the presence of Fe or other metal catalysts because ROOH will participate in a Fenton reaction leading to the formation of reactive alkoxy radicals.

According to above mechanism, oxygen free radical or lipid peroxidation reactions in plant membranes would selectively degrade unsaturated fatty acids and accumulate aldehydes, hydrocarbons, and cross-linked products. When examining the effects of environmental stresses on plant membranes, many studies have measured the products of lipid peroxidation, such as malondialdehyde and/or ethane.

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a peptide differ in their susceptibility to attack. Sulphur containing amino acids, and thiol groups specifically, are very susceptible sites. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges. Other forms of free radical attack on proteins are not reversible. For example, the oxidation of iron-sulphur centres by superoxide destroys enzymatic function (Gardner and Fridovich, 1991).

Many amino acids undergo specific irreversible modifications when a protein is oxidized. For example, tryptophan is readily cross-linked to form bityrosine products (Davies, 1987). Histidine, lysine, proline, arginine, and serine form carbonyl groups on oxidation. The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of

redox cycling, such as Fe. In these cases, the metal binds to a divalent cation binding site on the protein. The metal then reacts with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical that rapidly oxidizes an amino acid residue at or near the cation binding site of the protein (Stadtman, 1986).

Activated oxygen and agents that generate oxygen free radicals, such as ionizing radiation, induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects. Characterization of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986).

Degradation of the base will produce numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and –saturated products. The principle cause of single strand breaks is oxidation of the sugar moiety by the hydroxyl radical. *In vitro* neither hydrogen peroxide alone nor superoxide cause strand breaks under physiological conditions, and therefore, their toxicity *in vivo* is most likely the result of Fenton reactions with a metal catalyst.

1.2.2. Drought Stress

Water stress can arise as a result of two conditions, either because of excess of water or water deficit. Flooding is an example of excess of water, which primarily results in reduced oxygen supply to the roots. The more common water stress encountered is the water deficit stress known as the drought stress. Removal of water from the membrane disrupts the normal bilayer structure and results in the membrane becoming porous structure. Stress within the lipid bilayer may also result in displacement of membrane proteins and this contributes to loss of membrane integrity, selectivity, disruption of cellular compartmentalization and a loss of activity

of enzymes, which are primarily membrane based. (Tuteja *et al*., 2005) As a consequence of drought stress, many changes occur in the cell and these include change in the expression level of *LEA*/*dehydrin*-type genes, synthesis of molecular chaperones, which help in protecting the partner protein from degradation and proteinases that function to remove denatured and damaged proteins. This stress also leads to activation of enzymes involved in the production and removal of ROS (Cushman *et al*., 2000).

Another adaptive mechanism for protection against drought is the maintenance of turgor during periods of drought by adjusting the osmotic pressure of cells. There are two main routes whereby this can be achieved. Firstly, the cell can sequester ions into cellular compartments. Secondly, specialized osmolytes such as proline, glycine betaine, mannitol, trehalose, ononitol and ectoine can be synthesized to readjust cellular osmotic potential. These osmolytes are also active in scavenging ROS, especially if they are targeted to the chloroplast (Shen *et al*., 1997).

Proline and sugars can coat protein molecules, exclude solute from their surfaces and thereby reduce the rate of unfolding (Hoekstra *et al.,* 2001). During stress condition, tolerant plants synthesize large amounts of nonreducing disaccharides, such as trehalose, which can substitute for water by satisfying hydrogen bonding requirements of polar amino acid residues at protein surfaces, and maintain the folded active states of the proteins (Crowe *et al*., 1997).

General physiological effects of drought on plants are the reduction in vegetative growth, especially leaves growth. This water deficit condition results in slower cell division because of reduced cyclin-dependent kinase activity (Schuppler *et al*., 1998). Leaf growth is generally more sensitive

than the root growth. Reduced leaf expansion is beneficial to plants under water deficit condition, as less leaf area is exposed resulting in reduced transpiration.

The first response of virtually all the plants to acute water deficit is the closure of their stomata to prevent the transpirational water loss (Mansfield *et al*., 1990). Closure of stomata may result from direct evaporation of water from the guard cells with no metabolic involvement. This process of stomatal closure is referred to as hydropassive closure. Stomatal closure may also be metabolically dependent and involve processes that result in reversal of the ion influxes that cause stomatal opening. This process of stomatal closure, which requires ions and metabolites, is known as hydroactive closure. This seem to be ABA regulated.

Plant growth and response to a stress condition is largely under the control of hormones. Hormones, in particular ABA along with cytokinins and ethylene, have been implicated in the root–leaf signaling. Transport of ABA into root xylem is modulated by environmental factors such as xylem pH (S. Wilkinson *et al*., 2002). Under the water deficit condition the pH of xylem sap increases therefore promoting the loading of ABA into the root xylem and its transport to the leaf (Hartung *et al*., 2002). Environmental conditions that increase the rate of transpiration also result in an increase in the pH of leaf sap, which can promote ABA accumulation and lead to reduction in stomatal conductance (Davies *et al*., 2002). In addition, ABA promotes the influx of K+ ions from the guard cells, which results in the loss of turgor pressure leading to stomata closure.

Stomatal closure in response to a water deficit stress primarily results in decline in the rate of photosynthesis. Very severe drought conditions result in limited photosynthesis due to decline in Rubisco activity (Bota *et al*., 2004).

1.2.3. Salinity Stress

Salinity is one of the major environmental stress and is substantial constraint to crop production. The occurrence of the salinity land depends on various factors like amount of evaporation (leading to increase in salt concentration), or the amount of precipitation (leading to decrease in salt concentration) and weathering of rocks also affects salt concentration. Furthermore, agricultural lands that have been heavily irrigated are highly saline.

High salt concentration can alter the basic texture of the soil leading to decreased soil porosity, reduced soil aeration and water conductance. High salt depositions generate a low water potential zone in the soil making it increasingly difficult for the plant to acquire both water and nutrients. Therefore, salt stress results in a water deficit condition in the plant and is the same form of a physiological drought.

Salt stress has four basic affects on plant cells. Firstly, this stress arise from the disruption of ionic equilibrium due to dissipation of membrane potential. Secondly, Na has deleterious effect on the functioning of some of the enzymes (Niu *et al*., 1995) Thirdly, high concentration of Na cause osmotic imbalance, membrane disorganization, reduction in growth, inhibition of cell division, and expansion. Finally, high Na levels result in reduction photosynthesis and production of reactive oxygen species (Yeo, 1998).

The major ions for salt stress signaling are Na^+, K^+, H^+, and Ca^{2+} which brings homeostasis in the cell. K^+ has role in opening and closing of stomata. İt is essential co-factor for many enzymes like the pyruvate kinase.

High salinity results in increased cytosolic Ca^{2+} that is transported from the apoplast as well as the intracellular compartments (Knight *et al*., 1997). This transient increase in cytosolic Ca^{2+} initiates the stress signal transduction leading to salt adaptation.

A major consequence of NaCl stress is the loss of intracellular water. To prevent this water loss from the cell and protect the cellular proteins, plants accumulate many metabolites that are also known as “compatible solutes.” Frequently observed metabolites with an osmolyte function are sugars, mainly fructose and sucrose, sugar alcohols and complex sugars like trehalose and fructans. In addition charged metabolites like glycine betaine proline and ectoine are also accumulated (McCue and Hanson, 1990).

1.2.4. Cold Stress

Cold is one the major environmental limitation to crop productivity and to the distribution of wild species. In the world, approximately annual losses of $ 10-100 million or higher is due to low temperature damage (Pearce, 1999). As well as the economic effect of limitation, climate change is to alter the geographical distribution of some wild and crop species. Exposure to low temperature causes change in morphological, biochemical, and physiological characters since this stress increases in production of ROS. Cold stress was explained in detail in section 1.4.).

1.3. Antioxidant Defense Mechanisms

Hydroxyl radicals are much more reactive than other radicals. The main risk for a cell that produces superoxide and hyrogen peroxide may be posed by the intermediates' interaction, leading to the generation of highly reactive hyroxyl radicals. Since there are no known scavengers of hyroxyl radicals, the only way to avoid oxidative damage through this radical is to control the reactions that lead to its generation. Therefore, cells must evolve sophisticated strategies to keep low concentrations of superoxide, hyrogen peroxide (Apel *et al.,* 2004).

1.3.1. Non-enzymatic Systems

Nonenzymatic antioxidants consist of ascorbate, glutathione, tocopherol, flavonoids, alkoloids, and carotenoids. Among these antioxidants, the major ones are ascorbate and glutathione (GSH).

Glutathione (GSH) is a tripeptide (Glu-Cys-Gly) whose antioxidant function is facilitated by the sulphydryl group of cysteine (Rennenberg, 1982). In other words, its function is mediated by sulfhydryl group of cysteine, which forms a disulfide bond with a second molecule of GSH to form oxidized glutathione (GSSG) upon oxidation (Hausladen and Alscher, 1993). GSH is main storage form of sulfur, and it act as a potent detoxifier of xenobiotics through GSH-conjugation, and can serve as a precursor of phytochelatins (May *et al.,* 1998). Together with its oxidized form (GSSG) glutathione serve as a redox balance in the cellular compartments.

GSH has been found virtually in all cell compartments: cytosol, endoplasmic reticulum, vacuole, and mitochondria (Jimenez *et al.,* 1998). Its levels decline with tissue age and vary among growth environments. GSH can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger.

L-ascorbic acid (Vitamin C) is a water-soluble vitamin needed for the growth and repair of tissues in all parts of the body and is abundant in plants. Ascorbate has been found in the chloroplast, cytosol, vacuole and extra-cellular compartments of the cell. About 20-40% of the ascorbate in the mesophyll leaf cell is in the chloroplast. It exists mostly in the reduced form (90 % of the ascorbate pool) in leaves and chloroplasts (Smirnoff, 2000); and its intracellular concentration can build up to millimolar range (e.g. 20 mM in the cytosol and 20–300 mM in the chloroplast stroma (Foyer and Lelandais, 1996).

$2O^{-2} + 2H^{+}$ + ascorbate -----→$2H_2O_2$ + dehydroascorbate

Vitamin C can directly scavenge superoxide, hydroxyl radicals and singlet oxygen and reduce H_2O_2 to water via ascorbate peroxidase reaction (Noctor and Foyer, 1998). AA regenerates tocopherol from tocopheroxyl radical providing membrane protection (Thomas *et al*., 1992). In addition, AA carries out a number of non-antioxidant functions in the cell. It has been implicated in the regulation of the cell division, cell cycle progression from G_1 to S phase (Liso *et al*., 1988; Smirnoff, 1996) and cell elongation (De Tullio *et al*., 1999).

Tocopherols are essential components of biological membranes where they have both antioxidant and non-antioxidant functions (Kagan, 1989). There are four tocopherol and tocotrienol isomers which structurally consist of a chroman head group and a phytyl side chain giving vitamin E compounds amphipathic character (Kamal-Eldin and Appelqvist, 1996). Relative antioxidant activity of the tocopherol isomers *in vivo* is > ß > > which is due to the methylation pattern and the amount of methyl groups attached to the phenolic ring of the polar head structure. Chloroplast membranes of higher plants contain –tocopherol as the predominant

tocopherol isomer, and are hence well protected against photooxidative damage. The antioxidant properties of tocopherol are the result of its ability to quench both singlet oxygen and peroxides (Fryer, 1992).

Vitamin E is a chain-breaking antioxidant, i.e. it is able to repair oxidizing radicals directly, preventing the chain propagation step during lipid autoxidation (Serbinova and Packer, 1994). In addition, tocopherols act as chemical scavengers of oxygen radicals, especially singlet oxygen (via irreversible oxidation of tocopherol), and as physical deactivators of singlet oxygen by charge transfer mechanism (Fryer, 1992).

Carotenoids are organic pigments that are naturally occurring in plants that belong to the category of tetraterpenoids (i.e. they contain 40 carbon atoms). Carotenoids are synthesised from geranylpyrophosphate from the isoprenoid pathway in plastids, and thus have common precursors to chlorophyll and tocopherol. In terms of its antioxidant properties carotenoids can protect the photosystems in one of four ways: by reacting with lipid peroxidation products to terminate chain reactions (Burton and Ingold, 1984); by scavenging singlet oxygen and dissipating the energy as heat (Mathis and Kleo, 1973); by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen; or by the dissipation of excess excitation energy through the xanthophyll cycle.

Phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxycinnamate esters and lignin) abundant in plant tissues (reviewed by Grace and Logan, 2000). Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans *et al*., 1997). Another mechanism underlying the antioxidative

properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora *et al*., 2000).

1.3.2. Enzymatic Systems

Enzymatic ROS scavenging mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT).

1.3.2.1. Superoxide Dismutase

Superoxide dismutase (SOD) was first isolated by Mann and Keilis (1938) and its catalytic function was discovered by McCord and Fridovich (1969). Within a cell, the superoxide dismutases (SODs) constitute the first line of defence against ROS. O_2^- is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol (Elstner, 1991). This being the case, it is not surprising to find that SODs are present in all these subcellular locations. While all compartments of the cell are possible sites for $O_2^{-\cdot}$ formation, chloroplasts, mitochondria and peroxisomes are thought to be the most important generators of ROS (Fridovich, 1986).

SOD catalyses the dismutation of superoxide to hydrogen peroxide. The reaction has a 10000-fold faster rate than spontaneous dismutation (Bowler *et al*., 1992). Based on the metal co-factor used by the enzyme, SODs are classified into three groups: iron SOD (FeSOD), manganese SOD (MnSOD), and copper-zinc SOD (Cu/Zn SOD), and these SODs are located in different compartments of the cell. Fe SODs are located in the chloroplast, MnSODs in the mitochondrion and the peroxisome, and Cu/Zn SODs in the chloroplast, the cytosol, and possibly the extracellular

space. According to amino acid sequences information, Mn and Fe SODs are more ancient types of SODs whereas Cu/ZnSODs have no sequences similarity to Mn and FeSODs. (Kanematsu and Asada, 1990).

The group of FeSODs probably constitute the most ancient SOD group. It has been suggested that iron was probably the first metal used as a metal cofactor at the active site of the first SOD because of an abundance of iron in soluble Fe (II) form at that time (Bannister *et al*., 1991). While the levels of O_2 in the environment increased, the mineral components of the environment were oxidized. The decrease in available Fe (II) in the environment caused a shift to the use of a more available metal, Mn (III). FeSOD is found both in prokaryotes and in eukaryotes and it is inactivated by H_2O_2 and is resistant to KCN inhibition.

There are two distinct groups of FeSOD. The first group is a homodimer formed from two identical 20 kDa subunit proteins, with 1–2 gram atom of iron in the active centre. This type of FeSOD has been isolated from *Escherichia coli* (Yost and Fridovich, 1973). The second FeSOD group, found in most higher plants, is a tetramer of four equal subunits with a molecular weight of 80–90 kDa. Members of this group contain 2–4 gram atoms of iron in the active centre.

As mentioned previously, as the levels of O_2 in the environment increased, the amount of available Fe (II) in the environment decreased, causing a shift to the more available metal, Mn (III). As a consequence, MnSODs are likely to be second enzyme. MnSODs occur in mitochondria and peroxisomes that carry only one metal atom per subunit. These enzymes cannot function without the Mn atom present at the active site. Even though Mn and FeSODs have a high similarity in their primary, secondary, and tertiary structure, these enzymes have diverged sufficiently that Fe (II) could not restore the activity of MnSOD and vice versa (Fridovich, 1986).

MnSOD is either a homodimeric or a homotetrameric enzyme with one Mn (III) atom per subunit. The enzyme is not inhibited by potassium cyanide (KCN) or inactivated by H_2O_2 and is present in both eukaryotes and prokaryotes. Plant MnSODs have approximately 65% sequence similarity to one another, and these enzymes also have high similarities to bacterial MnSODs (Bowler *et al*., 1994). Although MnSOD is known as the mitochondrial enzyme of eukaryotes, a Mn-containing SOD has also been located in the peroxisomes.

According to a theory, when the atmosphere was completely replenished with oxygen, Fe (II) was almost completely unavailable and insoluble Cu (I) was converted into soluble Cu (II). At this stage, Cu (II) began to be used as the metal cofactor at the active sites of SODs. Since FeSODs and Mn have similar electrical properties, the transition from the use of iron to the use of manganese required little change in SOD protein structure. Thus, Mn and FeSODs are structurally very similar. However, the electrical properties of Cu/ZnSODs differ greatly from those of Fe and MnSODs. Therefore, a major change in the structure of the protein occurred after Cu become a metal cofactor (Bannister *et al*., 1991). Fe and MnSODs are present both in prokaryotic and in eukaryotic organisms whereas Cu/ZnSODs have been found mostly in eukaryotes.

Cu/ZnSODs are found throughout the plant cell. There are two different groups of Cu/ZnSODs. The first group consists of cytoplasmic and periplasmic forms, which are homodimeric. The second group comprises the chloroplastic and extracellular Cu/ZnSODs, which are homotetrameric (Bordo *et al*., 1994). The active sites of each subunit function independently. When these subunits are separated and then coupled with an inactive subunit, newly formed enzymes show full activity, providing evidence that the functional interactions between the subunits are not essential for full catalytic activity (Fridovich, 1986).

Cu/ZnSOD exists in both chloroplastic and cytosolic forms. Deduced amino acid sequences of these two isoforms show approximately 68% similarity, whereas there is approximately 90% similarity among the chloroplastic Cu/ZnSODs (Cu/ZnSOD$_{chl}$) and 80–90% similarity among the cytosolic Cu/ZnSODs (Cu/ZnSOD$_{cyt}$).

1.3.2.2. Catalase

Catalase is a heme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in ß-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. Multiple forms of catalase have been described in many plants. For instance, maize has three isoforms termed cat-1, cat-2 and cat-3, that are on separate chromosomes and are differentially expressed and independently regulated (Scandalias, 1990). Cat-1 and cat-2 are localized in peroxisomes and the cytosol, whereas cat-3 is mitochondrial. It is interesting to note that catalase is very sensitive to light and has a rapid turnover rate similar to that of the D1 protein of PSII (Hertwig *et al*., 1992). This may be a result of light absorption by the heme group or perhaps hydrogen peroxide inactivation.

Detoxification of H_2O_2 is mediated by catalase, which is mostly localized in peroxisomes. However, catalase possesses a very low afinity for H_2O_2. That's why H_2O_2 can also be removed by the Halliwell-Asada pathway, originally described in the chloroplasts (Nakano *et al.,* 1980). In a recent investigation on the relationship between H_2O_2 metabolism and the senescence process in mitochondria and peroxisomes have indicated the presence of ascorbate-glutathione cycle components in both organelles (Jumenez *et al.* 1998).

1.3.2.3. Peroxidase

Peroxidases are heme-containing enzymes that use hydrogen peroxide as the electron acceptor to catalyse a number of oxidative reactions. Peroxidases are found in bacteria, fungi, plants and animals and can be viewed as members of a superfamily consisting of 3 major classes (Welinder, 1992). Class III comprises the secretory plant peroxidases, which have multiple tissue-specific functions e.g., removal of hydrogen peroxide from chloroplasts and cytosol; oxidation of toxic compounds; biosynthesis of the cell wall; defence responses towards wounding; indole-3-acetic acid (IAA) catabolism; ethylene biosynthesis. Peroxidases follow the reaction scheme:

$Fe^{3+} + H_2O_2 \rightarrow [Fe^{4+}=O]R'$ (Compound I) + H_2O

$[Fe^{4+}=O]R'$ + substrate $\rightarrow$ $[Fe^{4+}=O]R$ (Compound II) + oxidised subst.

$[Fe^{4+}=O]R$ + substrate $\rightarrow$ $Fe^{3+} + H_2O$ + oxidised substrate.

In this mechanism, the enzyme reacts with one equivalent of H_2O_2 to give $[Fe^{4+}=O]R'$ (compound I). This is a two-electron oxidation/reduction reaction where H_2O_2 is reduced to water and the enzyme is oxidized. One oxidizing equivalent resides on iron, giving the oxyferryl (Nelson R.E *et al.,* 1994) intermediate, while in many peroxidases the porphyrin (R) is oxidized to the porphyrin pi-cation radical (R'). Compound I then oxidizes an organic substrate to give a substrate radical (Li *et al.,* 1994)

1.4. Cold Stress Injury

Each plant has its unique temperature requirements, which are optimum for its growth and development. This temperature condition may be optimum for one plant, however, it may be stressful for another plant. That's why plants are divided to three cold-stress categories:

1. chill-susceptible: damaged by temperatures below 12°C.
2. chill-tolerant but freezing susceptible: able to acclimate to temperatures below 12°C but unable to survive freezing.
3. freeze-tolerant: able to acclimate to survive temperatures significantly below freezing (Pearce, 1999).

The natures of chill- and freezing stresses are different. Chill-stress is a direct effect of low temperatures on cells, however, freezing often acts indirectly damaging cells by dehydration. The duration of cold stress varies from plant to plant and also depends on the sensitivity of a plant to cold stress. There are various phenotypic symptoms in response to chilling stress including reduced leaf expansion, wilting, chlorosis (yellowing of leaves).

The real cause of freeze-induced injury to plants is the ice formation rather than low temperatures. Therefore, dehydrated tissues such as seeds and fungal spores can survive at very low temperatures without any symptoms of injury. Under this conditions, freezing temperatures induce the formation of ice in the intercelular spaces and cell walls of plant tissue. Extracellular ice formation occurs since the intercellular fluid has a higher freezing point than the cytoplasm. During freezing, the ice-state of water has a much lower water potential than liquid solution (Guy, 1990). Therefore, when ice forms extracellulary, there is a sudden drop in water potential outside the cell. As a result, water from the cytoplasm moves through the plasma membrane by osmosis, leading to cellular dehydration.

The major negative effect of freezing is that it induces severe membrane damage (Steponkus *et al*., 1993). This damage is largely due to the acute dehydration associated with freezing. Membrane lipids are primarily composed of two kinds of fatty acids, namely, unsaturated and saturated fatty acids. Lipids containing saturated fatty acids solidify at temperatures higher than those containing unsaturated fatty acids. Therefore, the

relative proportion of unsaturated fatty acids in the membrane strongly influences the fluidity of the membrane (Steponkus *et al*., 1993). Chilling sensitive plants usually have a higher proportion of saturated fatty acids and, therefore, a higher transition temperature. Chilling resistant species on the other hand are marked by higher proportion of unsaturated fatty acids.

Furthermore, ROS produced in response to freeze stress contribute to membrane damage. Generally, chilling results of loss in membrane integrity, which leads to solute leakage. The integrity of intracellular organelles is disrupted, because of loss of compartmentalization, impairing of photosynthesis and reduction of protein assembly,

1.4.1. Cold Acclimation

The primary environmental factors responsible for triggering increased tolerance against freezing, is the phenomenon known as ‘cold acclimation. It is the process where certain plants increase their freezing tolerance upon prior exposure to low non-freezing temperatures. The primary function of cold acclimation is to stabilize the membranes against freeze injury. It results in increase in proportion of unsaturated fatty acids (Williams *et al*., 1988).

Cold acclimation provide in physical and biochemical restructuring of cell membranes through changes in the lipid composition and induction of other non-enzymatic proteins that change the freezing point of water. Addition of solutes decreases the freezing point of water to a more negative value, thus preventing ice formation. Accumulation of sucrose and other simple sugar that occurs with cold acclimation also contribute to the stabilization of membrane since these molecules can protect membranes against freeze-damage (Mahajan and Tuteja, 2005).

1.4.2. Cold-Induced Genes

Low temperatures activate a number of cold-induced genes. Some of these genes encode proteins with known enzymatic functions, such as alcohol dehydrogenase (Jarillo *et al.,*1993), phenylalanine ammonia lyase, chalcone synthase (Leyva *et al*., 1995), the fatty acid desaturase (Gibson *et al*., 1994), lipid transfer protein (Hughes and Pearce, 1988), a translation initiation factor (Dunn *et al*.,1993), a thiol protease (Shaffer and Fischer, 1988), catalases (Prasad *et al.,*1994), and pyrroline-5-carboxylate synthase (Yoshiba *et al*.,1995). Some of them show similarity to a group of proteins involved in dehydration such as dehydrin- or LEA-like proteins (Gilmour *et al*.,1992), antifreeze proteins (Kurkela and Franck, 1990), heat shock proteins or molecular chaperones (Anderson *et al*.,1994). Some encode various signal transduction or regulatory proteins such as MAP kinases (Jonak *et al*.,1996) and calcium-dependent protein kinases (Tahtiharju *et al*.,1997). This finding provides strong support that a fundamental role of cold-inducible genes is to protect the plant cells against cellular dehydration.

1.4.3. Signalling Processes in Cold Stress

Cold acclimation contains a temperature sensor to perceive the low temperatures and a signal transducer to activate the biochemical and gene expression events required for increased freezing tolerance. It is still unclear how plants perceive the decreasing temperature, however, some evidence indicates that changes in cellular calcium levels may be involved in temperature sensing. It means that the change in cytosolic calcium levels is a necessary first step in a temperature sensing mechanism, which enables the plant to withstand future cold stress (Monroy and Dhindsa, 1995).

Furthermore, calcium channel blockers inhibit the expression of *cor* genes at low temperature whereas calcium ionophores causes calcium influx and induces the expression of COR genes in warm temperatures (Knight *et al*.,1996).

The plant hormone ABA has also been shown to mediate the development of freezing tolerance. Four important evidence suggest that ABA can play a critical role in the signal transduction of cold acclimation. First, ABA treatment at normal growth temperatures can increase the freezing tolerance in *Arabidopsis* (Lang *et al*., 1994). Second, endogenous ABA levels increase in certain plants in response to low temperatures (Chen *et al*., 1983). Third, ABA-deficit mutants are impaired in developing freezing tolerance during cold acclimation, however, the freezing tolerance can be restored to wild-type level by adding ABA into the culture medium (Heino *et al*., 1990). Lastly, COR genes can be induced by ABA. However, all of the ABA-insensitive mutants examined can cold acclimate to the same level as wild type (Gilmour and Thomashow, 1991). For this result, possible explanation is that different ABA receptor or pathway mediate cold acclimation. This means that there are low-temperature signalling pathways independent of ABA.

According to some evidence, protein kinase and phosphatases have function in transduction of low temperature signals during cold acclimation in plants (Anderberg *et al*., 1992).

1.5. Response of Superoxide Dismutase to Environmental Stress

As mentioned above, O_2 is an abundant ROS that is formed by univalent electron transfer to O_2 and can contribute to the synthesis of $OH^{\cdot}$, so control of this ROS is essential (Cadenas, 1989). SODs catalyze the conversion of $O_2^{-\cdot}$ to H_2O_2. The importance of SOD has been demonstrated by analysis of mutants in microbes and animals (Carlioz and

Touati, 1986). To date the protective role of SOD in plants has been explored by transgenic approaches, primarily through overexpression or by correlation of SOD expression to the degree of oxidative stress resistance (Bowler *et al*., 1994; Alscher *et al*., 1997). However, both approaches have given inconclusive and sometimes contradictory results about the role of SOD in plant oxidative stress responses.

One of the earliest papers dealing with antioxidative response is by Monk's study. They compared the flooding-tolerant species *Iris pseudacorus* with the flooding-sensitive *Iris germanica* and SOD activity increased strongly in tolerant species. However, the activities of antioxidative enzymes including SOD were reduced in submerged rice plants (Usimaru *et al*., 1992).

As another approach, transgenic tobacco plants overexpressing chloroplastic Cu/ZnSOD showed increased resistance to oxidative stress caused by high light and low temperatures (Gupta et al. 1993a, 1993b). In another study, transgenic alfalfa plants expressing either MnSOD or FeSOD had increased winter survival rates and yields (McKersie et al. 1999, 2000). However, this was not associated with protection against primary injury caused by freezing, and the FeSOD transgenic alfalfa did not show greater tolerance to oxidative stress (McKersie *et al*., 2000). According to authors, FeSOD overexpression may reduce secondary injury symptoms and thereby enhance recovery from the stress experienced during the winter.

Additionally, transgenic tobacco plants were produced which expressed the Cu/ZnSOD from petunia in addition to the native forms of the enzyme (Teppermann and Dunsmuir, 1990). The transgenic tobacco leaf discs expressed 30 to 50-fold more Cu/ZnSOD than controls, however, they were not protected against methyl-viologen-mediated inhibition of CO_2 assimilation, chlorophyll bleaching and ozone toxicity (Teppermann *et.al*.,

1991). According to authors, elevating SOD alone was not enough to protect against oxygen toxicity and it would be necessary to increase simultaneously the enzymes contained H_2O_2 detoxification. On the contary, overproduction of MnSOD resulted in production against methyl violegen (Bowler *et al*., 1991). Finally, lots of the studies may need to understand the pathway of protection system in plants.

1.6. Aim of the Study

The aim of this research was to clarify the role of SOD isozymes of barley cultivars under chilling and freezing stress conditions.

For this purpose, the following analyses were performed on leaf and root tissues of two barley cultivars, Tarm-92 and Zafer-160 differing in their cold stress response.

i) Plants were exposed to different cold stress regime.

ii) SOD isozyme activities were determined by negative activity staining technique.

iii) Cu/ZnSOD expression levels under cold stress conditions were determined by RT-PCR.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

In this study, Turkish cultivars of barley (*Hordeum vulgare* L.), Tarm-92 (winter type) and Zafer-160 (spring type), were used. The seeds were obtained from the Turkish Ministry of Agriculture and Rural Affairs.

2.1.2. Chemicals

The chemicals and their suppliers were listed in Appendix A.

2.2. Methods

2.2.1. Growth of Plants

Seeds were surface-sterilized with 30% sodium hypochloride solution by shaking for 13 minutes. Then, they were washed and imbibed in distilled water for overnight at room temperature. Imbibed seeds were transferred to plastic trays containing perlite. Approximately 15-20 seeds were grown for 8 days in growth chamber at 23°C with 16 hours light and 8 hours dark photo-cycle at a light intensity of 40 µmol $m^{-2}s^{-1}$.

2.2.2. Application of Cold Stresses

At the 8th day of growth, chilling stress was performed at 4°C for 1, 3 and 7 days. Before freezing treatment, at the end of the 8th day of growth, plants were cold-acclimatized (process of increasing plant resistance to cold injury through regular cold exposure) for 3 days at 4°C and then were exposed to -3°C for 3 hours (Nuve ES110 Cooled incubator). Subsequently, plants were moved at 4°C for 4 days for recovery. In second freezing stress, plants were grown at 23°C for 8 days and moved at 4°C for 3 days and then, at -3°C for 30 minutes for acclimation. After this, plants were exposed to -7°C for 90 minutes. Subsequently, they were incubated at 4°C for 4 days for recovery.

2.2.3. Determination of SOD Isozyme Activities

SOD activity and SOD patterns of treated and control plants were observed by running one dimensional polyacrylamide gel electrophoresis. Bands were determined by using negative activity staining technique according to Beauchamp and Fridovich (1971).

2.2.3.1. Preparation of Leaf Crude Extracts

Approximately 20 mg of leaf tissues from treated and control plants were weighed. Each sample was homogenized with liquid nitrogen and suspended 800 µl homogenization buffer containing 9 mM Tris-HCl, pH:6.8 and 13,6% glycerol. The homogenates were transferred into eppendorf tubes and centrifuged at 14000 rpm for 5 minutes in an microcentrifuge (Thermo IEC). Supernatants were separated and used for SOD assay.

2.2.3.2. Preparation of Root Crude Extracts

Approximately, 0.5 g of root tissues from control and treated plants were ground with liquid nitrogen and suspended in 750 µl grinding buffer consisting of 0.2 M sodium phosphate buffer (pH:7.8) and 2 mM Na_2EDTA. The homogenate were transferred into new eppendorf tubes and centrifuged at 10000 g for 30 minutes. Supernatants were separated and used for SOD assay.

2.2.3.3. Protein Determination

The protein concentrations of crude extracts were determined according to Bradford method (Bradford,1976). This reagent was prepared according to protocol (Appendix B). The concentrated Bradford reagent (5X) was diluted 5 times (1X) before use.

Ten µl of the leaf samples and 40 µl of the root samples were diluted to 500µl distilled water in test tubes. 5 ml Bradford reagent was added. After that, the tube was vortexed and left at room temperature for 10 minutes. The absorbance at 595 nm was measured in Shimadzu UV mini 1240 spectrophotometer against a blank solution containing 500 µl distilled water and 5 ml Bradford reagent. Bovine Serum Albumin (BSA) was used as a standard at following concentrations: 5, 10, 20, 30, 40, 50, 60, 70 µg/ml.

2.2.3.4. One Dimensional Native Polyacrilamide Gel Electrophoresis (1-D PAGE)

Five ml 12% separating gel and 2.5 ml 5% stacking gel were prepared to carry out 1-D PAGE. The preparation of these gels was shown in Appendix H. Equal amounts of proteins (for leaf samples 50 mg and for

root samples 25 mg) were loaded per well. 5 µl standard SOD protein was applied in one of the wells to determine the intensity of SOD isozymes and electrophoresis was achieved under constant current at 6 mA in stacking and 8 mA separating gel in a Amersham Minigel Apparatus at 4°C for approximately 3 hours.

2.2.3.5. Negative Activity Staining

At the end of electrophoresis, gel was transferred into a glass container and 25 ml of negative activity stain containing solution 50 mM potassium phosphate buffer (pH:7.5), 0.1 mM EDTA, 0.2% v/v N,N,N',N'-tetramethyl ethylene diamine (TEMED), 3 mM Riboflavin and 0.25 mM Nitroblue Tetrazolium was added. Gel was incubated at dark for 45 minutes at room temperature by gentle shaking. After incubation, gel was exposed to light and washed with distilled water for several times until dark blue color appear.

The photograph of the gel was taken at Vilber Gel Imager and Scion Imaging Software program was used for densitometric analysis. The changes in SOD isozymes were evaluated from band intensities. The unit of SOD activity was determined by running a SOD standart (specific activity = 3350 units/mg).

2.2.3.6. Determination of SOD Isozymes

Samples were electrophoresed on polyacrylamide gel under non-denaturing conditions. After that, gel was cut into three, in other words, each piece contains one leaf and one root sample. 50 mM potassium phosphate buffer (pH:7.8) containing 0.5 mM Na_2EDTA was prepared and one of the gels was incubated for 30 minutes only in buffer as a control, and the other two gels were incubated for 30 minutes in buffer containing

either 5 mM KCN or 5 mM H_2O_2 for inhibition. After that, gels were treated with negative activity stain. Cu/ZnSOD is sensitive to both KCN and H_2O_2, FeSOD is sensitive only to H_2O_2 , MnSOD is resistant to both inhibitors.

2.2.4. RNA Extraction

Approximately, 0.5 g tissues from control and treated plants were ground with liquid nitrogen and were transferred 100 mg of the ground tissues to precooled eppendorf tubes. After that, 1 ml of TRIzol reagent was added and vortexed for 15 minutes in a block shaker at room temperatures, then centrifuged in eppendorf centrifuge for 5 minutes at room temperatures at maximum speed. 900 µl of the supernatant was taken to a new tube and added 180 µl chloroform, incubated at room temperature for 6 minutes. At the end of the incubation, it was centrifuged in eppendorf centrifuge for 15 minutes at 4°C at maximum speed. 450 µl from the upper phase was transferred into a new eppendorf tube, and added 200 µl chloroform, and then incubated at room temperature for 3 minutes. After that, it was spinned for 5 minutes at room temperature at maximum speed and pipetted 400 µl from the upper phase and added 1 volume isopropanol and then, incubated for 10 minutes at room temperature. Tube was centrifuged for 10 minutes at room temperatures at maximum speed and supernatant was removed. The pellet was washed with 1 ml 75% ethanol and left for 3 minutes at room temperatures and spinned down for 5 minutes at room temperature at maximum speed, and removed the supernatant. In order to remove ethanol, it was centrifuged for an additional 15 seconds and air dried for 10 minutes. Finally, 50 µl DEPC-water added and it was incubated for 15 minutes at 65°C to dissolve pellet.

2.2.4.1. Determination of RNA Quality and Quantity

The RNA concentration was determined by measuring absorbance at 260 nm on a spectrophotometer in 10 mM Tris/HCl pH:8 (one absorbance unit = 40 µg/ml RNA). The 260/280 ratio was taken and values between 1.9 to 2.1 were considered acceptable.

The integrity of the RNA was tested by agarose gels. For this, RNase-free 1% agarose gel in TAE was prepared. The diluted RNA sample was loaded and run for 20-40 minutes at 80-100 V. The rRNA bands were seen clearly without any obvious smearing patterns.

2.2.5. Relative RT-PCR

RNA was reverse transcribed into cDNA using Moloney Murine Leukemia virus reverse transcriptase (Fermentas, Burlington, Canada) as recommended by the manufacturer and stored at -20°C until needed. After cDNA production, all of the parameters were optimized for PCR reactions, and performed in 50 µl reaction mixture with the following:

- 3 µl of cDNA from control and treated plants (5 µg RNA for leaf tissues and 2.5 µg for root tissues were used for cDNA production.)
- 0.05 U/µl *Taq* DNA Polymerase (Applichem, Ottoweg, Germany)
- 10X *Taq* DNA Polymerase buffer
- 2 mM dNTPs
- 25 mM $MgCl_2$
- 10 µM of primers Sod-fwd (5'- TCATCAGCATCACCATGGAC-3') and Sod-rev (5'- ATGTCAACTGGACCGCACTT-3') to amplify *HvSod.*

In this study, elongation factor 1-beta was used as a internal control. The primers Ef-fwd (5'-CCAAGAATCCAGCAGCAACA-3') and Ef-rev (5'-TAAGCTCATGCCTG TGGGTTA-3') to amplify *HvEF-1β.*

Primers were synthesized by IDT (Integrated DNA Technologies). The sequence was determined using the software Primer 3 (developed by Steve Rozen, Helen J. Skaletsky, 1996, 1997

Amplification conditions were as follows: 94°C for 5 minutes; 33 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 1 minute; and a final extension at 72°C for 10 minutes. RT-PCR were loaded onto Ethidium Bromide-stained, 2 % agarose gels in TAE. A 1kbp DNA ladder molecular weight marker (Fermentas, SM0371) was run on every gel to confirm expected molecular weight of the amplification product.

Images of the RT-PCR ethidium bromide-stained agarose gels were photographed with Vilber Gel Imager and quantification of the bands was performed by Scion Corporation. The optical density was determined for both *HvSod* and *HvEF-1α* and a ratio of *HvSod* band intensity to *HvEF-1α* band intensity was calculated.

2.2.6. Sequencing Reactions

50 ng RT-PCR product was sequenced by ABI 310 DNA sequencer at Central Laboratory at Middle East Technical University.

2.2.6.1. Sequence and Homology Analysis

NCBI databanks were searched for finding homolog sequences that have been previously available. tblastx (Appendix G) and blastn (Appendix F)

sequence analysis were done by using NCBI and TIGR Gene Index databases. Besides, the sequence alignments were obtained using ClustalX 1.81 program.

2.2.7. Statistical Analysis

All data in the present study were obtained from 4 or 5 independent measurements. Statistical differences between treatments and controls were analyzed with analysis of variance (ANOVA) with one-way classification technique using MINITAB program. Differences were considered significant when P value was less than 0.05 ($P < 0.05$).

CHAPTER 3

RESULTS

Barley cultivars (Tarm-92-winter type, Zafer-160-spring type) were exposed to cold stress both chilling at 4°C and freezing stress at -3°C and -7°C for different time periods. Their responses to cold stress were determined by measuring the SOD isozyme patterns and activities. Transcript levels of Cu/ZnSOD gene were also measured by RT-PCR.

3.1. Effect of Chilling Stress on Superoxide Dismutase Enzyme Activities in Leaf Tissues

The SOD isozyme activities and patterns were determined by non-denaturing PAGE activity staining technique. In this study, 3 isozymes in both leaf and root tissues were detected according to their inhibition by KCN and H_2O_2.

The main characteristic feature of one of these isoyzmes was resistance to both KCN and H_2O_2. Therefore, it was identified as manganase superoxide dismutase isozymes (MnSOD). All of the other isozymes were sensitive to both KCN and H_2O_2. Therefore, they were identified as cupper-zinc superoxide dismutase isozymes (Cu/ZnSOD). In this study, these Cu/ZnSODs were named as x and 2 according to increased relative mobility (Figure 3.1).

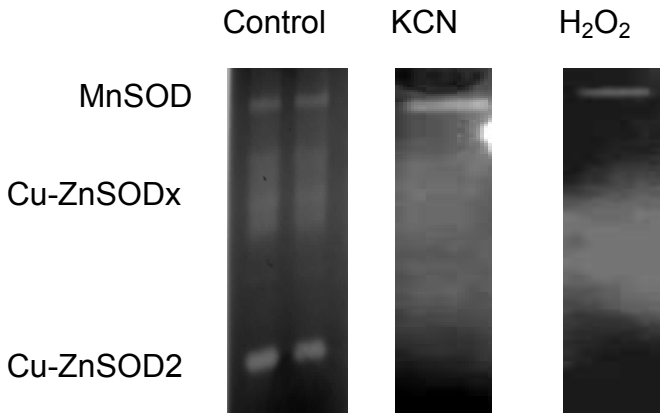


Figure 3.1. SOD isozymes: MnSOD, Cu/ZnSODx, Cu/ZnSOD2

In order for determination changes of SOD isozyme activities, crude extracts that contain equal amounts of protein (50 mg/well for leaf and 25 mg/well for root extracts) were loaded on a native-PAGE and stained for SOD activity. After stained by negative activity staining, Scion İmage program was used for determining the relative activities of the SOD isozymes.

For calculation SOD isozyme activities, it was applied known activity standard of SOD from bovine erythrocytes into one well of the gel. The activity (pixel intensity) of the standard was used as a reference for the activities of other isozymes of SOD (Figure 3.1).

Crude extracts prepared from control and chilled stress plants were electrophoresed and bands corresponding to SOD isozymes were obtained by negative activity staining in Chapter 2. The figure 3.2 and 3.3 represents the SOD isozymes patterns of leaf tissues obtained from both control and chilled plants in Tarm-92 and Zafer-160 cultivars.

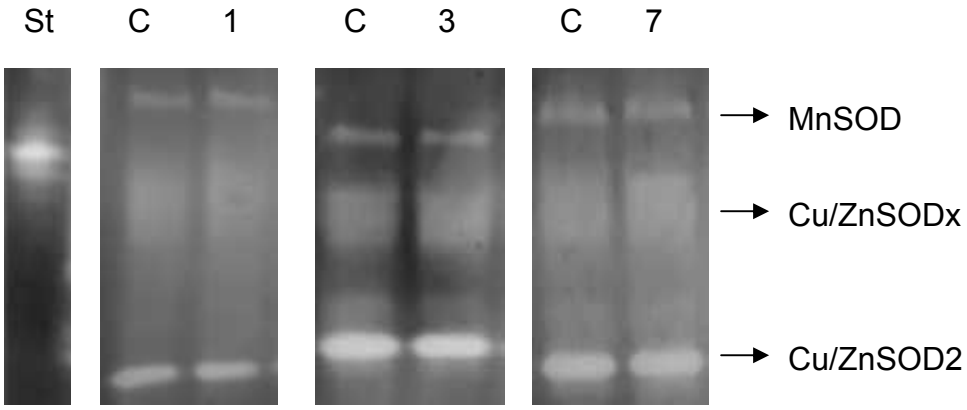


Figure 3.2. Activities of SOD isozymes in leaf tissues of Tarm-92. **St**: 0.5 unit SOD standard from bovine erythrocytes, **C**: Control, **1**: Treated plant for 1 day at 4°C, **3**: Treated plant for 3 day at 4°C, **7**: Treated plant for 7 day at 4°C.

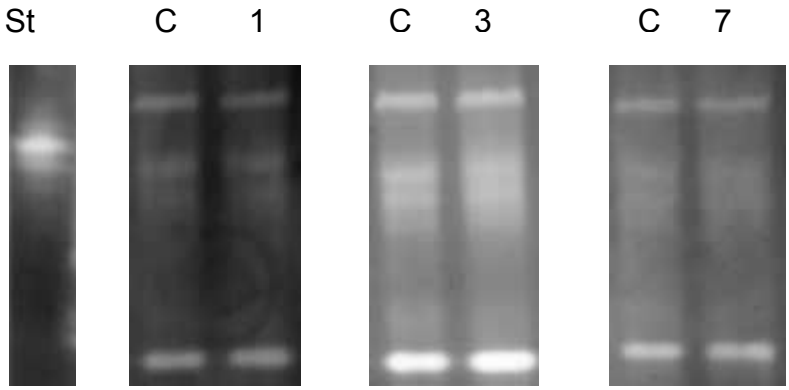


Figure 3.3. Activities of SOD isozymes in leaf tissues of Zafer-160. **St**: 0.5 unit SOD standard from bovine erythrocytes, **C**: Control, **1**: Treated plant for 1 day at 4°C, **3**: Treated plant for 3 day at 4°C, **7**: Treated plant for 7 day at 4°C.

3.1.1. Evaluation of Superoxide Dismutase Isozyme Activities by Densitometric Analysis

Band intensities were analyzed by using Scion densitometer image program, after gel photographs were taken in Figure 3.2 and 3.3. According to this analysis, MnSOD activity in leaf tissues of Tarm-92 cultivar was not significantly different than controls for 1 and 7 days, however, 3 days treatment led to a significant increase in MnSOD activity leaf tissues of Tarm-92 (Figure 3.4). In leaf tissues of Zafer-160 cultivar, the activity of MnSOD was not significantly different than controls at all stress conditions (Figure 3.4).

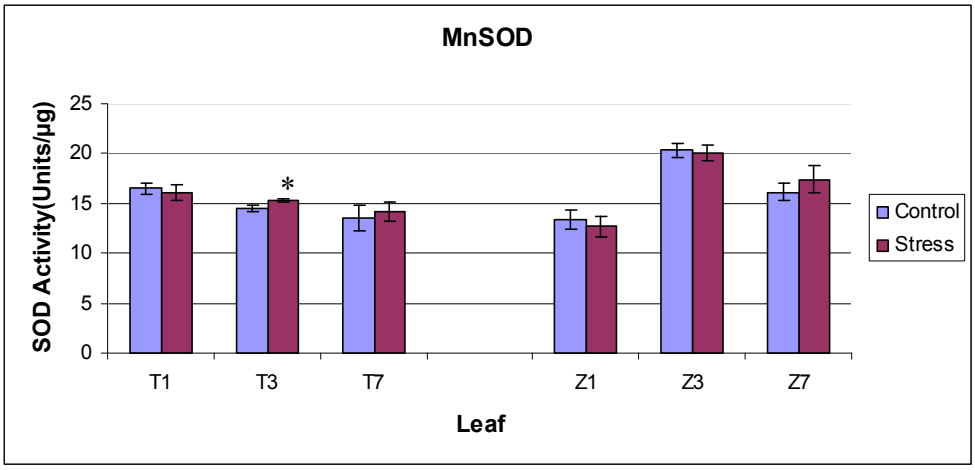


Figure 3.4. MnSOD activity (units/µg protein) in leaf tissues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=4). * Asterisk shows significant difference ($p < 0.05$) with respect to control.

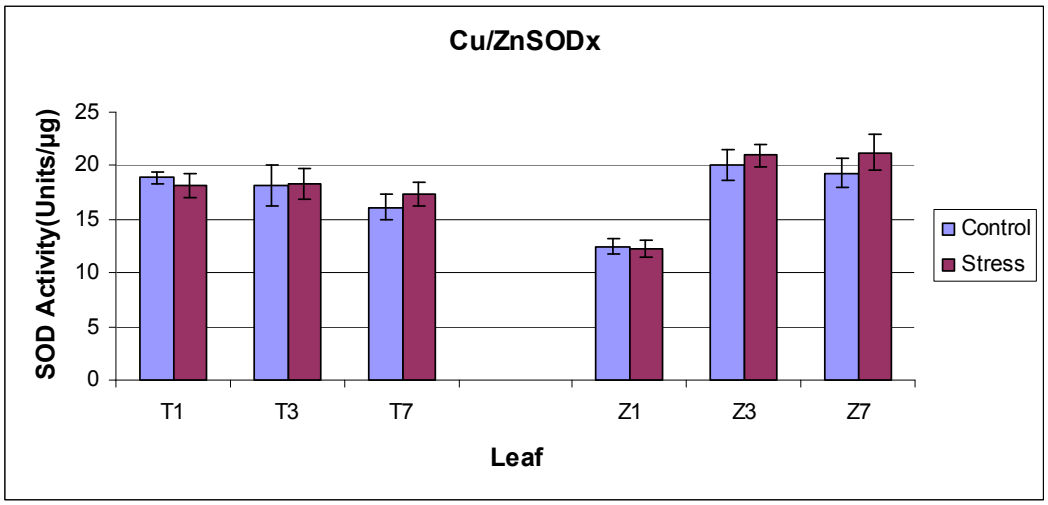


Figure 3.5. Cu/ZnSODx activity (units/µg protein) in leaf tissues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=4).

Cu/ZnSODx activity in leaf tissues of both cultivars was not significantly different than controls at all stress conditions (for 1, 3, 7 days treatments at 4°C) (Figure 3.5).

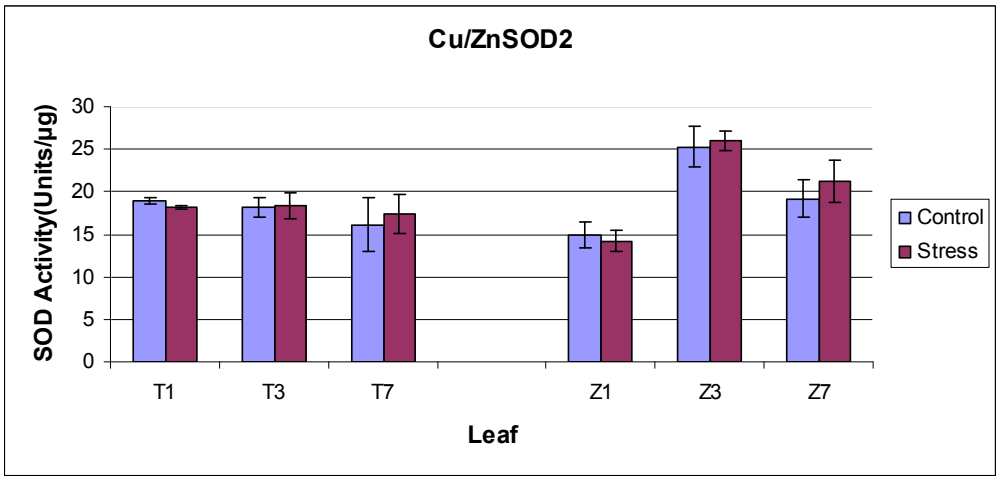


Figure 3.6. Cu/ZnSOD2 activity (units/µg protein) in leaf tissues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=4).

Chilling stress did not cause any significant change in the activity of Cu/ZnSOD2 isozyme in leaf tissues of both cultivars for 1, 3, 7 days at 4°C (Figure 3.6).

3.2. Effect of Chilling Stress on Superoxide Dismutase Enzyme Activities in Root Tissues

Under chilling stress condition, the changes in SOD activity of the root tissues of both cultivars were given in Figure 3.7 and 3.8.

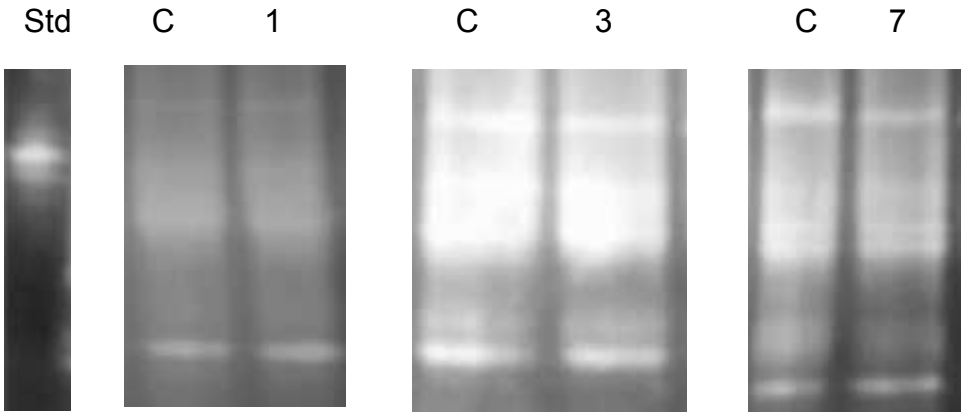


Figure 3.7. Activities of SOD isozymes in root tissues of Tarm-92 under chilling stress. **Std**: 0.5 unit SOD standard from bovine erythrocytes**, C**: Control, **1**: Treated plant for 1 day at 4°C, **3**: Treated plant for 3 day at 4°C, **7**: Treated plant for 7 day at 4°C.

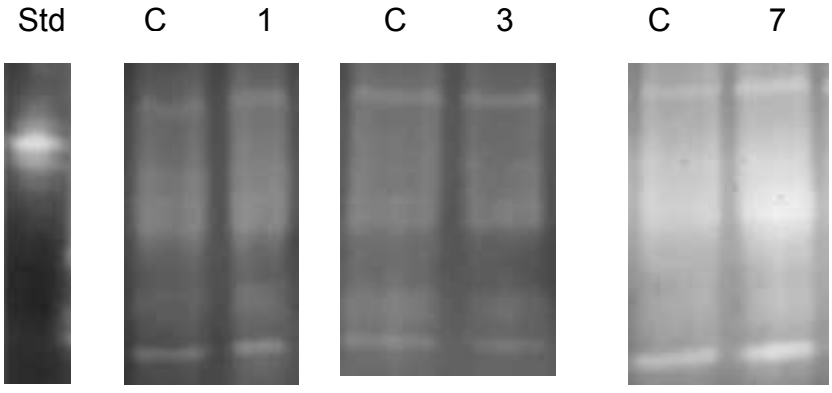


Figure 3.8. Activities of SOD isozymes in root tissues of Zafer-160 under chilling stress. **Std**: 0.5 unit SOD standard from bovine erythrocytes, **C**: Control, **1**: Treated plant for 1 day at 4°C, **3**: Treated plant for 3 day at 4°C, **7**: Treated plant for 7 day at 4°C.

3.2.1. Evaluation of Superoxide Dismutase Isozyme Activities by Densitometric Analysis

Like the leaf tissues, all root gel photographs was used for pixel intensity and taken data for control and chilled root tissues of both cultivars (Figure 3.7 and 3.8). Accordingly, in root tissues of Tarm-92, 1 and 3 days treatments did not cause significant change in the activity of MnSOD isozyme, whereas 7 days treatments led to a significant decrease in the activity of MnSOD isozyme at 4°C. In addition, MnSOD activity in root tissues of Zafer-160 was not significantly different than controls at all stress conditions (1, 3, 7 days at 4°C) (Figure 3.9).

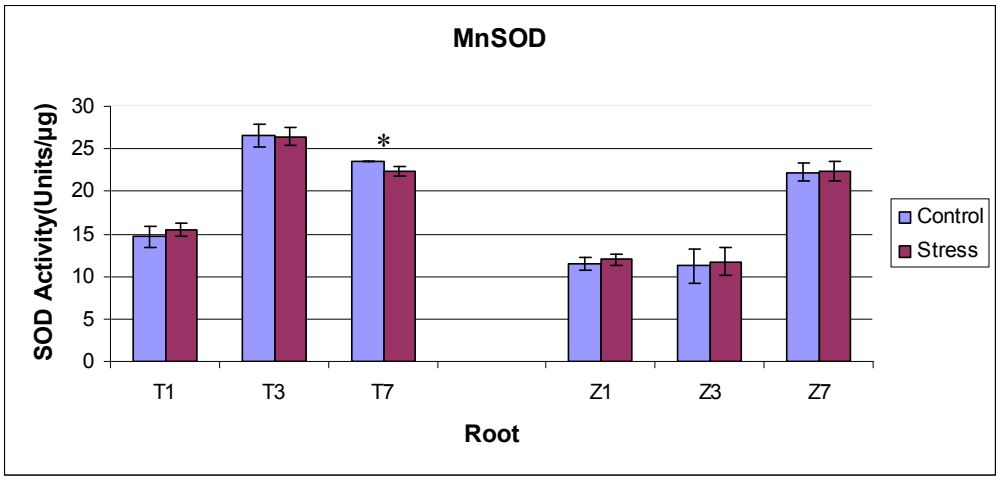


Figure 3.9. MnSOD activity (units/µg protein) in root tissues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=4). * Asterisk shows significant difference ($p < 0.05$) with respect to control.

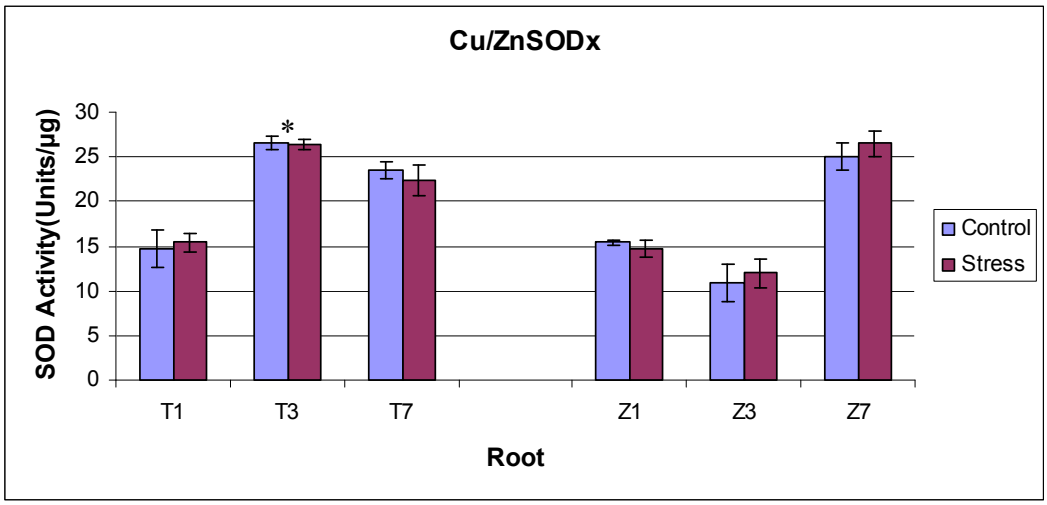


Figure 3.10. Cu/ZnSODx activity (units/µg protein) in root tisuues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=4). * Asterisk shows significant difference ($p<0.05$) with respect to control.

Cu/ZnSODx activity in root tissues of Zafer-160 did not significantly change under chilling stress conditions. 1 and 7 days treatments did not led to a significant change in root tissues of Tarm-92, however, only, its activity was significantly decreased at 3 days treatment in root of Tarm-92 (Figure 3.10).

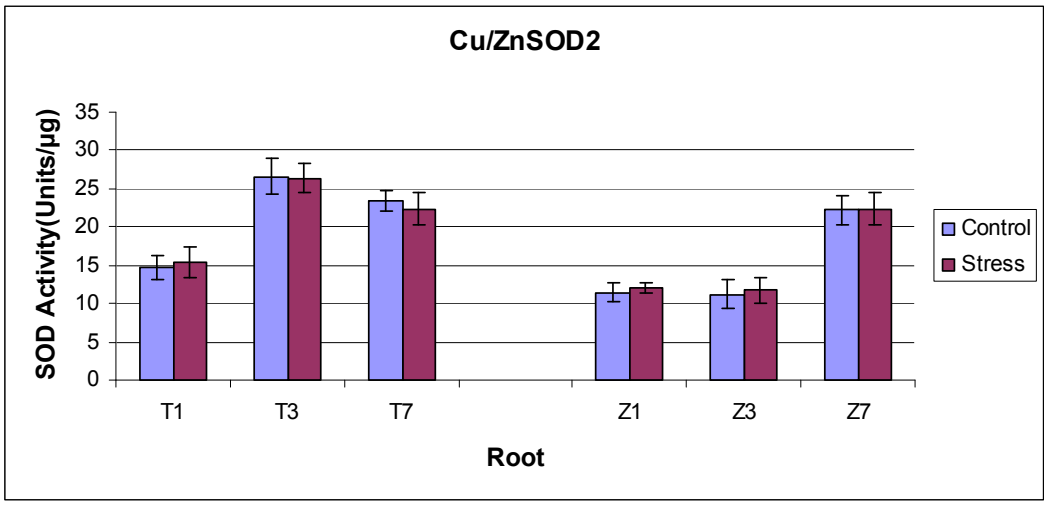


Figure 3.11. Cu/ZnSOD2 activity (units/µg protein) in root tissues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=4).

Chilling stress did not cause any significant change in the activity of Cu/ZnSOD2 isozyme in root tissues of both cultivars for 1, 3, 7 days at 4°C (Figure 3.11).

Table 3.1. Overall view of SOD isozyme activities under chilling stress. **+**; significantly increase, **-**; significantly decrease, **n.c**; no change. (In this table, all data were compared to controls of each treatment).

Chilling Stress	MnSOD	Cu/ZnSODx	Cu/ZnSOD2
1 Day Tarm-Leaf	n.c	n.c	n.c
3 Days Tarm-Leaf	+	n.c	n.c
7 Days Tarm-Leaf	n.c	n.c	n.c
1 Day Zafer-Leaf	n.c	n.c	n.c
3 Days Zafer-Leaf	n.c	n.c	n.c
7 Days Zafer-Leaf	n.c	n.c	n.c
1 Day Tarm-Root	n.c	n.c	n.c
3 Days Tarm-Root	n.c	+	n.c
7 Days Tarm-Root	+	n.c	n.c
1 Day Zafer-Root	n.c	n.c	n.c
3 Days Zafer-Root	n.c	n.c	n.c
7 Days Zafer-Root	n.c	n.c	n.c

Table 3.1 shows overall view of SOD isozyme activities under chilling stress condition. According to this table, interestingly, Cu/ZnSOD2 isozyme activity of both cultivars did not have any significant change in all treatments under chilling stress.

3.3. Effect of -3°C Freezing Stress on Superoxide Dismutase Enzyme Activities

The figure 3.12 represents the SOD isozymes patterns of leaf tissues obtained from Tarm-92 and Zafer-160 cultivars for -3°C freezing stress condition. In addition, the changes in SOD activity of the root tissues of both cultivars were given in figure 3.13.

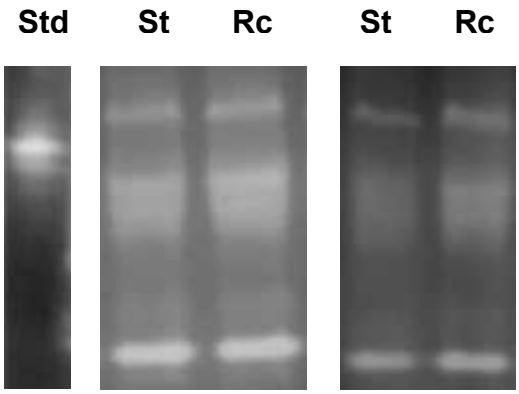


Figure 3.12. Activities of leaf tissues of SOD isozymes for freezing stress (-3°C). **Std**; 0.5 unit SOD standard from bovine erythrocytes, **St**; -3°C treated plants, **Rc**; After -3°C treatment, 4°C exposed plants. **A**; Activities of SOD isozymes of leaf tissues from Tarm-92. **B**; Activities of SOD isozymes of leaf tissues from Zafer-160.

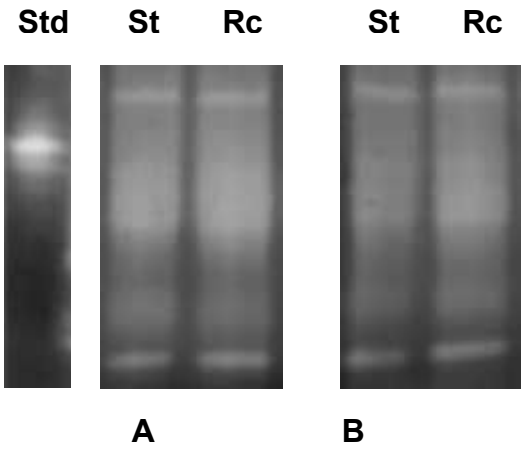


Figure 3.13. Activities of root tissues of SOD isozymes for freezing stress (-3°C). **Std**; 0.5 u SOD standard from bovine erythrocytes**, St**; -3°C treated plants, **Rc**; After -3°C treatment, 4°C exposed plants. **A**; Activities of SOD isozymes of root tissues from Tarm-92. **B**; Activities of SOD isozymes of root tissues from Zafer-160.

3.3.1. Evaluation of Superoxide Dismutase Isozyme Activities by Densitometric Analysis

Under -3°C freezing stress condition, 1-D Native PAGE studies were done in order to understand the change of SOD isozymes (figure 3.13 and 3.14). Pixel intensity analysis was used for these gel photographs. Accordingly, in leaf tissues of Tarm-92, MnSOD activity of cold acclimatized and -3°C treated plants was significantly different than controls and its activity of recovered plants was significantly different than cold-acclimatized plants. However, MnSOD activity of -3°C treated plants was not significantly different than recovered and cold-acclimatized plants in leaves of Tarm-92 (Figure 3.14).

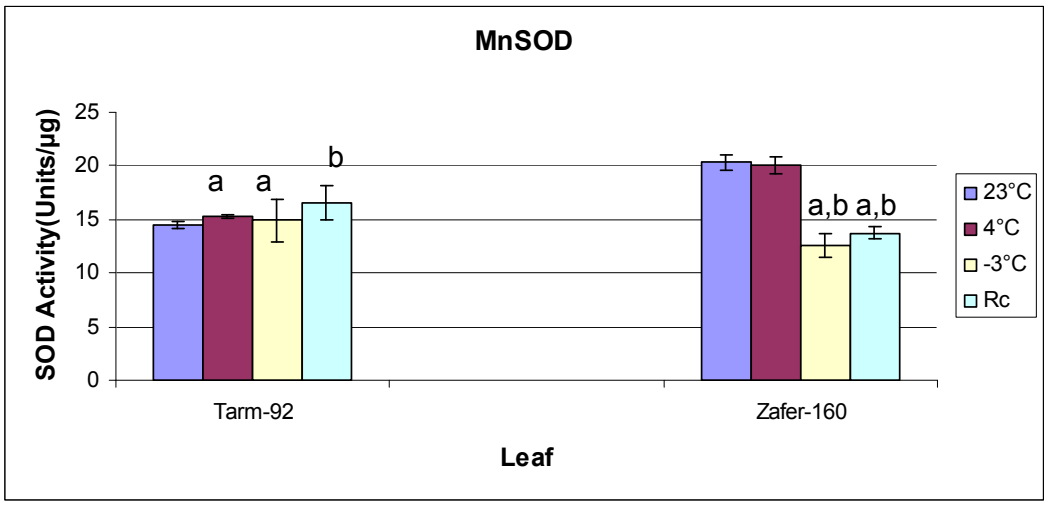


Figure 3.14. MnSOD activity (units/µg protein) in leaf tissues for freezing stress (-3°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plant. Bars indicate the mean values ± SEM (n=4).

In leaf tissues of Zafer-160, MnSOD activity of -3°C treated and recovered plants was significantly decreased than controls and cold-acclimatized plants. However, there were no significant difference between recovered and -3°C treated plants for MnSOD activity, and cold-acclimization did not cause any change in MnSOD activity rather than controls (Figure 3.14).

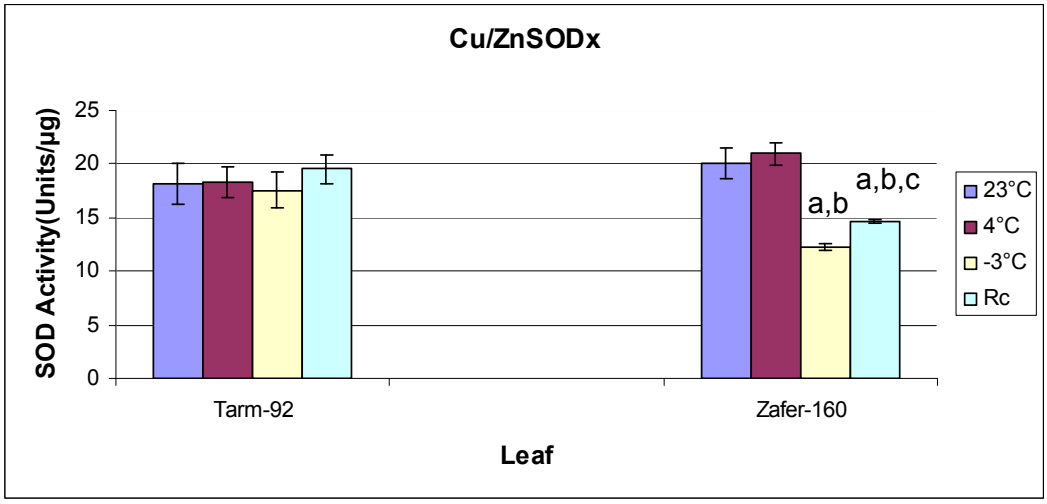


Figure 3.15. Cu/ZnSODx activity (units/µg protein) in leaf tissues for freezing stress (-3°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control.[b] shows significant difference ($p<0.05$) with respect to cold acclimatized plants. [c] shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

Cu/ZnSODx activity in leaf tissues of Tarm-92 did not significantly change in all treatments for freezing stress. However, Cu/ZnSODx activity of -3°C treated and recovered plants was significantly decreased than controls and cold-acclimatized leafs of Zafer-160.

After freezing stress, recovering on leafs of Zafer-160 was significantly increased in Cu/ZnSODx activity, whereas there were no significant difference between cold-acclimatized and control leafs of Zafer-160 for this isozyme activity(Figure 3.15).

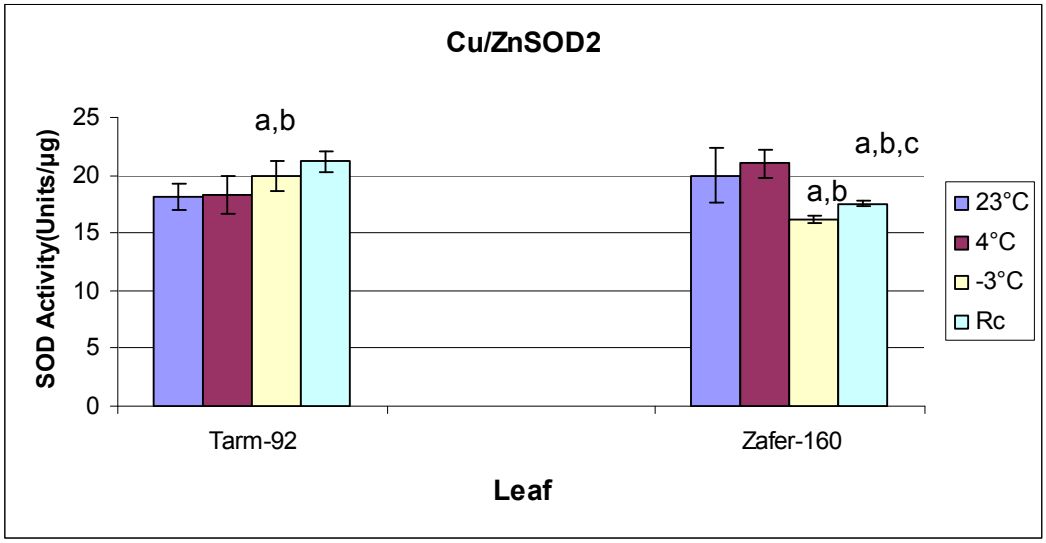


Figure 3.16. Cu/ZnSOD2 activity (units/µg protein) in leaf tissues for freezing stress (-3°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold acclimatized plants. [c] shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

In leaf tissues of Tarm-92, there were no significant difference between control and cold acclimatized plants of Cu/ZnSOD2 activity, and its activity in recovered leafs of Tarm-92 was not significantly different than -3°C

treated ones. However, Cu/ZnSOD2 activity of -3°C treated leafs of Tarm-92 was significantly higher than control and cold-acclimatized ones (Figure 3.16).

Cu/ZnSOD2 activity of cold-acclimatized leaf tissues of Zafer-160 was not significantly different than controls, however, -3°C treatment led to a significant decrease in the activity of Cu-ZnSOD2 isozyme. After freezing treatment, recovering at 4°C caused significant change in the activity of Cu/ZnSOD2 rather than controls, cold acclimatized, and , -3°C treated leafs of Zafer-160 (Figure 3.16).

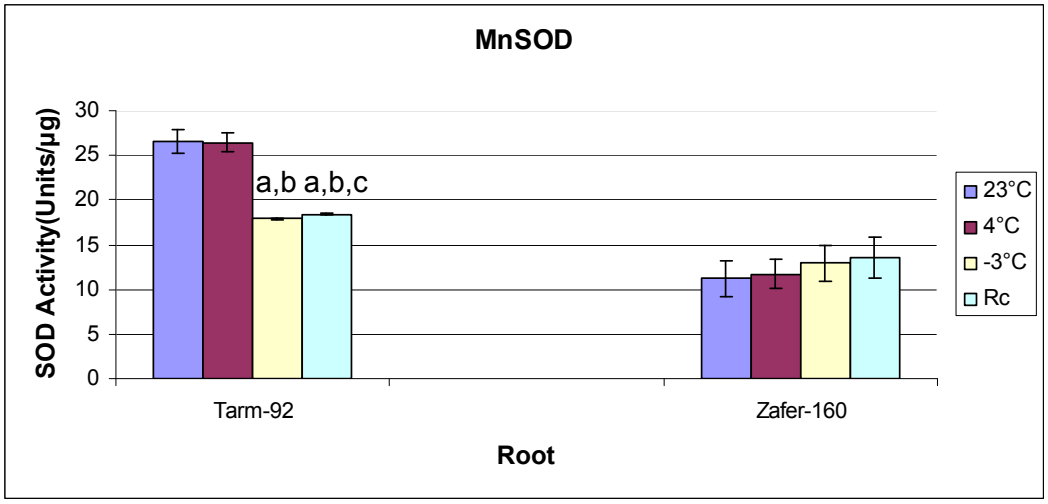


Figure 3.17. MnSOD activity (units/µg protein) in root tissues for freezing stress (-3°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control.[b] shows significant difference ($p<0.05$) with respect to cold acclimatized plants. [c] shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

In root tissues of Zafer-160, all treatments for freezing stress did not cause any significant change in the activity of MnSOD isozyme, however, in recovered and -3°C treated roots of Tarm-92, MnSOD activity was lower than controls and cold-acclimatized ones. Also, there were no significant diference between controls and cold-acclimatized root tissues of Tarm-92 (Figure 3.17).

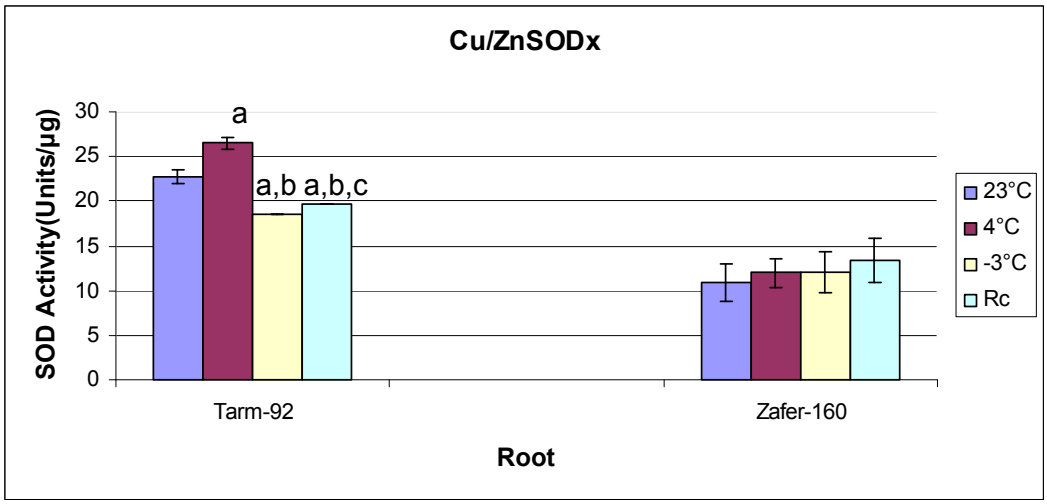


Figure 3.18. Cu/ZnSODx activity (units/µg protein) in root tissues for freezing stress (-3°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. [c] shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

Like MnSOD, freezing stress and recovering did not led to a significant change in the activity of Cu/ZnSODx in root tissues of Zafer-160. On the other hand, recovered roots of Tarm-92 Cu/ZnSODx activity was

significantly different than -3°C treated ones. In recovered and -3°C treated roots of Tarm-92, Cu/ZnSODx activity was significantly lower than controls and cold-acclimatized ones. Also, there were significantly difference between controls and cold-acclimatized root tissues of Tarm-92 (Figure 3.18).

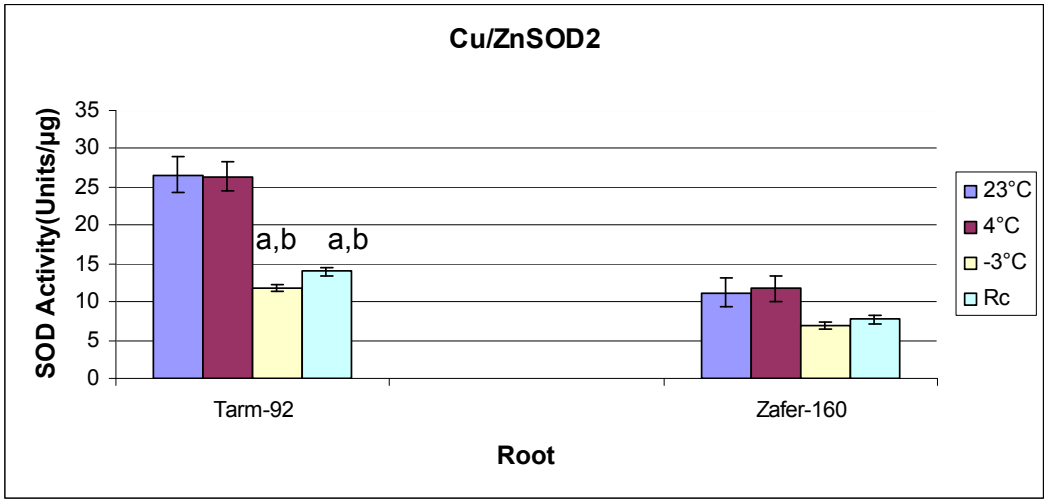


Figure 3.19. Cu/ZnSOD2 activity (units/µg protein) in root tissues for freezing stress (-3°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. Bars indicate the mean values ± SEM (n=4).

Like the other isozymes, in root tissues of Zafer-160, there were no significant difference between all conditions for Cu/ZnSOD2 activity. However, in recovered and -3°C treated roots of Tarm-92, Cu/ZnSOD2 activity was significantly lower than controls and cold-acclimatized ones. Also, in cold-acclimatized roots of Tarm-92, Cu/ZnSOD2 activity was not

significantly different than controls and its activity of recovered plants did not have significant change after freezing treatment at -3°C (Figure 3.19).

3.4. Effect of -7°C Freezing Stress on Superoxide Dismutase Enzyme Activities

The change in activities of SOD isozymes from leafs of both cultivars was given in figure 3.20. Also, figure 3.21 represented the SOD isozymes patterns of root tissues obtained from Tarm-92 and Zafer-160 cultivars for -7°C freezing stress condition.

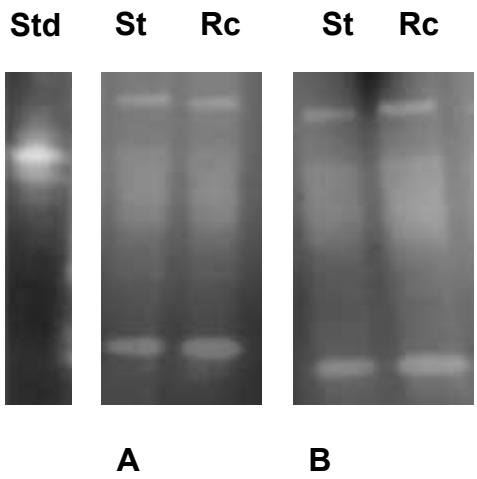


Figure 3. 20. Activities of SOD isozymes from leaf tissues for freezing stress (-7°C). **Std**; 0.5 unit SOD standard from bovine erythrocytes, **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants. **A**; Activities of SOD isozymes of leaf tissues from Tarm-92. **B**; Activities of SOD isozymes of leaf tissues from Zafer-160.

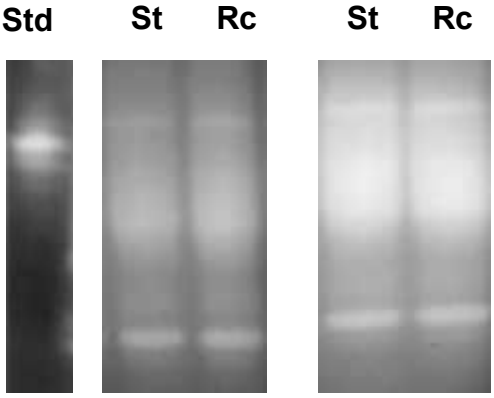


Figure 3.21. Activities of SOD isozymes from root tissues for freezing stress (-7°C). **Std**; 0.5 unit SOD standard from bovine erythrocytes, **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants. **A**; Activities of SOD isozymes of root tissues from Tarm-92. **B**; Activities of SOD isozymes of root tissues from Zafer-160.

3.4.1. Evaluation of Superoxide Dismutase Isozyme Activities by Densitometric Analysis

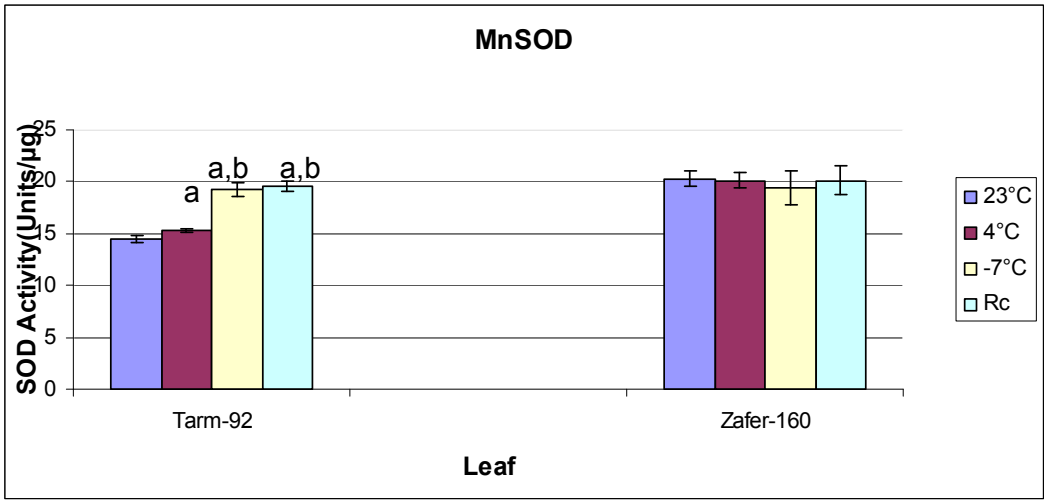


Figure 3.22. MnSOD activity (units/µg protein) in leaf tissues for freezing stress (-7°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold acclimatized plants. Bars indicate the mean values ± SEM (n=4).

Like the other freezing stress condition, in order for evaluating the changes in activity of SOD isozymes under -7°C freezing stress, gel photographs were used for pixel intensity (Figure 3.20 and 3.21). Accordingly, in leaf tissues of Zafer-160, cold-acclimatization, freezing stress at -7°C and recovering did not cause any significant change in MnSOD activity. However, the activity of recovered and -7°C treated leaf tissues of Tarm-92 was significantly higher than controls and cold-acclimatized plants. In addition, MnSOD activity of cold-acclimatized leafs

of Tarm-92 was significantly different than controls, whereas there were no significant difference between recovered and -7°C treated ones (Figure 3.22).

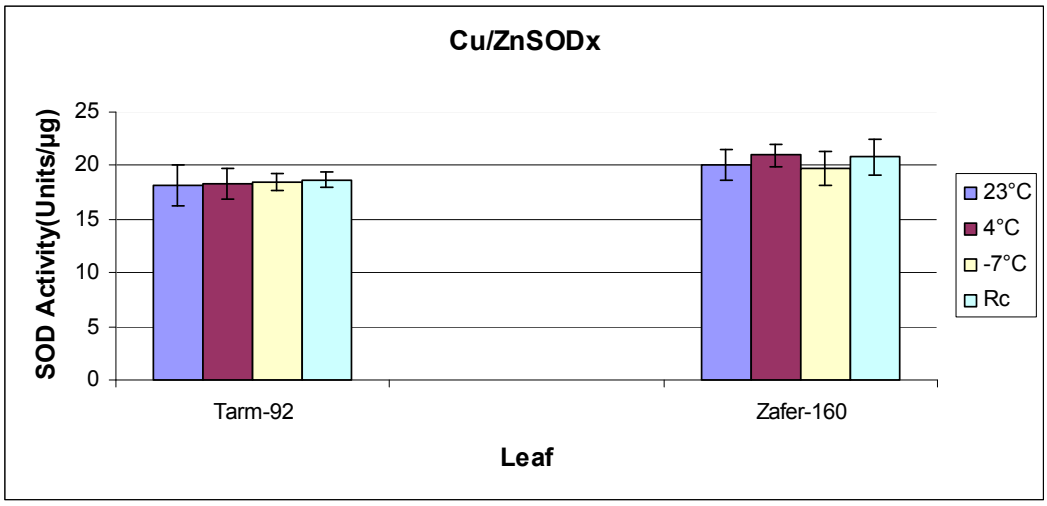


Figure 3.23. Cu/ZnSODx activity (units/µg protein) in leaf tissues for freezing stress (-7°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). Bars indicate the mean values ± SEM (n=4).

In leaf tissues of both cultivars, there were no significant difference between Cu/ZnSODx activities at all treatments (cold-acclimatization at 4°C, freezing stress at 7°C, and recovering at 4°C) (Figure 3.23).

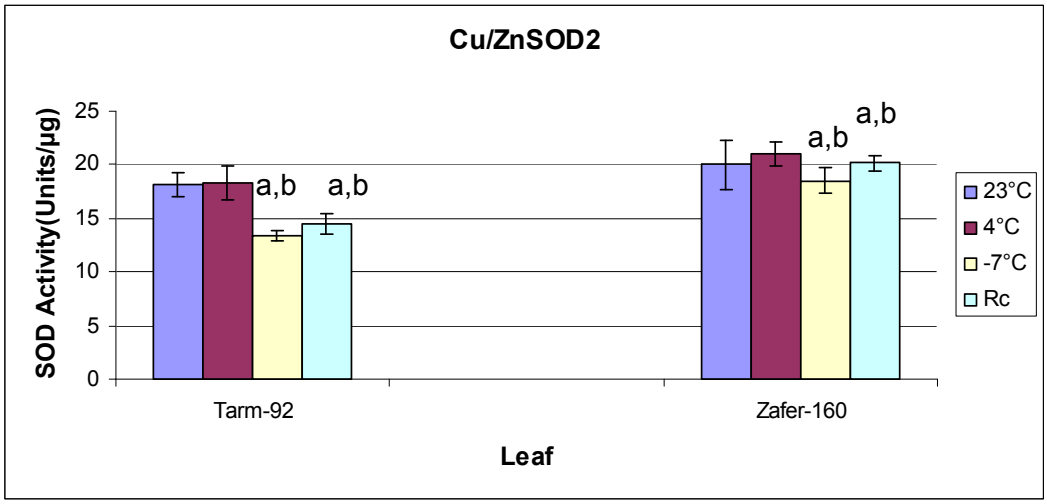


Figure 3.24. Cu/ZnSOD2 activity (units/µg protein) in leaf tissues for freezing stress (-7°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. Bars indicate the mean values ± SEM (n=4).

Cu/ZnSOD2 activity of -7°C treated and recovered leafs of Tarm-92 was significantly lower than controls and cold-acclimatized ones, however, after freezing stress, recovering at 4°C did not led to a significant change in this isozyme activity. Also, there were no significant difference between controls and cold-acclimatized leafs of Tarm-92 for the activity of Cu/ZnSOD2 (Figure 3.24).

Cu/ZnSOD2 activity of -7°C treated and recovered leafs of Zafer-160 was significantly different than controls and cold-acclimatized ones, however,

there were no significant difference between -7°C treated and recovered leafs of Zafer-160. Also, Cu/ZnSOD2 activity of cold- acclimatized leafs of Zafer-160 was not significantly different than controls (Figure 3.24).

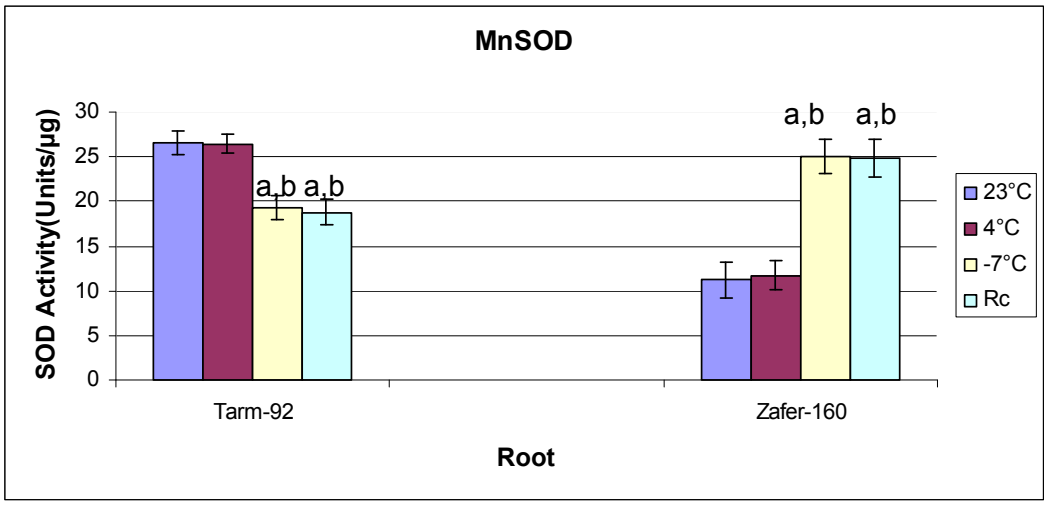


Figure 3.25. MnSOD activity (units/µg protein) in root tissues for freezing stress (-7°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. Bars indicate the mean values ± SEM (n=4).

MnSOD activity of -7°C treated and recovered root tissues of Tarm-92 was significantly lower than cold-acclimatized and control plants, however, its activity in recovered roots of Tarm-92 was not significantly different than -7°C treated ones, and there were no significant difference between cold-acclimatized and controls of root tissues of Tarm-92 (Figure 3.25).

Unlike the Tarm-92, MnSOD activity of -7°C treated and recovered root tisues of Zafer-160 was significantly higher than cold-acclimatized and control plants, whereas recovering did not led to a significant change in its activity after freezing stress at -7°C. Also, MnSOD activity in cold acclimatized roots of Zafer-160 was not different than controls (Figure 3.25).

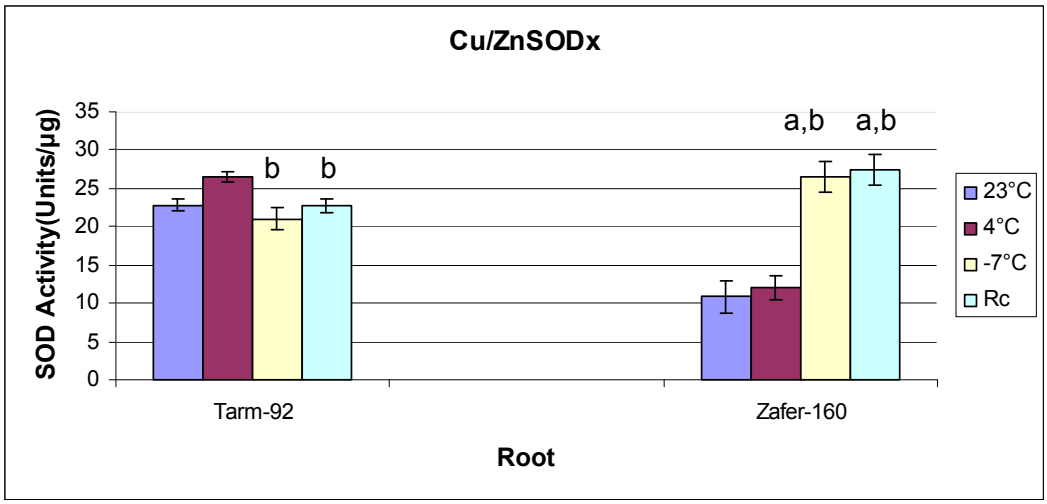


Figure 3.26. Cu/ZnSODx activity (units/µg protein) in root tissues for freezing stress (-7°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. Bars indicate the mean values ± SEM (n=4).

In recovered and -7°C treated roots of Tarm-92, Cu/ZnSODx activity was only significantly different than cold-acclimatized ones, however, there were no significant difference between its activity of recovered and -7°C

treated plants, and cold-acclimatization did not cause significant change in the activity of Cu/ZnSODx in this cultivars (Figure 3.26).

Cu/ZnSODx activity of -7°C treated and recovered root tisues of Zafer-160 was significantly higher than cold-acclimatized and control plants, whereas its activity in recovered roots of Zafer-160 was not significantly different than -7°C treated ones. Also, there were no significant difference between cold-acclimatized and controls of root tissues of zafer-160 (Figure 3.26).

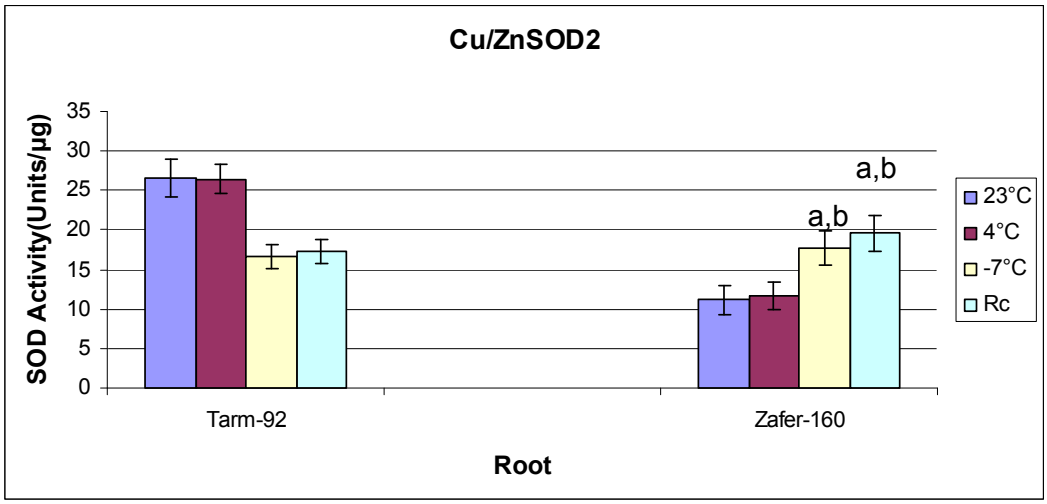


Figure 3.27. Cu/ZnSOD2 activity (units/µg protein) in root tissues for freezing stress (-7°C). (After plants were grown at 23°C for 8 days, they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. Bars indicate the mean values ± SEM (n=4).

In root tissues of Tarm-92, cold-acclimatized, freezing stress at -7°C and recovering did not cause any significant change in Cu/ZnSOD2 activity. However, Cu/ZnSOD2 activity of -7°C treated and recovered root tissues

of Zafer-160 was significantly higher than cold-acclimatized and control plants. In addition, Cu/ZnSOD2 activity in cold acclimatized roots of Zafer was not different than controls and after freezing treatment, recovering did not led to a significant change in its activity in root tissues of Zafer-160 (Figure 3.27).

Table 3.2. The overall view of SOD isozyme activities under freezing stresses conditions. +; significantly increase, -; significantly decrease, n.c; No Change, a shows significant difference ($p<0.05$) with respect to 23 ºC control. b shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. c shows significant difference ($p<0.05$) with respect to freezing stress treated plants.

Freezing Stress	MnSOD			Cu/ZnSODx			Cu/ZnSOD2		
	4	-3	Rc	4	-3	Rc	4	-3	Rc
-3°C Tarm-Leaf	a+	a+	b+	n.c	n.c	n.c	n.c	ab+	n.c
-3°C Zafer-Leaf	n.c	Ab-	ab-	n.c	ab-	Ab-c+	n.c	ab-	ab-c+
-3°C Tarm-Root	n.c	Ab-	ab-	a-	ab-	ab-c+	n.c	ab-	ab-
-3°C Zafer Root	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c
-7°C Tarm-Leaf	a+	Ab+	ab+	n.c	n.c	n.c	n.c	ab-	ab-
-7°C Zafer-Leaf	n.c	n.c	n.c	n.c	n.c	n.c	n.c	ab-	b-
-7°C Tarm-Root	n.c	Ab-	ab-	n.c	b-	b-	n.c	n.c	n.c
-7°C Zafer-Root	n.c	Ab+	ab+	n.c	ab+	Ab+	n.c	ab+	ab+

Table 3.2 shows the overall view of SOD isozyme activities under freezing stresses conditions.

3.5. Total RNA Isolations for RT-PCR

Total RNA isolations were performed from leaf and root tissues of Tarm-92 (winter type) and Zafer-160 (spring type) barley cultivars according to the protocols presented in section 2.2.4.

High quality and intact RNA preparation was the most critical step for RT-PCR. Therefore, the isolation of RNA was very important for the rest of the experiment. In this study, TRI Reagent that combined phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate inhibition of RNase activity, was used. Unlike other gene expression techniques, although RT-PCR was more tolerant of partially degraded RNA because of analysis of smaller regions of RNA, integrity of RNA was checked before gene expression analysis.

When working with RNA, some precautions must be taken since autoclaving is not effective at eliminating RNase in solution and RNases simply reactivate as the solution cools down. Therefore, all the solutions used in RNA isolations were prepared by using DEPC treated ddH_2O. However, high level or residual of DEPC can inhibit some enzymatic reactions or chemically alter (carboxymethylate) RNA. 0.1% DEPC is probably sufficient to inhibit most RNases.

It is crucial to determine concentrations of isolated RNA since these concentrations can result in false positives in RT-PCR when the RNA levels of control and treated samples were compared. Therefore, all of the concentrations of RNA were measured attentively.

After RNA isolations, they were run on 1% agarose gel in order to assess the integrity of total RNA (Figure 3.28). In this gel, 25S and 18S ribosomal RNA (rRNA) were seen clearly. This means that mRNAs were intact. The integrity of RNA samples were checked in agarose gel, and then quantification of RNA samples were done by U.V Vis spectrophotometer (Varian Cary 100).

In this study, total RNA isolation was done successfully for RT-PCR. Total RNA was isolated from leaf tissues of Tarm-92 (Figure 3.28) and was isolated from leaf tissues of Zafer-160 (Figure 3.29). In addition, total RNA was isolated from root tissues of Tarm-92 and Zafer-160 (Figure 3.30-3.31).

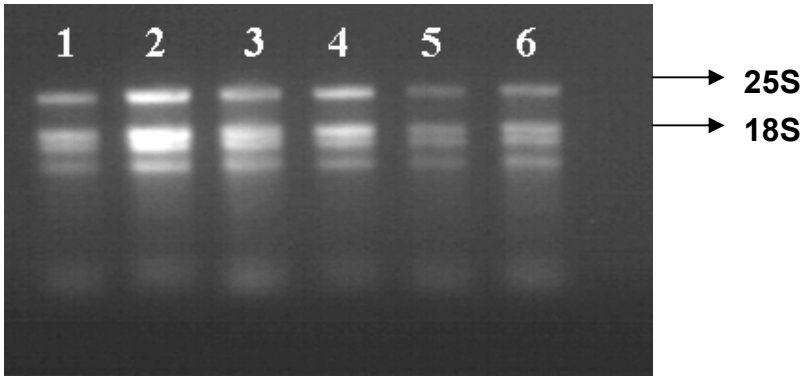


Figure 3.28. The view of total RNA isolated from leaf tissues of Tarm-92 on 1% agarose gel. The control plant for 1 day of chilling after 8 days of growth. **2.** Treated plant for 1 day at 4°C. **3.** The control plant for 3 days of chilling after 8 days of growth. **4.** Treated plant for 3 days at 4°C. **5.** The control plant for 7 days of chilling after 8 days of growth. **6.** Treated plant for 7 days at 4°C.

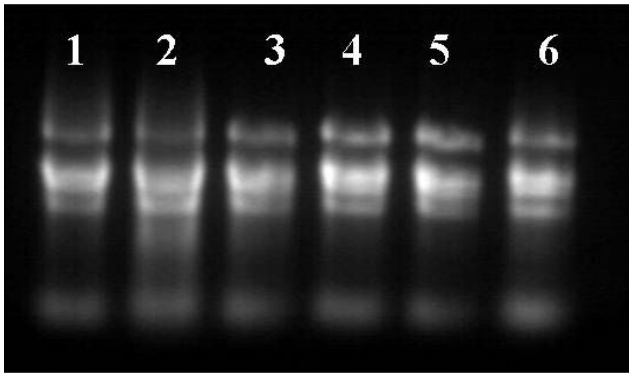


Figure 3.29. The view of total RNA isolated from leaf tissues of Zafer-160 on 1% agarose gel.**1.** The control plant for 1 day of chilling after 8 days of growth. **2.** Treated plant for 1 day at 4°C. **3.** The control plant for 3 days of chilling after 8 days of growth. **4.** Treated plant for 3 days at 4°C. **5.** The control plant for 7 days of chilling after 8 days of growth. **6.** Treated plant for 7 days at 4°C.

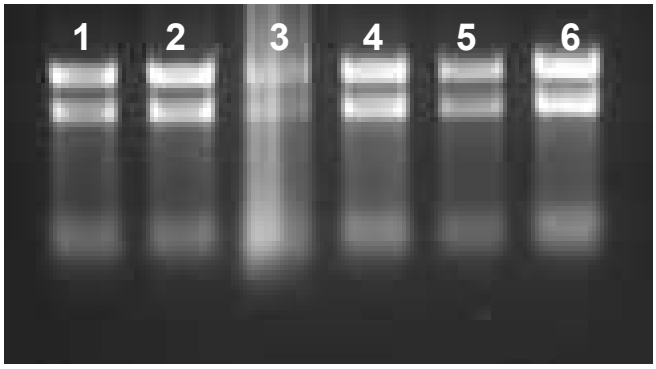


Figure 3.30. The view of total RNA isolated from root tissues of Tarm-92 on 1% agarose gel. **1.** The control plant for 1 day of chilling after 8 days of growth. **2.** Treated plant for 1 day at 4°C. **3.** The control plant for 3 days of chilling after 8 days of growth. **4.** Treated plant for 3 days at 4°C. **5.** The control plant for 7 days of chilling after 8 days of growth. **6.** Treated plant for 7 days at 4°C.

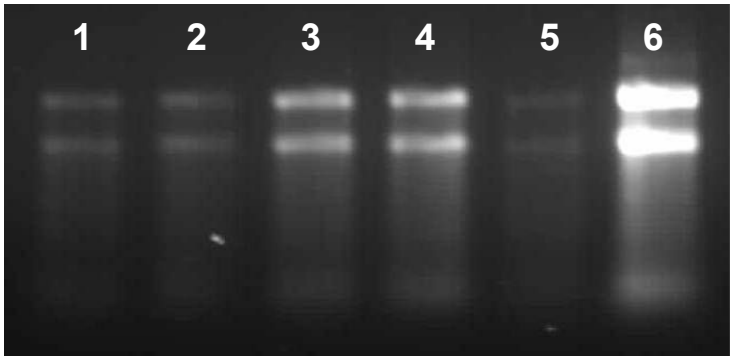


Figure 3.31. The view of total RNA isolated from root tissues of Zafer-160 on 1% agarose gel.**1.** The control plant for 1 day of chilling after 8 days of growth. **2.** Treated plant for 1 day 4°C. **3.** The control plant for 3 days of chilling after 8 days of growth. **4.** Treated plant for 3 days 4°C. **5.** The control plant for 7 days of chilling after 8 days of growth. **6.** Treated plant for 7 days 4°C.

For -3°C and -7°C freezing stresses, total RNA isolations were done successfully in leaf and root tissues of both barley cultivars These isolations were seen in Appendix C.

3.6. The Optimization of Relative RT-PCR

The optimization of relative RT-PCR was critical for expression analysis. No RT-PCR optimization was shown in figure 3.32a, and positive control was illustrated in figure 3.32b. In this optimization, isolated RNA was directly used as template for PCR amplification and checked whether there were any genomic DNA contamination.

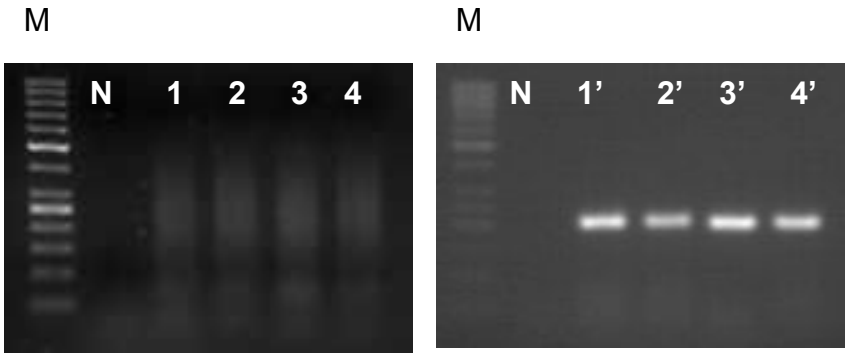


Figure 3.32. The optimization of RT-PCR for genomic DNA contamination. **A**; No-RT-PCR Study. **B**; Positive Control. **M**; Marker (DNA Ladder), **N**; Negative Control, **1-2**; Results of No-RT-PCR for Cu/ZnSOD gene **3-4**; Results of No-RT-PCR for internal control (*Ef-1*). **1’-2’**; Positive Control for 1 and 2 (expected size 180 bp), **3’-4’**; Positive Control for 3 and 4 (expected size 170 bp).

The second important optimization was the determination of cycle numbers for RT-PCR. For this purpose, figure 3.33 shows different cycles of RT-PCR for Cu/ZnSOD and internal control genes. In this study, the cycle number of Cu/ZnSOD and internal control was determined as 33 cycle for expression analysis with two replicate studies.

Figure 3. 33. The optimization of cycle numbers of Cu/ZnSOD and internal control. **M**; Marker (DNA Ladder). **1**. 25 cycle of Cu/ZnSOD **2**. 30 cycle of Cu/ZnSOD **3**. 33 cycle of Cu/ZnSOD **4**. 35 cycle of Cu/ZnSOD **5**. 25 cycle of internal control **6**. 30 cycle internal control **7**. 33 cycle of internal control **8**. 35 cycle of internal control.

After optimization, Cu/ZnSOD and internal control RT-PCR bands were sequenced in order to understand whether there were any contaminations. For these purposes, tblastx and blastn sequence analysis programs were used and any contaminations were not detected. (Appendix F and G). The sequence results of RT-PCR product for Cu/ZnSOD and internal control were given in Appendix E. In addition, all RT-PCR products of internal control were given in Appendix D.

3.7. The Results of RT-PCR for Cu/ZnSOD gene in Exposed to Chilling Stress

RNA was successfully isolated from control and chilled plants, and then it was converted to cDNA, and then used as a template for PCR. The RT-PCR products of leaf and root tissues in both cultivars were seen clearly in Figure 3.34-35. RT-PCR studies were also done for root tissues in Tarm-92 (Figure 3.36) and Zafer-160 cultivars (Figure 3.37).

Figure 3.34. RT-PCR results for Cu/ZnSOD of leaf tissues from Tarm-92 on 2% agarose gel. **M**; Marker (DNA Ladder), **N**; Negative Control. **1.** The control plant for 1 day of chilling after 8 days growth. **2.** Treated plant for 1 day at 4°C. **3.** The control plant for 3 days of chilling after 8 days growth. **4.** Treated plant for 3 days at 4°C. **5.** The control plant for 7 days of chilling after 8 days growth. **6.** Treated plant for 7 days at 4°C.

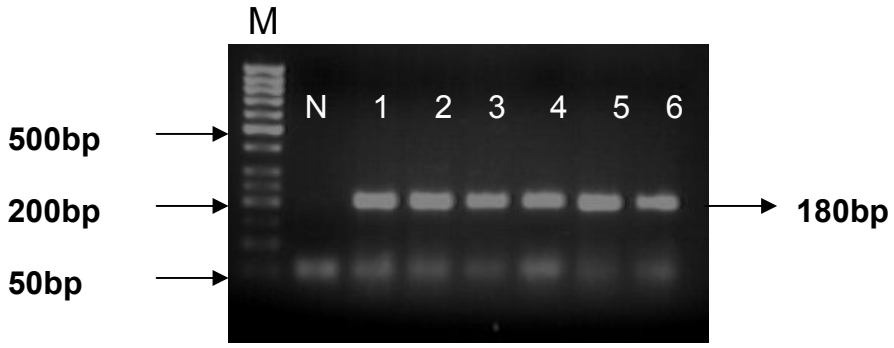


Figure 3.35. RT-PCR results for Cu/ZnSOD gene of leaf tissues from Zafer-160 on 2% agarose gel. **M**; Marker (DNA Ladder), **1.** The control plant for 1 day of chilling after 8 days growth. **2.** Treated plant for 1 day at 4°C. **3.** The control plant for 3 days of chilling after 8 days growth. **4.** Treated plant for 3 days at 4°C. **5.** The control plant for 7 days of chilling after 8 days growth. **6.** Treated plant for 7 days at 4°C.

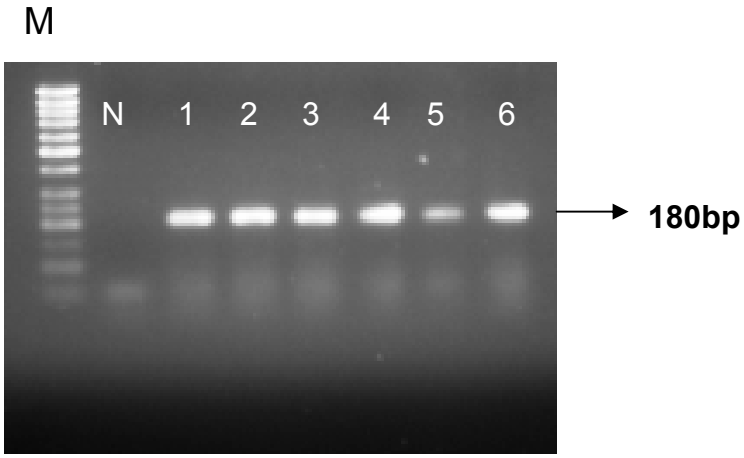


Figure 3.36. RT-PCR results for Cu/ZnSOD gene of root tissues from Tarm-92 on 2% agarose gel. **1**.The control plant for 1 day of chilling after 8 days growth. **2.** Treated plant for 1 day at 4°C. **3.** The control plant for 3 days of chilling after 8 days growth. **4.** Treated plant for 3 days at 4°C. **5.** The control plant for 7 days of chilling after 8 days growth. **6.** Treated plant for 7 days at 4°C.

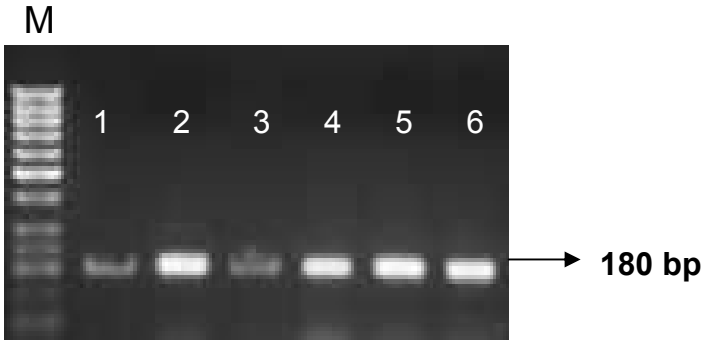


Figure 3.37. RT-PCR results for Cu/ZnSOD gene of root tissues from Zafer-160 on 2% agarose gel. **M**; Marker (DNA Ladder),. **1.** The control plant for 1 day of chilling after 8 days growth. **2.** Treated plant for 1 day at 4°C. **3.** The control plant for 3 days of chilling after 8 days growth. **4.** Treated plant for 3 days at 4°C. **5.** The control plant for 7 days of chilling after 8 days growth. **6.** Treated plant for 7 days at 4°C.

3.7.1. Evaluation of Cu/ZnSOD Gene Expression by Densitometric Analysis

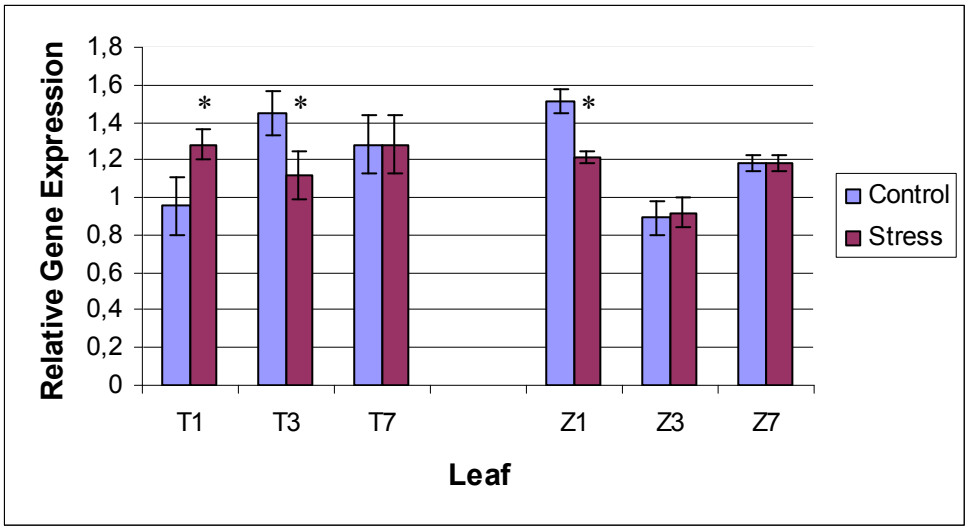


Figure 3.38. Relative RT-PCR analysis of Cu/ZnSOD gene of leaf tissues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=4). * Asterisk shows significant difference ($p<0.05$) with respect to control.

Pixel intensities of Cu/ZnSOD and internal control genes were taken and Cu/ZnSOD results were normalized by using results of internal control intensities. According to this analysis, in leaves, after 1 day treatment, chilling at 4°C significantly increased the expression level of the Cu/ZnSOD gene, however, for 3 days treatment, its level significantly decreased in Tarm-92. In addition, chilling stress at 4°C did not cause any significant change in the expression of this gene in 7-days treated Tarm-92 (Fig.3.38).

In leaf tissues of Zafer -160, only 1 day treatment led to a significant decrease in the expression level of the Cu/ZnSOD gene, on the other hand, Cu/ZnSOD did not significantly change in 3 and 7 days treatment at 4°C (Fig.3.38).

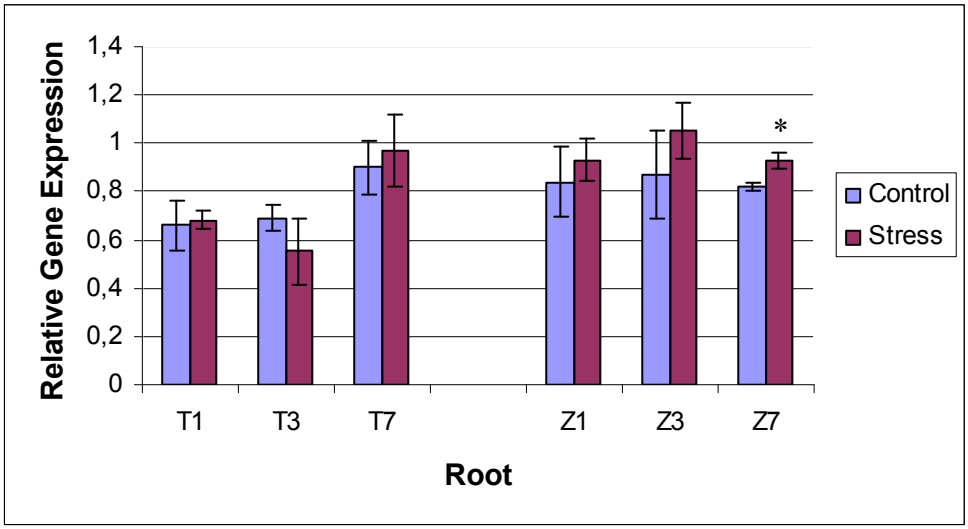


Figure 3.39. Relative RT-PCR analysis of Cu/ZnSOD gene of root tissues under chilling stress. **T1**; Treated Tarm-92 for 1 day at 4°C, **T3**; Treated Tarm-92 for 3 day at 4°C, **T7**; Treated Tarm-92 for 7 day at 4°C, **Z1**; Treated Zafer-160 for 1 day at 4°C, **Z3**; Treated Zafer-160 for 3 day at 4°C, **Z7**; Treated Zafer-160 for 7 day at 4°C. Bars indicate the mean values ± SEM (n=5). * Asterisk shows significant difference ($p<0.05$) with respect to control.

In roots, chilling stress at 4°C did not affect the expression level of Cu/ZnSOD of Tarm-92 barley cultivar for 1, 3, and 7 days treatments. In roots of Zafer-160, there were no significant difference between the control and treated plants for 1 and 3 days treatment, hovewer, 7 days treatment resulted in significant increase in the expression level of Cu-Znsod gene (Figure 3.39).

3.8. The Results of RT-PCR for Cu/ZnSOD gene in Exposed to Freezing Stress

RT-PCR products were seen clearly in leaves and roots of both cultivars for -3°C freezing stress (Figure 3.40-41). In addition, RT-PCR was successfully done in leafs and roots of both cultivars for -7°C freezing stress (Figure 3.42-43).

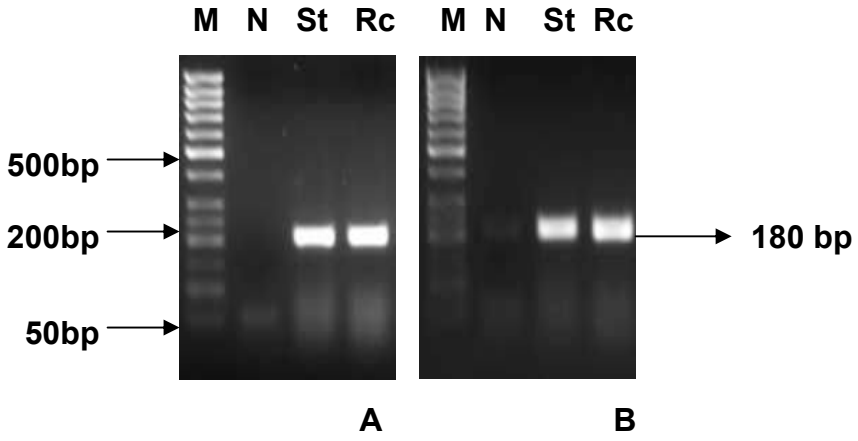


Figure 3.40. Appearance of RT-PCR products from leaf tissues of Cu/ZnSOD gene under freezing stress (-3°C). **M**; Marker (DNA Ladder), **N**: Negative Control. **St**; -3°C treated plants, **Rc**; After -3°C treatment, 4°C exposed plants. **A**; RT-PCR products of leaf tissues of Tarm-92. **B**; RT-PCR products of leaf tissues of Zafer-160.

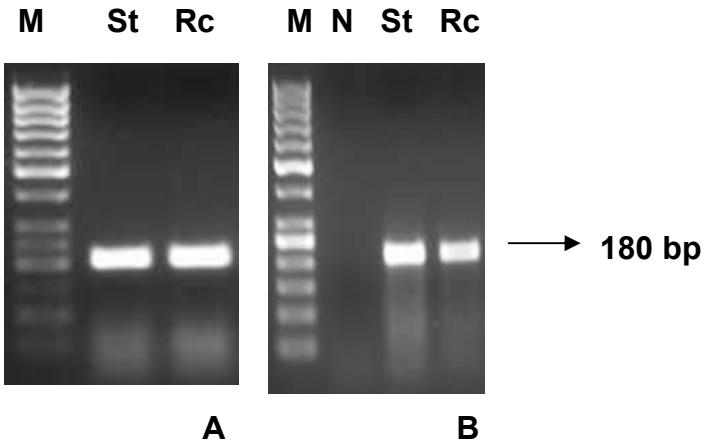


Figure 3. 41. Appearance of RT-PCR products from root tissues of Cu/ZnSOD gene under freezing stress (-3°C). **M**; Marker (DNA Ladder), N; Negative Control. **St**; -3°C treated plants, **Rc**; After -3°C treatment, 4°C exposed plants. **A**; RT-PCR products of root tissues from Tarm-92. **B**; RT-PCR products of root tissues from Zafer-160.

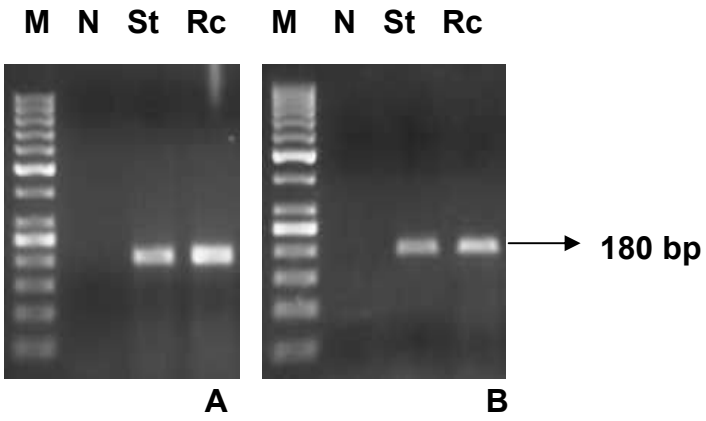


Figure 3.42. Appearance of RT-PCR products from leaf tissues of Cu/ZnSOD gene under freezing stress (-7°C). **M**; Marker (DNA Ladder), **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants. **A**; RT-PCR products of leaf tissues from Tarm-92. **B**; RT-PCR products of leaf tissues from Zafer-160.

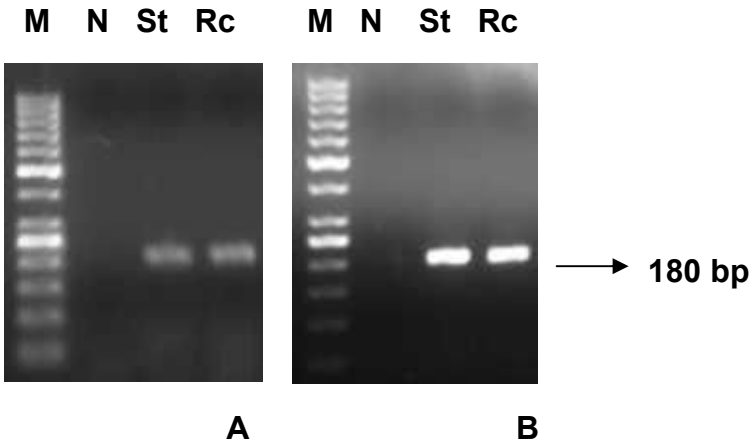


Figure 3.43. Appearance of RT-PCR products from root tissues of Cu/ZnSOD gene under freezing stress (-7°C). **M**; Marker (DNA Ladder), **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants. **A**; RT-PCR products of root tissues from Tarm-92. **B**; RT-PCR products of root tissues from Zafer-160.

3.8.1. Evaluation of Cu/ZnSOD Gene Expression by Densitometric Analysis

The gel photographs of RT-PCR were used for intensity analysis under freezing stress conditions (Figure 3.40-43). Accordingly, in the expression level of Cu/ZnSOD of Tarm-92 leaves, there were significant difference between control and cold acclimatized plants at 4°C, -3°C treated, and recovered Tarm-92 at 4°C. -3°C treatment did not cause any significant change than cold-acclimatized Tarm-92 in the expression level of Cu/ZnSOD. In addition, after freezing treatment at -3°C, recovery at 4°C did not lead to any significant change in the expression level of Cu/ZnSOD gene of leaves of Tarm-92 (Figure 3.44).

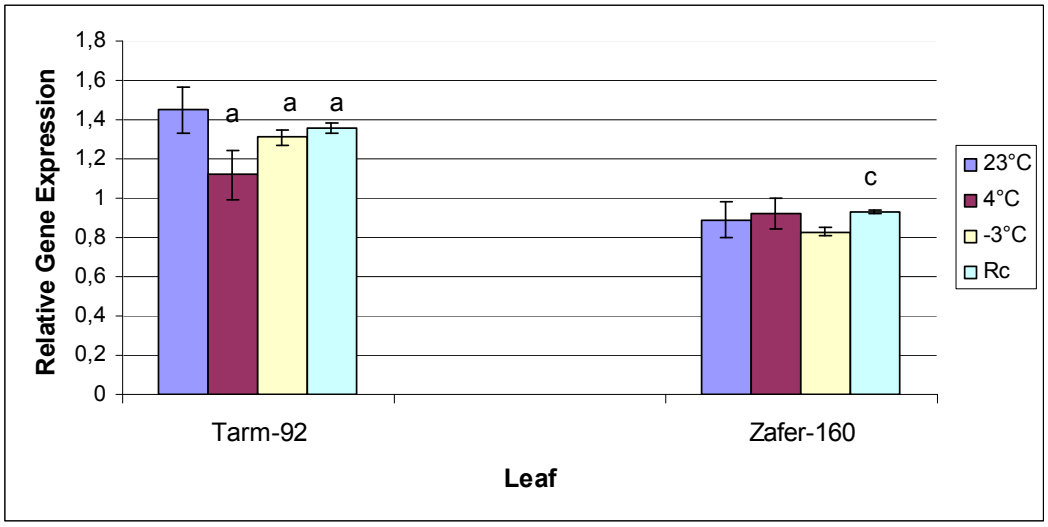


Figure 3.44. Relative RT-PCR analysis of Cu/ZnSOD gene of leaf tissues for freezing stress (-3°C). (The plants were grown at 23°C for 8 days, then they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were incubated at 4°C for 4 days for recovery). ^{a} shows significant difference ($p<0.05$) with respect to 23ºC control. ^{c} shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

In leaf tissues of Zafer-160, there were no significant difference between control and cold-acclimatized plants at 4°C, -3°C treated Tarm-92, and recovered of this cultivar at 4°C. Only, after -3°C treatment, recovery at 4°C result in change significant increase in the expression level of Cu/ZnSOD gene (Figure 3.44).

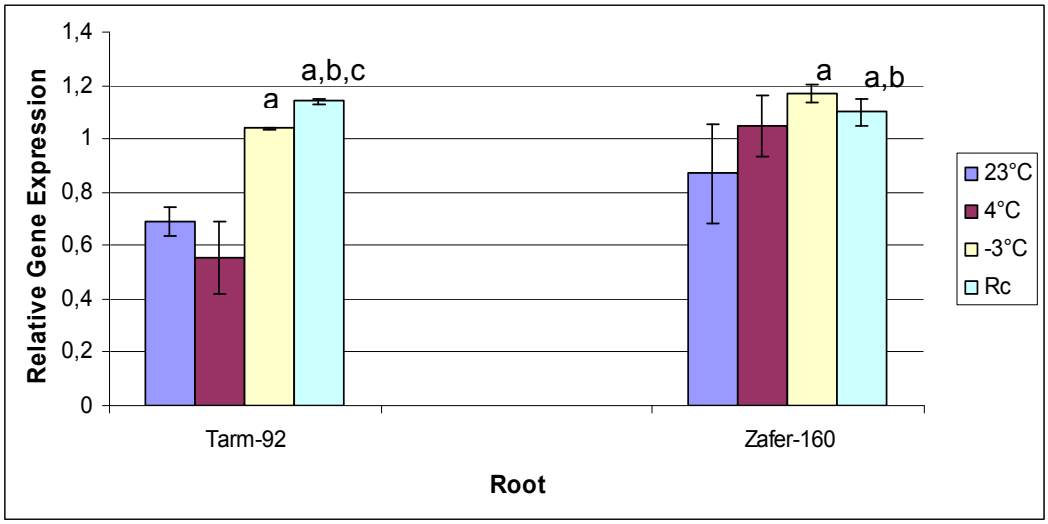


Figure 3. 45. Relative RT-PCR analysis of Cu/ZnSOD gene of root tissues for freezing stress (-3°C). (The plants were grown at 23°C for 8 days, then they were cold-acclimatized for 3 days at 4°C and were exposed to -3°C for 3 hours. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23 ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. [c] shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

In root, the expression levels Cu/ZnSOD of Tarm-92 cultivar at 4°C were not significantly different than controls, however, the expression levels of this gene at -3°C were significantly higher than controls. Recovered plants

at 4°C led to significant difference than controls, cold-acclimatized plants and -3°C treatment plants (Figure 3.45).

In cold-acclimatized Zafer-160 cultivars, the expression levels of Cu/ZnSOD gene were not significantly different than control plants in roots. Recovered plants were significantly different than controls and cold-acclimatized plants for the expression levels of Cu/ZnSOD gene in roots of Zafer-160 cultivars. In addition, the expression levels of this gene of -3°C treated plants were significantly higher than control plants in roots of Zafer-160 (Figure 3.45).

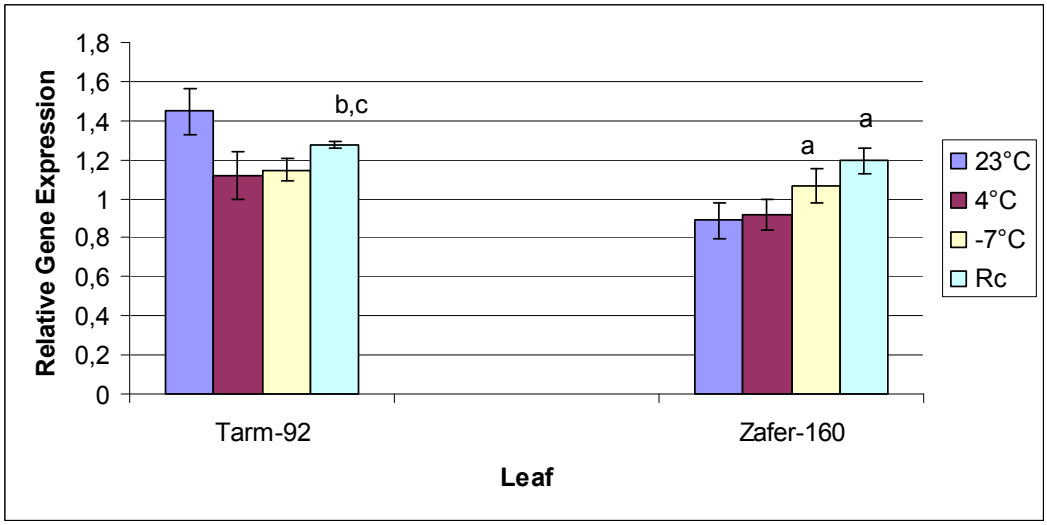


Figure 3. 46. Relative RT-PCR analysis of Cu/ZnSOD gene of leaf tissues for freezing stress (-7°C). (The plants were grown at 23°C for 8 days, then they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. [c] shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

In leaves of Tarm-92, the expression levels of Cu/ZnSOD of -7°C treated cultivars were not significantly different than controls and cold-acclimatized plants. In addition, there were no significant difference between controls and cold-acclimatized Tarm-92 cultivars in leaves of the transcript levels of this gene. However, in recovered Tarm-92 cultivars, the expression levels of Cu/ZnSOD gene were significantly higher than -7°C treated and cold-acclimatized plants (Figure 3.46).

In Zafer-160 cultivars, the transcription levels of the recovered leaves were not significantly different than controls, cold-acclimatized and -7°C treated cultivars. However, the leaves of control plants were significantly different than -7°C treated and recovered Zafer-160 cultivars for the expression levels of Cu/ZnSOD gene (Figure 3.46).

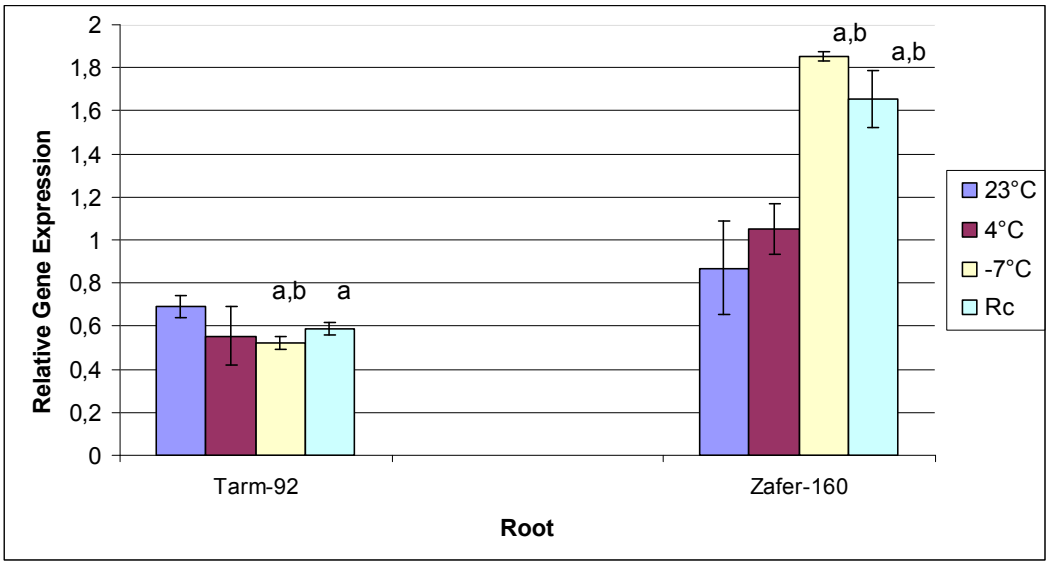


Figure 3.47. Relative RT-PCR analysis of Cu/ZnSOD gene of root tissues for freezing stress (-7°C). (The plants were grown at 23°C for 8 days, then they were cold-acclimatized for 3 days at 4°C and hardened at -3°C for 30 minutes, and then they were exposed to -7°C for 90 minutes. Subsequently, plants were moved at 4°C for 4 days for recovery). [a] shows significant difference ($p<0.05$) with respect to 23ºC control. [b] shows significant difference ($p<0.05$) with respect to cold-acclimatized plants. [c] shows significant difference ($p<0.05$) with respect to -3°C treated plants. Bars indicate the mean values ± SEM (n=4).

The transcription level of the Cu/ZnSOD in control root tissues of Tarm-92 were not significantly different than cold-acclimatized plants and recovered roots of Tarm-92 cultivars were not significantly different than -7°C treated plants for its expression levels of its gene. However, the expression levels of Cu/ZnSOD of control plants were significantly different than -7°C treated and recovered roots of Tarm-92 cultivars. In addition, there were significant difference cold-acclimatized and -7°C treated roots of Tarm-92 cultivars for its expression levels of its gene (Figure 3.47).

The expression levels of the Cu/ZnSOD gene of recovered and -7°C treated roots of Zafer-160 were significantly higher than control and cold-acclimatized plants. On the other hand, cold-acclimatized roots of Zafer-160 were not significantly different than control plants for its expression levels of this gene and after freezing treatment at -7°C, recovering process at 4°C did not cause any significant changes in the expression levels of this gene (Figure 3.47).

CHAPTER 4

DISCUSSION

Abiotic stress greatly affect cereal crop yield in many growing areas of the world. Among these stresses, low temperature or cold is a major environmental limitation to crop productivity and the distribution of the wild species, since climate change affect the geographical distribution of some wild species. However, plants have acquired adaptive strategies to cope with abiotic stresses. Therefore, the understanding or identification of biochemical events and candidate genes for cold stress is essential for production and agriculture in the world.

Physiological and biochemical analysis determine general features of cold-tolerance. Solute accumulation and changes in membrane lipid composition are well-known examples. In the last 20 years, molecular analysis and genetic studies have provided different information and approaches. For instance, 54 cold-inducible genes were identified using a cDNA microarray containing approximately 7000 independent full-lenght *Arabidopsis* cDNA clones (Seki *et al*.,2002). Functions of their gene products have been predicted from comparisons of sequence homology with known proteins. In other words, some of them are functional proteins, others are regulatory ones. Water channel proteins, chaperones, proteases, late embryogenesis-abundant (LEA) proteins, and enzymes that are involved with the synthesis of osmoprotectants are well-known examples. Examples of stress-related regulatory proteins include transcription factors, protein kinases, and enzymes for phosphoinositide turnover, and enzymes for the synthesis of the plant hormone absisic acid (ABA) (Shinozaki *et al*.,2003).

Superoxide dismutases are key antioxidants, which play important roles in protecting cells against the toxic effects of superoxide radicals produced in different cellular loci (Fridovich, 1995). This SOD enzyme system seems to be regulated in plants on the gene expression level by both environmental and developmental stimuli (Bowler *et al*.,1994). However, occurrence of this regulation remains unclarified since there has been contradictory results in expression and activity of SOD isozymes under stress conditions. For instance, in chilled maize and tomato plants, SOD activity decreased especially sensitive genotypes (Pinhero *et al*.,1997). However, SOD activity was correlated with plant cold-resistance (Wang *et al*., 1995). Other researchers reported that SOD activity in leaves of either chilling-sensitive or cold-resistant plants did not change during chilling (Shen W.Y *et al*.,1999). In addition, a similarity SOD activity was noted between genotypes differing in cold-resistance (Bruggemann, W *et al*.,1999). Factors affecting the extent of chilling injury also did not influence the SOD activity (Skrudlik *et al*.,2000).

Like this activity changes of SOD isozymes, conflicting results were explored via transgenic approaches. Overexpression of tobacco chloroplasts SOD did not alter tolerance toward oxidative stress, however, expression of a pea chloroplast SOD in tobacco increased resistance to methyl viologen-induced membrane damage (Allen RD, 1995). Also, the transgenic tobacco leaf discs expressed 30- to 50- fold more Cu/ZnSOD than controls, however, they were not protected against methyl-viologen mediated inhibition of CO_2 assimilation, nor chlorophyll bleaching during photo inhibitory conditions (Tepperman and Dunsmuir, 1990). In contrast, overexpression of chloroplast Cu/ZnSOD in transgenic tobacco plants showed decreased level of cellular damage in the presence of methyl viologen.

The reports concerning the involvement of antioxidant enzymes under stress conditions have had contradictory results, especially in the

response of plants to cold stress. One of the antioxidant enzymes, superoxide dismutase is critical for protecting cells, however, it is unclear how SOD activity and expression change in plants under cold stress conditions of varying durations. Thus, the aim of this study was to clarify whether SOD isozymes had critical function in plant cold-resistance.

In this study, two barley cultivars (Tarm-92-winter type) and Zafer-160-spring type) were used since barley was chosen as the model plant. In other words, genetic variation of barley has played a primary role in determining positive adaptation to environmental stresses and extreme climatic conditions (Stanca *et al*., 1992). In addition, barley growing has been shifting for a number of years from spring to autumn sowing since winter varieties are higher-yielding than spring cultivars.

4.1. Effect of Chilling Stress on SOD isozymes

Exposure to low temperature may led to the increased generation of ROS in plants. The ROS may deliver signals for detection of the changed environment (Prasad *et al*., 1994). Exposure of winter cereals to cold acclimation temperatures induced an increased tolerance to oxidative stress (Bridger *et al*., 1994). In addition, the activities of several antioxidant enzymes may increase during cold acclimation (Scebba *et al*., 1999).

Over production of superoxide anion radicals leads to oxidative stress, which decrease SOD activity under chilling stress (Pinhero *et al*., 1997). However, the increase in the superoxide anion radicals would induce *de nova* synthesis of SOD isozymes (Tsang *et al*., 1991). In addition, SOD activity increased in leaves of tolerant maize cultivar exposed to chilling, whereas its activity reduced in sensitive one (Aroca *et al*., 2001).

Because of these contradictory results, some authors believe that SOD does not play a significant role in plants, especially under chilling stress

conditions. However, others claim that the protection mediated by SOD against environmental stresses show differences according to the stress conditions, species, cultivars, and tissues.

According to our chilling experiment results, Cu/ZnSODx and Cu/ZnSOD2 isozyme activities in leaves of both cultivars were not significantly different than controls for all days treatment. However, in leaves of Tarm-92, the transcript level of Cu/ZnSOD was significantly increased after 1 day treatment at 4°C, whereas for 3 days treatment its level significantly decreased in this cultivars. Like this enzyme activity, chilling stress did not cause any significant change in the expression level of this gene 7 days-treated leaves of Tarm-92. In addition, only 1 day treatment resulted in significant decrease in its gene expression in leaves of Zafer-160.

While the expression level of Cu/ZnSOD gene in roots of Tarm-92 was not affected by chilling stress and its level only increased in 7 days treated roots of Zafer-160, Cu/ZnSODx activity only was significantly decreased in 3 days treated Tarm-92. Like the expression level of this gene, Cu/ZnSOD2 activity was not significantly different under chilling stress condition in both cultivars.

Under chilling stress condition, we obtained contradictory results in the activity and transcript level of Cu/ZnSOD isozyme. Besides, chilling stress did not affect in activities of SOD isozyme. This means that these cultivars may tolerate to the chilling stress in terms of SOD activity and expression levels since barley (*Hordeum vulgare* L.) can be considered a good genetic model for its tribe (Triticeae) to study plant response to adverse environmental conditions. In other words, its wide range of adaptability, the availability of a wide range of genetic stocks, and the extended collinearity with other members of the tribe are additional advantages as a model (Hayes et al. 2003). Second possible explanation is that the constant level of Cu/ZnSOD may be enough to protect from damage by

superoxide during chilling stress. However, the expression level of Cu/ZnSOD gene was more variable than its activity since the gene that encodes a Cu/ZnSOD is a developmentally regulated gene in many plants. Although two cultivars of barley were not affected excessively by chilling stress, their developments may be affected under this cold stress.

4.2. Effect of Freezing Stress on SOD isozymes

There are limited number of studies related with effect of freezing stress to antioxidant systems. Generally, these studies have focused on the *cor* genes (cold-responsive genes) and determine whether they have roles in freezing tolerance. A great number of *cor* genes have been isolated and characterized from a variety of plant species, but only some of them code for proteins whose biochemical function is known, or can be at least predicted based on sequence similarity (Jung et al., 2003). Besides, the analysis of plants either overexpressing or antisense for a given *cor* gene demonstrated that the accumulation of protecting molecules or the degradation of dangerous metabolites is associated with cold and/or frost tolerance (Iba, 2002). However, the exact function of many stress-related gene products still remains unclear. In addition, there was no reports in superoxide dismutase under freezing stress.

According to our results, after cold-acclimatized at 4°C, the expression level of Cu/ZnSOD and activity of Cu/ZnSOD2 were increased in -3°C treated leaves of Tarm-92, whereas there was no change in activity Cu/ZnSODx. In leaf tissues of Zafer-160, after freezing stress treatment at -3°C, the transcript level of Cu/ZnSOD and activities of Cu/ZnSODx and Cu/ZnSOD2 were increased in recovered plants. In addition, freezing stress at -3°C led to a significant decrease in their activities of leaves of Zafer-160.

While the transcript level of Cu/ZnSOD increased significantly, the activities of Cu/ZnSODx and Cu/ZnSOD2 decreased in root tissues of Tarm-92 at -3°C. In roots of Zafer-160, the expression level of Cu/ZnSOD increased significantly, whereas the activities of Cu/ZnSODx and Cu/ZnSOD2 did not have any change at -3°C.

In leaf tissues of both cultivars, the expression level of Cu/ZnSOD was not correlated to the activity of Cu/ZnSODx and Cu/ZnSOD2 under freezing stress at -7°C. In other words, its transcript level increased, while their activities decreased.

There were only positive correlations between the expression level of Cu/ZnSOD and activities Cu/ZnSODx and Cu/ZnSOD2 in roots of Zafer-160. In other words, both of them increased significantly under freezing stress condition at -7°C.

Freezing stress is more dangerous than chilling stress since it is the formation of ice crystals in the extracellular space that dehydrates the cell under freezing stress condition. Therefore, plants have to activate dehydration tolerance mechanisms because of preventing ice formation. Under this condition, solutes are secreted to decrease the freezing point of water. For example, the constitutively freezing tolerant mutant eskimo1 (*esk1*; Xin and Browse, 1998) has increased total solute accumulation and increased accumulation of the compatible solute proline, traits that are also likely to make esk1 more resistant to low-induced dehydration. In this study, the expression of Cu/ZnSOD and activities of SOD isozymes were variable in some cultivars and in some others, they were unvariable since addition of solutes decrease the freezing point of water, and accumulation of sugar and other simple sugars contribute to the stabilization of membrane when plants were exposed to freezing stress. In other words,

these may be first response to the this stress. Besides, in this study, proline accumulation was highly detected after freezing treatment (data not shown).

Under freezing stresses, in both cultivars, the activities of SOD isozyme and the expression levels of Cu/ZnSOD significantly increased, however, in Zafer-160, they continued to increased after these stresses. since in tarm-92, these activity and expression level may be enough to protect the their cells. In other words, these results suggest that Tarm-92 cultivar (winter type) may have more adaptability to freezing stress than Zafer-160 (spring type).

CHAPTER 5

CONCLUSION

In this study, the expression level of Cu/ZnSOD and the activities of superoxide dismutase isozymes in two barley cultivars differing in their susceptibility to cold stress were investigated under cold stresses. It was found that the changes of SOD isozymes activities and expression level display cultivar, tissue specificity and duration of stress. The changes of leaves SOD isozymes are different than its root SOD isozymes under same stress condition because these isozymes have different locations in different tissues.

Interestingly, all SOD isozymes which are independent metal cofactors and cellular location display a parallel behaviour as increase or decrease in activity. Chilling stress did not affect the activitİes and the expression levels of Cu/ZnSOD isozyme. Therefore, these activity and expression level may be enough to protect the plant cells under chilling stress. Furthermore, under freezing stress conditions, the increment of SOD activities and expression levels in Zafer-160 was higher than Tarm-92.

Although there are lots of studies of antioxidant system and stress physiology, still some important points remain unclear. Therefore, to understand how plants overcome cold stress, further studies of antioxidant enzyme systems are required.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Chemicals	Chemical Suppliers
Acrylamide	Sigma
Alcohol	Applichem
Ammonium Persulfate	Sigma
Bis-acrylamide	Sigma
Chloroform	Applichem
EDTA	Duchefa
Glycerol	Duchefa
Guanidine Thiocyanata	Sigma
Hydrogen Peroxide	Merck
Isopropanol	Merck
Mercaptoethanol	Sigma
Nitroblue Tetrazolium	Applichem
Phenol (water-saturated)	Applichem
Potassium Acetate	Merck
Potassium Cyanide	Merck
Potassium Dihydrogen Phosphate	Applichem
Riboflavin	Sigma
Sarcosyl	Sigma
Sodium Dihydrogen Phosphate	Merck
TEMED	Sigma
Tris Base	Duchefa

APPENDIX B

PREPARATION OF BRADFORD REAGENT

Equipments & Chemicals:

-Spectrophotometer

-Vortex

-Micropipettes

-Balance

-BSA

-Coomassie Brilliant Blue G-250

-Ethanol

-Phosphoric acid

Preparation of Solutions:

5X Bradford Reagent:

1-) Dissolve 500 mg of Coomassie Brilliant Blue G-250 in 250 ml of 95% ethanol

2-) Add 500 ml of 85% (w/v) phosphoric acid to the mixture.

3-) Dilute the solution to 1 L with dH_2O.

4-) Filter the solution and keep at the refrigerator.

Final concentrations: 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid.

APPENDIX C

TOTAL RNA ISOLATION FOR FREEZING STRESS

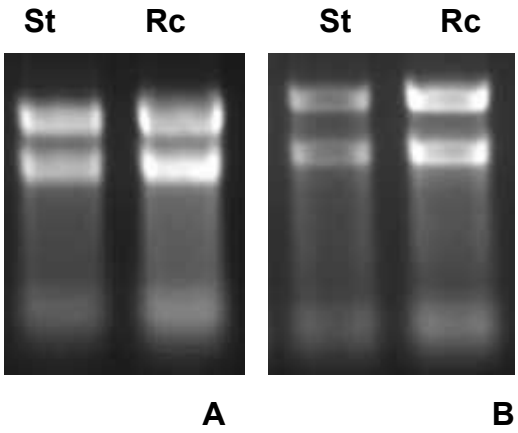

Figure A.1. The appearance of the total RNA isolated from leaf tissues for -3°C stress and recovery affect. St; -3°C treated plants, Rc; After -3°C treatment, 4°C exposed plants. **A**; The total RNA isolation of leaf tissues from Tarm-92. **B**; The total RNA isolation of leaf tissues from Zafer-160.

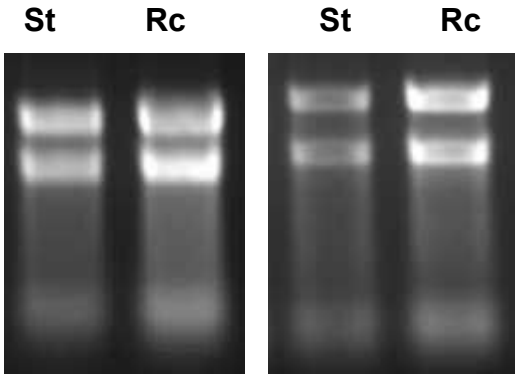

Figure A.2. The appearance of the total RNA isolated from root tissues for -3°C stress and recovery affect. **St**; -3°C treated plants, **Rc**; After -3°C treatment, 4°C exposed plants. **A**; The total RNA isolation of root tissues from Tarm-92. **B**; The total RNA isolation of root tissues from Zafer-160.

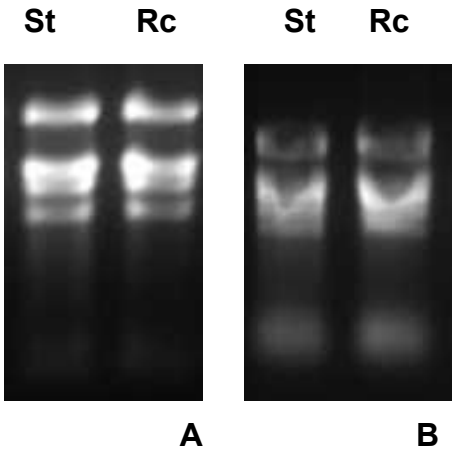

Figure A.3. The appearance of the total RNA isolated from leaf tissues for -7°C stress and recovery affect. **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants. **A**; The total RNA isolation of leaf tissues from Tarm-92. **B**; The total RNA isolation of leaf tissues from Zafer-160.

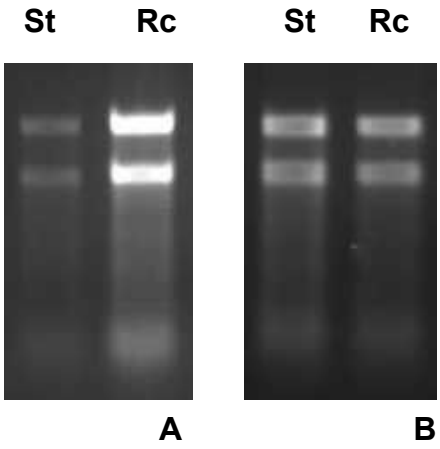

Figure A.4. The appearance of the total RNA isolated from root tissues for -7°C stress and recovery affect. **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants. **A**; The total RNA isolation of root tissues from Tarm-92. **B**; The total RNA isolation of root tissues from Zafer-160.

APPENDIX D

RESULTS OF RT-PCR FOR INTERNAL CONTROL

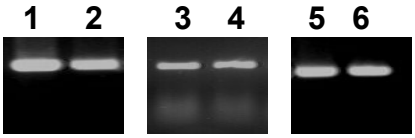

Figure A 5. RT-PCR results for internal control of leaf tissues from Tarm-92 on 2% agarose gel. **1**. The control plant for 1 day of chilling after 8 days growth. **2**. Treated plant for 1 day at 4°C. **3**. The control plant for 3 days of chilling after 8 days growth. **4**. Treated plant for 3 days at 4°C. **5**. The control plant for 7 days of chilling after 8 days growth. **6**. Treated plant for 7 days at 4°C.

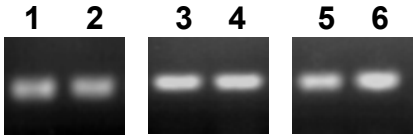

Figure A 6. RT-PCR results for internal control of leaf tissues from Zafer-160 on 2% agarose gel. **1**. The control plant for 1 day of chilling after 8 days growth. **2**. Treated plant for 1 day at 4°C. **3**. The control plant for 3 days of chilling after 8 days growth. **4**. Treated plant for 3 days at 4°C. **5**. The control plant for 7 days of chilling after 8 days growth. **6**. Treated plant for 7 days at 4°C.

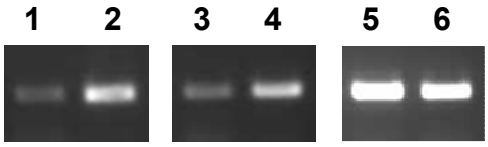

Figure A 7. RT-PCR results for internal control of root tissues from Tarm-92 on 2% agarose gel. **1**. The control plant for 1 day of chilling after 8 days growth. **2**. Treated plant for 1 day at 4°C. **3**. The control plant for 3 days of chilling after 8 days growth. **4**. Treated plant for 3 days at 4°C. **5**. The control plant for 7 days of chilling after 8 days growth. **6**. Treated plant for 7 days at 4°C.

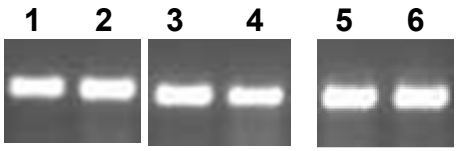

Figure A 8. RT-PCR results for internal control of root tissues from Zafer-160 on 2% agarose gel. **1**. The control plant for 1 day of chilling after 8 days growth. **2**. Treated plant for 1 day at 4°C. **3**. The control plant for 3 days of chilling after 8 days growth. **4**. Treated plant for 3 days at 4°C. **5**. The control plant for 7 days of chilling after 8 days growth. **6**. Treated plant for 7 days at 4°C.

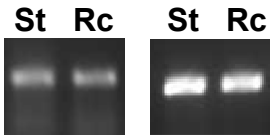

Figure A.9. RT-PCR products of internal control from leaf tissues of under freezing stress (-3°C). **St**; -3°C treated plants, **Rc**; After -3°C treatment, 4°C exposed plants.

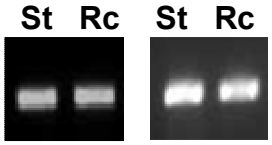

Figure A.10. RT-PCR products of internal control from root tissues of under freezing stress (-3°C). **St**; -3°C treated plants, **Rc**; After -3°C treatment, 4°C exposed plants.

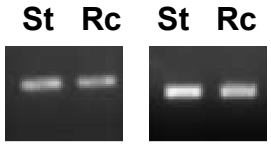

Figure A.11. RT-PCR products of internal control from leaf tissues of under freezing stress (-7°C). **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants.

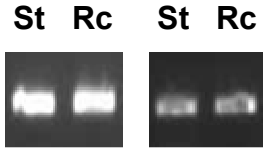

Figure A.12. RT-PCR products of internal control from root tissues of under freezing stress (-7°C). **St**; -7°C treated plants, **Rc**; After -7°C treatment, 4°C exposed plants.

APPENDIX E

SEQUENCE OF RT-PCR PRODUCTS FOR Cu/ZnSOD AND INTERNAL CONTROL GENE

Forward SOD:

ACGCCGNCNAATGATTGATNTATTCAGCAAGGNGGATATGGCAGTCN
TCNACATTGATGTNAGCAACACCATCCGCTCCAGCTGTCACATTTCC
GAGGTCACCAGCATGGCGGATTTCATCTTCGGGTGCCCCATGCACAT
CNACCAGCGTGGTTNAAGTGCGGNCCCAGTTGACATANNN

Reverse SOD:

CCNCTGNNATGTGCATGGGGCTTCTGAAGATGANATCCGCCATGCTG
GTTTCCTCGGAAATGTGACAGCTGGAGCGGATGGTGTTGCTAACATC
AATGTTACTGACTGCCATATCCCCCTTGCTGGACCACATTCAATCATT
GGCCGTGCTGTTGANGTCCATGGGGAATGCTGATGAA

Forward for internal control:

GCANAGACNAGACTTCNNTTAGGGNGNGGACACCGACTGNTNNACT
CGTNGNTGGGCGCCTCGCAGAGCACTTCCTCAATGAGAGTGTCGAC
AGACACGAGGTCATCAATGATCGTCAGCATGANCTGNAGCTTCTTGA
GGCTAAAAACCCACAGGNCATGAGCTAAAN

Reverse for internal control:

GAAGCTCAGACATTCANNCGANANNGAGGACCTCGNGTCTTTCGACA
CTCTCANTGAGGAAGTGCTCTGCGAGGCGCCCATCAACGAGTACGC
CAGTCGTGTGACATCGTTGCCTTCAACAAGATCTAGAATCTTTGTCCG
CTCACCTGTTGCTGCTNGGGATTCNNTGGANA

APPENDIX F

BLASTn SEQUENCES ANALYSIS FOR RT-PCR PRODUCT OF Cu/ZnSOD

```
>barley|TC146754 similar to UP|SOD4_MAIZE (P23345) Superoxide dismutase
[Cu-Zn]
            4A  , complete
         Length = 936

  Plus Strand HSPs:

 Score = 767 (121.1 bits), Expect = 3.5e-30, P = 3.5e-30
 Identities = 165/179 (92%), Positives = 165/179 (92%), Strand = Plus /
Plus

Query:     1 CCNCTGNN-ATGTGCATGGGGCTTCTGAAGATGANATCCGCCATGCTGGTTTCCTCGGAA
59
             || |||   |||||||||||||  | |||||||| ||||||||||||||||  ||||||||
Sbjct:   373 CCGCTGGTCATGTGCATGGGGCACCCGAAGATGAAATCCGCCATGCTGGTGACCTCGGAA
432

Query:    60 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   433 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
492

Query:   120 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGANGTCCATGGGGAATGCTGATGA
178
             ||||||||||||||||||||||||||||||||||  |||||||| || |||||||||
Sbjct:   493 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGTCGTCCATGGTGA-TGCTGATGA
550


>barley|TC146752 similar to UP|SOD4_MAIZE (P23345) Superoxide dismutase
[Cu-Zn]
            4A  , complete
         Length = 932

  Plus Strand HSPs:

 Score = 767 (121.1 bits), Expect = 3.5e-30, P = 3.5e-30
 Identities = 165/179 (92%), Positives = 165/179 (92%), Strand = Plus /
Plus

Query:     1 CCNCTGNN-ATGTGCATGGGGCTTCTGAAGATGANATCCGCCATGCTGGTTTCCTCGGAA
59
             || |||   |||||||||||||  | |||||||| ||||||||||||||||  ||||||||
Sbjct:   350 CCGCTGGTCATGTGCATGGGGCACCCGAAGATGAAATCCGCCATGCTGGTGACCTCGGAA
409

Query:    60 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   410 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
469

Query:   120 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGANGTCCATGGGGAATGCTGATGA
178
             ||||||||||||||||||||||||||||||||||  |||||||| || |||||||||
Sbjct:   470 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGTCGTCCATGGTGA-TGCTGATGA
527
```

barley|TC146753 similar to UP|SOD4_MAIZE (P23345) Superoxide dismutase [Cu-Zn]

4A , complete
Length = 835

Plus Strand HSPs:

Score = 767 (121.1 bits), Expect = 3.9e-30, P = 3.9e-30
Identities = 165/179 (92%), Positives = 165/179 (92%), Strand = Plus / Plus

```
Query:     1 CCNCTGNN-ATGTGCATGGGGCTTCTGAAGATGANATCCGCCATGCTGGTTTCCTCGGAA
59
             || |||   |||||||||||||  | |||||||| ||||||||||||||||  ||||||||
Sbjct:   330 CCGCTGGTCATGTGCATGGGGCACCCGAAGATGAAATCCGCCATGCTGGTGACCTCGGAA
389

Query:    60 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   390 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
449

Query:   120 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGANGTCCATGGGGAATGCTGATGA
178
             |||||||||||||||||||||||||||||||||||  |||||||| || |||||||||
Sbjct:   450 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGTCGTCCATGGTGA-TGCTGATGA
507
```

>**barley|BI946752 similar to PIR|S07007|S07 superoxide dismutase (EC 1.15.1.1)**
(Cu-Zn) 4 cytosolic [validated] - maize, partial (92%)
Length = 577

Plus Strand HSPs:

Score = 767 (121.1 bits), Expect = 5.7e-30, P = 5.7e-30
Identities = 165/179 (92%), Positives = 165/179 (92%), Strand = Plus / Plus

```
Query:     1 CCNCTGNN-ATGTGCATGGGGCTTCTGAAGATGANATCCGCCATGCTGGTTTCCTCGGAA
59
             || |||   |||||||||||||  | |||||||| ||||||||||||||||  ||||||||
Sbjct:   351 CCGCTGGTCATGTGCATGGGGCACCCGAAGATGAAATCCGCCATGCTGGTGACCTCGGAA
410

Query:    60 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   411 ATGTGACAGCTGGAGCGGATGGTGTTGCTAACATCAATGTTACTGACTGCCATATCCCCC
470

Query:   120 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGANGTCCATGGGGAATGCTGATGA
178
             |||||||||||||||||||||||||||||||||||  |||||||| || |||||||||
Sbjct:   471 TTGCTGGACCACATTCAATCATTGGCCGTGCTGTTGTCGTCCATGGTGA-TGCTGATGA
528
```

APPENDIX G

tBLASTx SEQUENCE ANALYSIS FOR RT-PCR PRODUCT GENE

```
gb|DQ244645.1| Zea mays clone 9859 mRNA sequence
Length=793
Score =  112 bits (240),  Expect = 1e-23
 Identities = 45/53 (84%), Positives = 48/53 (90%), Gaps = 0/53
(0%)
 Frame = +2/+3


Query  11   VHGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            VHGA ED IRHAG LGNVTAGADGVANINVTDCHIPL GP+SI+GRAV VHG+
Sbjct  345  VHGAPEDEIRHAGDLGNVTAGADGVANINVTDCHIPLTGPNSIVGRAVVVHGD
503


gb|DQ246113.1| Zea mays clone 19377 mRNA sequence
Length=693

Score = 97.3 bits (206),  Expect = 6e-19
 Identities = 39/52 (75%), Positives = 42/52 (80%), Gaps = 0/52
(0%)
 Frame = +2/+2

Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            HGA ED  RHAG LGNVTAG DGV N+N+TD  IPLAGPHSIIGRAV VH +
Sbjct  281  HGAPEDEDRHAGDLGNVTAGEDGVVNVNITDSQIPLAGPHSIIGRAVVVHAD
436


gb|DQ244320.1| Zea mays clone 4819 mRNA sequence
Length=793

Score = 97.3 bits (206),  Expect = 6e-19
 Identities = 39/52 (75%), Positives = 42/52 (80%), Gaps = 0/52
(0%)
 Frame = +2/+1

Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            HGA ED  RHAG LGNVTAG DGV N+N+TD  IPLAGPHSIIGRAV VH +
Sbjct  355  HGAPEDEDRHAGDLGNVTAGEDGVVNVNITDSQIPLAGPHSIIGRAVVVHAD
510


gb|AY103754.1| Zea mays PCO107770 mRNA sequence
Length=910
```

```
Score = 97.3 bits (206),  Expect = 6e-19
 Identities = 39/52 (75%), Positives = 42/52 (80%), Gaps = 0/52
(0%)
 Frame = +2/+1

Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            HGA ED  RHAG LGNVTAG DGV N+N+TD  IPLAGPHSIIGRAV VH +
Sbjct  403  HGAPEDEDRHAGDLGNVTAGEDGVVNVNITDSQIPLAGPHSIIGRAVVVHAD
558


gb|EF495351.1|  Pennisetum glaucum Cu-Zn superoxide dismutase
mRNA, complete
cds
Length=798

Score = 97.3 bits (206),  Expect = 6e-19
 Identities = 41/52 (78%), Positives = 43/52 (82%), Gaps = 0/52
(0%)
 Frame = +2/+1

Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            HGA ED  RHAG LGNVTAGADGVANINVTD  IPL GP+SIIGRAV VH +
Sbjct  319  HGAPEDENRHAGDLGNVTAGADGVANINVTDSQIPLTGPNSIIGRAVVVHAD
474

gb|L32834.1|ASNCZSD  Aspergillus japonicus (clone Assod4-g)
copper/zinc-superoxide
dismutase gene, 3' end
Length=393

Score = 92.7 bits (196),  Expect = 1e-17
 Identities = 37/52 (71%), Positives = 42/52 (80%), Gaps = 0/52
(0%)
 Frame = +2/+2

Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            HGA +D  RHAG LGN+TAGADGVAN+NV+D  IPL G HSIIGRAV VH +
Sbjct  107  HGAPQDENRHAGDLGNITAGADGVANVNVSDSQIPLTGAHSIIGRAVVVHAD
262


dbj|D01000.1|RICSODB Oryza sativa mRNA for copper/zinc-superoxide
dismutase, complete
cds, clone:RSODB
Length=667

Identities = 37/52 (71%), Positives = 42/52 (80%), Gaps = 0/52
(0%)
 Frame = +2/+1


Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
```

```
            HGA +D  RHAG LGN+TAGADGVAN+NV+D  IPL G HSIIGRAV VH +
Sbjct  223  HGAPQDENRHAGDLGNITAGADGVANVNVSDSQIPLTGAHSIIGRAVVVHAD
378
```

emb|X17564.1|ZMSOD4A Zea mays mRNA for superoxide dismutase-4A (Sod4A gene)
Length=691

```
Score = 97.3 bits (206),  Expect = 6e-19
 Identities = 41/52 (78%), Positives = 43/52 (82%), Gaps = 0/52
(0%)
 Frame = +2/+1

Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            HGA ED  RHAG LGNVTAGADGVANINVTD  IPL GP+SIIGRAV VH +
Sbjct  256  HGAPEDENRHAGDLGNVTAGADGVANINVTDSQIPLTGPNSIIGRAVVVHAD
411
```

gb|AF479059.1| Sandersonia aurantiaca copper/zinc superoxide dismutase mRNA,
complete cds
Length=799

```
Score = 89.5 bits (189),  Expect = 1e-16
 Identities = 36/52 (69%), Positives = 38/52 (73%), Gaps = 0/52
(0%)
 Frame = +2/+2

Query  14   HGASEDXIRHAGFLGNVTAGADGVANINVTDCHIPLAGPHSIIGRAVXVHGE
169
            HGA ED  RHAG LGNVTAG DG  N   +DC IPL GPHSIIGRAV VH +
Sbjct  227  HGAPEDENRHAGDLGNVTAGEDGNVNFTTSDCQIPLTGPHSIIGRAVVVHAD
382
```

gb|DQ058108.1| Oryza sativa (indica cultivar-group) cytoplasmic copper/zinc-superoxide
dismutase mRNA, complete cds
Length=698

APPENDIX H

PREPARATION OF SEPARATİNG AND STACKİNG GEL SOLUTIONS

Prepare seperating monomer solution by combining (for 5 ml):

1.65 ml dH_2O
1.25 ml seperating gel buffer
2 ml acrylamide-bis solution (30% T)
25 µl APS (10%)
2.5 µl TEMED

Prepare stacking monomer solution by combining (for 2.5 ml):

17 ml dH_2O

0.315 ml stacking gel buffer

0.415 ml acrylamide-bis solution (30% T)

10 µl APS (10%)

2.5 µl TEMED