AN ABSTRACT OF THE DISSERTATION OF

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Title: Overexpression of Antioxidant Genes in Transgenic Tomato Tolerant against Multiple Stresses.

Abstract approved:

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The effect of increased manganese superoxide dismutase (Mn-SOD) and cytosolic ascorbate peroxidase (cAPX) on various abiotic stresses was studied using transgenic tomato (*Lycopersicon esculentum*) plants. Transformants were selected *in vitro* based on kanamycin resistance, confirmed by PCR and northern blot analyses. Northern blots showed an enhanced gene expression of Mn-SOD and cAPX in transgenic *Mn-SOD* and *cAPX* plants, respectively. APX native gel assay showed APX activity in transgenic *cAPX* plants several fold greater than in wild-type (WT) plants in the absence of stress. An additional Mn-SOD enzyme activity band was detected by native PAGE in transformed *Mn-SOD* plants.

Germination of *cAPX* tomato plant seeds at low temperature (9 °C) was greater than of WT seeds. Leaf tissues of the *transgenic cAPX* plants had lower electrolyte leakage in response to cold temperatures (4 °C) than leaf tissues of WT plants. Transgenic *Mn-SOD* plants also showed resistance to the superoxide-generating herbicide methyl viologen (MV, 10^{-4} M). The total SOD activity was

1.5- to 2-fold higher and APX activity was 6- to 7-fold higher in transgenic *Mn-SOD* plants than in WT plants under MV stress, respectively. Germination of *Mn-SOD* tomato plant seeds in the presence of 150 mM NaCl was greater than in seeds from WT plants. Transgenic *Mn-SOD* shoots developed more roots under salt stress (200 mM and 250 mM NaCl, respectively). Both transgenic *Mn-SOD* and *cAPX* plant seedlings had enhanced ability to tolerate NaCl stress (200 mM and 250 mM). Total APX activity of transgenic plants was several fold higher than that of WT under salt stress (200 mM NaCl).

Leaves and fruits from *Mn-SOD* or *cAPX* transgenic tomato plants were more resistant to heat (40 °C) and UV-B (2.5 mW/cm^2 for 5 days) stresses compared to WT plants, respectively. Detached fruits from transgenic plants showed more resistance to sunscald in a field test. APX activity in leaves of the transgenic *cAPX* plants was several fold higher than that of WT plant leaves when exposed to the heat, drought, and UV-B stresses.

Overexpression of Antioxidant Genes in Transgenic Tomato Tolerant against Multiple Stresses

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Yueju Wang, Author

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Dr. Leslie H. Fuchigami was involved in overseeing this work from its onset and in the discussion, design and analysis of experiments; and proofreading and critical editing each of manuscript. Dr. Michael Wisniewski was involved in support, cAPX construct donation, data interpretation, and writing of each manuscript. Molecular and protein analysis was performed in the laboratory of Dr. Richard Meilan, who also contributed his knowledge and assisted in editing each manuscript. Minggang Cui assisted with plant stress experimental design, laboratory work and data collection. Dr. Abahaya M. Dandekar kindly provided Mn-SOD construct. Dr. Charles D. Boyer provided support and consul for my Ph.D. program.

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LIST OF ABBREVIATIONS

AA	ascorbic acid (vitamin C)
APX	ascorbate peroxidase
Chl	chlorophyll
cDNA	complementary DNA
Cu/Zn-SOD	copper/zinc superoxide dismutase
DHA	dehydroascorbic acid
DHAR	dehydroascorbate reductase
Fe-SOD	iron superoxide dismutase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulphide
MDHA	monodehydro-ascorbic acid
MDHAR	monodehydro-ascorbate-reductase
Mn-SOD	manganese superoxide dismutase
mRNA	messenger RNA
MV	methyl viologen (paraquat)
NADPH	reduced nicotinamide ademine dinucleotide phosphate
ROS	reactive oxygen species
SOD	superoxide dismutase
WT	wild-type plant

Overexpression of Antioxidant Genes in Transgenic Tomato Tolerant against Multiple Stresses

Chapter 1

Introduction and Literature Review

1.1 Oxidative Stress as Related to Plant Environmental Stress

Tomatoes (*Lycopersicon esculentum*) are of particularly great economic importance being the second most commonly grown vegetable crop in the world. The demand for tomatoes has increased substantially worldwide (Jones, 1999). In fact, tomatoes as sliced fruit and various sauces are among the fastest growing sectors in the vegetable industry. From a nutritional perspective, tomatoes are good sources of potassium and offer pro-vitamin A, vitamin C, lycopene, tomatine, some calcium and iron (Jones, 1999).

Tomato plants are susceptible to environmental stresses such as chilling (Karpinski et al., 2002) and heat (Picken, 1984). Tomato fruit suffers from sunscald-type lesions, thought to result from high light and high temperature stresses (Rabinowitch and Sklan, 1980; Renquist et al., 1989), with wide ranges of growth and yield losses found between cultivars. Much of the environmental stress damage is associated with oxidative damage at the cellular level.

Abiotic (extreme temperatures, salinity, and drought; Boyer, 1982) and biotic (pests and diseases; Dempsey et al., 1998) stresses can severely restrict the

geographic distribution of plants and limit crop yields. Plants may also be stressed by air pollutants (Robinson et al., 1998) and ultraviolet (UV) radiation (A-H-Mackerness, 2000). Plants are aerobic organisms that require oxygen for survival, but oxygen is inherently dangerous to plants, because it can be readily reduced to reactive oxygen species (ROS). ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), are associated with a number of physiological disorders in plants (Allen, 1995; Blokhina, et al., 2003; Bowler et al., 1992). Oxidative stresses may be induced by such environmental stresses as high temperatures (Sairam et al., 2000), ultraviolet radiation (A-H-Mackerness, 2000), pathogens (Mittler et al., 1998), freezing (Martinez et al., 2001), and chemicals (Donahue et al., 1997; Gossett et al., 1996; Iturbe-Ormaetxe et al., 1998). Oxidative stress can have a significant negative impact on crop production (Gosset et al., 1996; Hernández et al., 1999; Wise and Naylor, 1987).

ROS can induce deleterious functions in cells, such as lipid peroxidation, DNA mutation, and protein denaturation (Foyer et al., 1994; Scandalios, 1993, 1997). To maintain normal growth and prevent oxidative stress, ROSs need to be sequestered and eliminated from cells. Plants have developed both enzymatic [e.g., superoxide dismutase, (SOD, EC 1.15.1.1) and ascorbate peroxidase (APX, EC 1.11.1.11)] and non-enzymatic (such as ascorbate and glutathione) defense systems for protection against oxidative damage. SOD and APX have been identified as essential components in the organism defense mechanisms and have consequently been the subject of much research (Allen, 1995; Scandalios, 1993).

1.2 Reactive Oxygen Species and Oxidative Damage

Stresses that result in the production of toxic oxygen species are called oxidative stresses. Reactive oxygen species identified in living organisms are shown in Table 1.1.

Reactive Oxygen Species	Structure
Hydrogen peroxide	H_2O_2
Hydroxyl radical	$OH^{\cdot}$
Peroxyl radical	$HOO^{\cdot}$
Singlet oxygen	$^1O_2$
Superoxide anion	$O_2^{\cdot-}$

Table 1.1. Nomenclature for various reactive oxygen species.

Ground-state dioxygen is rather unreactive because dioxygen has two unpaired electrons with paralleled spins, imposing an energetic barrier to its reaction with non-radical compounds (the 'spin restriction') (Elstner, 1982). After physical or chemical activation (Perl-Treves and Perl, 2002), oxygen could be reduced sequentially to either superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($OH^{\cdot}$), all of which could cause biologically toxic effects on cellular systems. Metal ions are mainly present in cells in the oxidized form (Fe^{3+}). They are reduced in the presence of $O_2^{\cdot-}$ and, consequently, may catalyze

the conversion of H_2O_2 to HO^- by the Fenton or Haber–Weiss reactions (Fig. 1.1) (Vranová, et al., 2002).

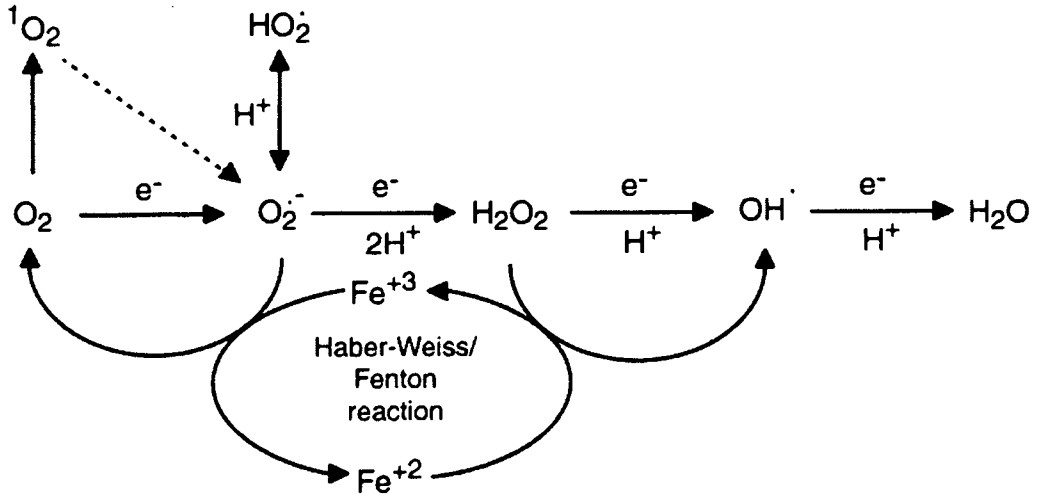

Figure. 1.1. The successive reduction of molecular oxygen to form superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and water (Vranová, et al., 2002).

Superoxide

Superoxide (O_2^-) is the first reduction product of ground-state oxygen. It can act as either an oxidant or a reductant. It is formed upon the reduction of one electron from oxygen, mediated by enzymes such as NADPH oxidase or xanthine oxidase, or from the respiratory chain. It may react to the form H_2O_2 and oxygen either spontaneously or by enzymatic dismutation (Asada, 1992, 1997).

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is the first stable ROS produced in the plant cell. H_2O_2 is not a free radical but reacts as either an oxidant or reductant in many

cellular reactions. H_2O_2 readily permeates membranes and aqueous compartments. It can diffuse over considerable distances and may inactivate sensitive enzymes at low concentrations. In the presence of metal reductants such as iron and copper, H_2O_2 can be converted to form the highly reactive hydroxyl radical (Vranová, et al., 2002).

Hydroxyl Radical

The hydroxyl radical ($OH^{\cdot}$) is the most reactive oxygen species and is responsible for modifications of all kinds of biological macromolecules and damage to living cells (Asada, 1997). $OH^{\cdot}$ reacts with organic substrates to form hydroxylated products that are further oxidized to stable, oxidized products. No specific scavenger is known in plants that can stop the action of $OH^{\cdot}$.

ROS can induce oxidative injury that causes cell death. It initiates a cascade of reactions that readily oxidize lipids, proteins and nucleic acids. ROS cause the polyunsaturation of lipids, resulting in their peroxidation. Lipid peroxidation in a cell can initiate chain reactions, seriously destroying membrane function. Lipid peroxidation can be divided into three stages: initiation, propagation and termination (Shewfelt and Purvis, 1995). The initiation phase includes activation of O_2, which produces hydroxyl radicals, followed by the desaturation of fatty acids (e.g., linoleate). Finally, $OH^{\cdot}$ acquires an H atom from the methylvinyl group on the fatty acid (reaction 1 following). The propagation phase includes the carbon radical product ($R^{\cdot}$) reacting with triplet oxygen to form a peroxyl radical

(reaction 2 following). The peroxyl radical formed is highly reactive and is able to propagate the chain reaction (reactions 2 and 3). Antioxidants can react with the chain-propagating radical and terminate the reaction.

$OH^- + RH \text{ -----> } R^{\cdot} + H_2O$	Initiation	(reaction 1)
$R^{\cdot} + O_2 \text{ ----- > } ROO^{\cdot}$	Propagation	(reaction 2)
$ROO^{\cdot} + RH \text{ ---- > } R^{\cdot} + ROOH$	Chain	(reaction 3)

Lipid peroxidation causes the loss of membrane integrity, which is among the key factors involved in the abiotic stress of plants (Blokhina et al., 2003; Chirkova et al., 1998). ROS can alter the purine and pyrimdine bases and the deoxyribose sugar, cleaving the phosphodiester backbone. This can cause a break in the DNA chain, resulting in deletions, mutations and other genetic defects. The hydroxyl ions can also cause cross-linking between DNA and proteins, resulting in potentially lethal cell damage (Scandalios, 1997).

Proteins may also be damaged by ROS, leading to structural changes and loss of enzyme activity. ROS attack proteins at many reactive sites during oxidative stress. ROS, such as OH^-, can fragment proteins in the plasma membrane. This fragmentation is associated with reactions at specific amino acids such as proline and histidine. Proteins may contain metal-binding sites that are especially susceptible to oxidative events through interaction of ROS with metals (Gardner and Fridovich, 1991). These reactions usually produce irreversible modifications in amino acids that might be involved in metal ion binding, e.g., histidine. Intracellular proteins have "reactive" sulfhydryl groups on specific cysteine residues that can be modified (oxidized) to specific forms (disulfides) that can be

reduced again by metabolic processes (Farr and Kogoma, 1991). Activated oxygen can extract a hydrogen atom from cysteine residues to form a thiol radical that will cross-link to a second thiol radical to form a disulphide bridge. Most protein degradation processes are irreversible (Gardner and Fridovich, 1991).

1.3 Sites of Reactive Oxygen Species in Plant

Sites such as chloroplasts, mitochondria, endoplasmic reticulum, and peroxisomes can produce ROS in plant cells (Elstner and Osswald, 1994). Some ROS are produced during normal metabolism (Vranová et al., 2002). Major sources of ROS, such as chloroplasts and mitochondria, are described next.

Production of ROS in Chloroplasts

In plant cells, the major sources of ROS are the chloroplasts. They harvest light energy, produce reductant, such as NADP, and form singlet oxygen and superoxide radicals (Elstner and Osswald, 1994; Salin, 1987). By the Mehler reaction, photosystem I (PSI) can reduce oxygen. Without enough electron acceptors, e.g. when the Calvin cycle can not consume enough NADPH, PSI is thought to significantly contribute to the production of free radicals such as $O_2^{\cdot}$, which may dismutate to H_2O_2 and directly inhibit CO_2 fixation (Bowler et al., 1992; Foyer et al., 1992). Evidence also indicates that photosystem II (PSII), under high-light intensities, produces $O_2^{\cdot}$ and H_2O_2 (Landgraf et al., 1995). Photoactivated chlorophyll can also excite oxygen from the triplet to the singlet form when the captured light energy is not consumed in the electron transport

systems. This can occur when the plants are under environmental stresses such as drought, heat, and herbicides (Elstner and Osswald, 1994; Foyer, 2002).

Production of ROS in Mitochondria

The mitochondrial respiratory chain is a powerful source of ROS (Poyton and McEwen, 1996). Stresses that increase ROS in plants also induce alternative path respiration. This has led to the hypothesis that this type of respiration may function to mitigate ROS damage in plant cells (Bowler et al., 1991; Mackenzie and McIntosh, 1999). Oxygen is normally reduced to water by cytochrome oxidases in the electron transport chain of plant mitochondria. When oxygen receives only one electron, the free radical superoxide is formed. This, in turn, can form a number of other reactive of species (Elstner and Osswald, 1994). Plant mitochondria also have a specific location, namely the cyanide-insensitive alternative site, at which H_2O_2 and O_2^- are produced (McKersie and Leshem, 1994).

Production of ROS at Other Sites

Other sites, such as microsomes, peroxisomes, and cell walls, can also produce ROS (Elstner and Osswald, 1994). Microsomes are derived from the endoplasmic reticulum. Various oxidative processes, including oxidation, hydroxylations, and desaturation, occur on the smooth endoplasmic reticulum (McKersie and Leshem, 1994). Peroxisomes and glyoxysomes contain xanthine oxidase as an O_2^-- and H_2O_2-producing enzyme in addition to glycolate oxidase

and fatty acid β-oxidase as H_2O_2-producing enzymes. In the plasma membrane and the apoplast compartment, superoxide-generating NAD(P)H oxidase activity has been clearly identified. ROS is also produced by pathways enhanced during abiotic stresses, such as glycolate oxidase in peroxisomes during photorespiration (del Río et al., 2002). NADPH oxidases, amine oxidases and cell wall-bound peroxidases are also found to be regulated and participate in the production of ROS during processes such as programmed cell death and pathogen defense (Mittler, 2002).

1.4 Defense Against Oxidative Systems

Plants have developed enzyme and non-enzyme systems to defend themselves against ROS produced under normal and stressful conditions (Allen, 1995; Blokhina et al., 2003; Bowler et al., 1992). Enzyme systems include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and catalase (CAT). Non-enzyme systems include water- or lipid-soluble molecules, such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, and reduced glutathione (GSH).

1.4.1 Enzyme Antioxidant Systems

Superoxide Dismutase

Superoxide dismutase is one of the most important enzymes in the front line of defense against oxidative stress (Alscher et al., 2002). The enzyme is present in

all aerobic organisms and in all subcellular compartments susceptible to oxidative damage (Bowler et al., 1992). SODs are a group of metal-containing enzymes and are classified into three types according to their metal cofactor requirements: iron SOD (Fe-SOD), which is localized solely in the chloroplast; copper/zinc SOD (Cu/Zn-SOD), which is localized in the chloroplast, the cytosol, and possibly the extracellular spaces; and manganese SOD (Mn-SOD), which is found mainly in the mitochondria and peroxisome (Alscher et al., 2002; Bowler et al., 1992). SOD catalyzes dismutation of two superoxide anions (O_2^-) into H_2O_2 and O_2. This reaction has a 10,000-fold faster rate than spontaneous dismutation (Bowler et al., 1992; Elstner and Osswald, 1994). The reaction is as follows:

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

SODs are also found in plant glyoxisomes and in the extracellular spaces (Alscher et al., 2002). SODs are differentially regulated in response to diverse environmental stresses including paraquat (Iannelli et al., 1999; Iturbe-Ormaetxe et al., 1998); UV-B (Balakumar et al., 1997); heat (Sairam et al., 2000); salt (Hernández et al., 1995, 2000; Sairam and Srivastava, 2002; Sreenivasulu et al., 2000); flooding (Ushmaru et al., 2001); drought (Mittler and Zilinskas, 1994; Zhang and Kerkham, 1996); chilling (Pastori et al., 2000; Pinhero et al., 1997); and ozone (Noormets et al., 2000). All three enzymes are nuclear encoded. Genes SODs have been cloned from many plant species, such as tomato (Galun and Ori, 1995); pineapple (Lin et al., 2000); mustard (Liu et al., 1998); cotton (Kim and Triplett, 1998); rubber (Miao and Gaynor, 1993); maize (Zhu and Scandalios,

1993); rice (Kaminaka et al., 1999); wheat (Wu et al., 1999); and *Arabidopsis* (Abarca et al., 1999). The effects of SOD overexpression in transgenic plants have been reported to induce resistance to paraquat (Bowler et al., 1991; Perl et al., 1993; Sen Gupta et al., 1993a, b; Slooten et al., 1995; Van Camp et al., 1996); ozone (Pitcher and Zilinskas, 1996; Van Camp et al., 1994); low temperature (McKersie et al., 1993, 1996, 1999); salt stress (Tanaka et al., 1999); and other stresses (Allen, 1995; Alscher et al., 2002; Bowler et al., 1992).

Ascorbate Peroxidase

Ascorbate peroxidase exist as isoenzymes and play an important role in the metabolism of H_2O_2 in higher plants. APX catalyzes the conversion of H_2O_2 to water, with ascorbate serving as the electron donor (Asada, 1992). The reaction is as follows:

$$\text{Ascorbate} + H_2O_2 \xrightarrow{\text{APX}} \text{dehydroascorbate} + 2H_2O$$

Together with SOD, APX participates in the primary defense against oxidative toxicity. APX has been identified in many higher plants (Shigeoka et al., 2002). cDNAs encoding various APX isoenzymes have been isolated from various plant species and are well characterized (Caldwell, et al., 1998; Gadea et al., 1999; Santos et al., 1996; Yamaguchi et al., 1996). APX isoenzymes are classified according to their subcellular localization. These sites include the stroma (sAPX) and thylakoid membrane (tAPX) in chloroplasts; microbodies (mAPX); the cytosol (cAPX) (Bunkelmann and Trelease, 1996; Chen and Asada, 1989;

Ishikawa et al., 1991, 1998; Miyake et al., 1993; Yamaguchi et al., 1995a,b); and the mitochondrial membrane (mitAPX) (Leonardis et al., 2000). APX isoenzymes differ with regard to properties such as molecular weight, electron donor specificity, lability in the absence of ascorbate, and affinity for ascorbate and H_2O_2. Nevertheless, APX isoenzymes show 50% to 70% amino acid homology with each other (Shigeoka, 2002).

In general, APX activity increases in plants exposed to various environmental stresses, and this elevated activity correlates with increases in the activity of other antioxidant enzymes, such as CAT, SOD, and GR, suggesting that the components of ROS-scavenging systems are co-regulated (Allen, 1995; Pastori and Trippi, 1992; Sen Guota, 1993; Shigeoka et al., 2002).

Glutathione Reductase

Glutathione reductase (EC 1.6.4.2) is a flavoprotein that catalyzes the reduction of oxidized glutathione (GSSG) to gluthione (GSH) using NADPH as the reducing cofactor according to the following reaction:

$$GSSG + NADPH \xrightarrow{GR} 2GSH + NADP^+$$

GR is necessary for maintaining high GSH/GSSG ratios in cells. High GR maintains the pool of glutathione in the reduced state, allowing GSH to be used by dehydroascorbate reductase (DHAR) to reduce dehydroascorbate (DHA) to ascorbate (AA) (Foyer and Halliwell, 1976). GSH plays important roles in a variety of cellular functions, including protection against oxidative stress,

detoxification of xenobiotics and heavy metals, sulfur metabolism, and regulation of gene expression (Mullineaux and Creissen, 1997; Noctor and Foyer, 1998). GR activity is a key enzyme in the ascorbate-glutathione pathway, which operates to scavenge active oxygen species produced under both normal and stress conditions (Foyer and Halliwell, 1976). GR genes have been isolated from a variety of organisms including several plant species, such as pea and *Arabidopsis* (Kubo et al., 1998; Mullineaux et al., 1996). Like SOD and APX, GR activities increase in response to environmental stresses, such as chilling, salt, ozone, and paraquat (Allen, 1995; Anderson et al., 1995; O'Kane et al., 1996; Sairam and Srivastava, 2002; Strohm et al., 1999). Overexpression of GR genes has resulted in transgenic plants that are more resistant to paraquat, sulfur dioxide, and ozone than control plants (Aono et al., 1995; Foyer et al., 1994, 1995, 1997; Strohm et al., 1999).

For plant survival under stressful conditions, antioxidants that work together provide better defense and regeneration of active, reduced forms (Noctor and Foyer, 1998). The ascorbate-glutathione cycle, also called Foyer-Halliwell-Asada cycle (Fig. 1.2), is an efficient way for plant cells to dispose of H_2O_2 in certain cellular compartments where this metabolite is produced and no CAT is present (Halliwell and Gutteridge, 2000). This cycle makes use of the non-enzymatic antioxidants ascorbate and glutathione in a series of reactions catalysed by four antioxidative enzymes: APX, MDHAR, DHAR, and GR. MDHAR catalyses the

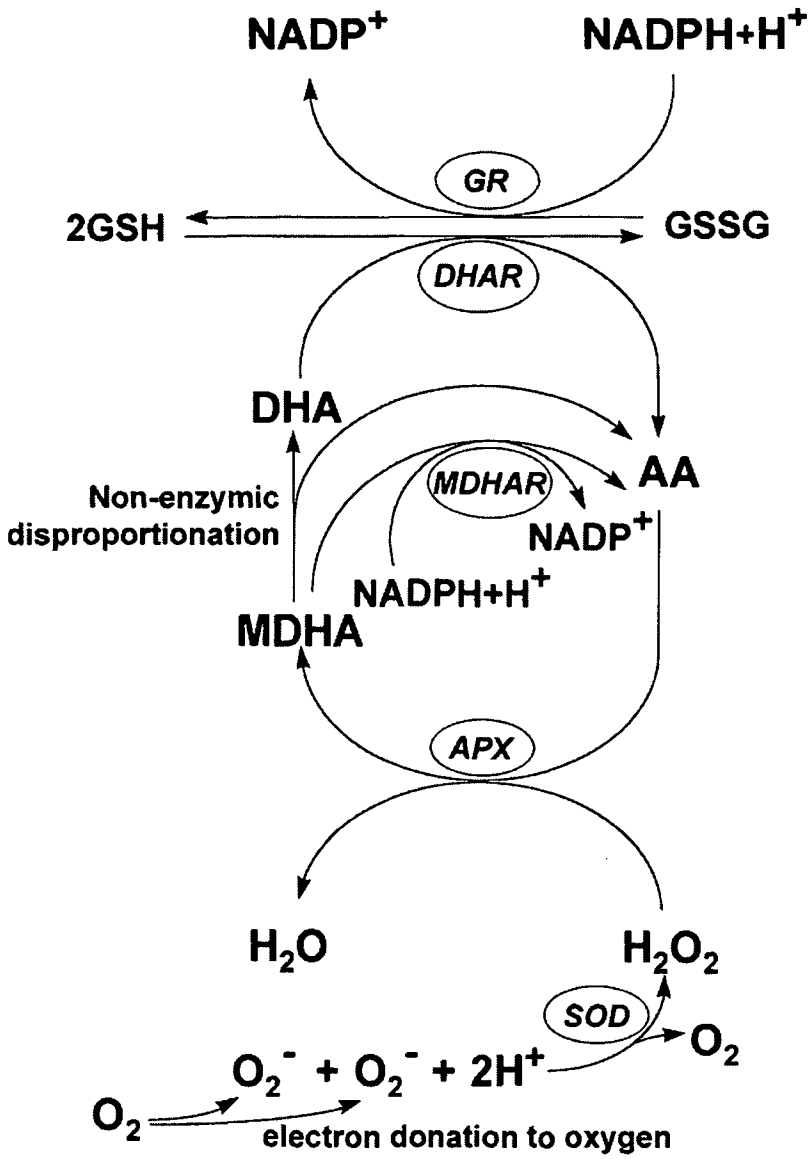

Figure. 1.2. The ascorbate-glutathione cycle (Noctor and Foyer, 1998).

reduction of monodehydroascorbate (MDHA) by NADPH. Alternatively, MDHA can dissociate into ascorbate and dehydroascorbate (DHA); the latter is re-reduced by DHAR, with GSH as an electron donor; GR then re-reduces the oxidized glutathione back to its reduced form (Halliwell and Gutteridge, 2000).

Catalase

Catalase (EC 1.11.1.6) is a tetrameric heme-containing enzyme found in plant peroxisomes and glyoxisomes (Willekens et al., 1995). Together with APX, catalase is important in the removal of H_2O_2 or oxidized substrates, such as methanol, formaldehyde and formic acid. It is one of the major enzyme components of cellular mechanisms of defense against oxidative stress, catalyzing the conversion of two molecules of H_2O_2 to H_2O and O_2 as shown in the following reaction (Scandalios, 1997).

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

For plant CAT, several cellular roles have been described. CAT neutralizes H_2O_2, which is produced during photorespiration and acylCoA oxidation of fatty acids (Willekens et al., 1995). CAT exists in different forms in different plant species. These forms have been cloned from maize (Scandalios, 1990); winter rye (Schmidt and Feierabend, 2000); cotton (Ni, 1990); pea (Isin and Allen, 1991); and rice (Morita et al., 1994). CAT isozymes are rapidly induced by UV-B; ozone (Willekens et al., 1994); chilling (El-Saht, 1998); and salt (Bueno et al., 1998). It

has been suggested that CAT play a role protecting cells against pollutant stress (Willekens et al., 1994).

Peroxidase

Peroxidase (POX; EC 1.11.1.7) is an oxidoreductase that is known to catalyze the oxidation of numerous substrates through the associated reduction of hydrogen peroxide (Asada, 1992). POX is a heme-containing glycoprotein encoded by a large multigene family in plants. In plants, the main roles of peroxidase are attributed to lignification, defense against pathogen attack, and the possible cross-linking of cell wall components (Hiragal et al., 2001; Ye et al., 1990). Together with CAT, POX plays an important role in the regulation of ROS concentration in the cell (Foyer et al., 1994).

1.4.2 Non-enzyme Antioxidant Systems

Ascorbic acid (AA, vitamin C) is an important antioxidant in plant and animal systems (Noctor and Foyer, 1998). It has been shown to react with hydrogen peroxide, superoxide and hydroxyl radicals (Nocto and Foyer, 1998). All plants can synthesize AA. AA has an essential role in several physiological processes in plants and is implicated in root elongation (Cordoba-Pedregosa et al., 1996); cell vacuolarization (McCann and Roberts, 1994); cell wall expansion and the cell cycle (Potter et al., 2000). AA can directly scavenge oxygen free radicals with and without enzyme catalysts, and can indirectly scavenge them by recycling α-tocopherol (vitamin E) to the reduced form (Arrigoni, 1994).

Tocopherols are essential components that have both antioxidant and non-antioxidant functions on membranes (Fryer, 1992). The major isomer of vitamin E is α-tocopherol, a phenolic antioxidant present in both plants and animals (Bramley et al., 2000). Vitamin E is a lipid-soluble molecule that can quench both singlet oxygen and peroxides, resulting in the scavenging of oxygen free radicals, lipid peroxy radicals, and singlet oxygen (Blokhina et al., 2003).

Carotenoids, lipid-soluble molecules located in both photosynthetic and non-photosynthetic plant tissues, can protect plants against oxidative damage (Inzé and Van Montagu, 2002). Carotenoids function as accessory pigments in light harvesting and detoxify various forms of activated oxygen and triplet chlorophyll produced during photosynthesis (Young, 1991).

Glutathione is a tripeptide (Glu-Cys-Gly). It is an important component of the ascorbate-glutathione cycle and participates in the removal of H_2O_2, which may accumulate during high-temperature-induced oxidative stress (Noctor and Foyer, 1998). It can react chemically with either singlet oxygen, O_2^-, or OH^-, functioning as a free radical scavenger (Alscher, 1989). GSH is widely distributed in organisms and has diverse functions in protein synthesis, sulfur storage, and protection against a variety of stresses. During oxidative stress, GSH is oxidized and regenerated by GR (Noctor and Foyer, 1998).

1.5 Environmental Stresses and the ROS-scavenging Systems

Plant cells can produce ROS in cells under both non-stressed and stressed conditions (Allen, 1995; Alscher et al., 2002; Scandalios, 1993). Under non-

stressed conditions, the formation and removal of ROS is kept in balance by the plant antioxidant defense systems (Alscher et al., 2002). When plants are under stress from herbicides, UV-B, salt, heat, chilling, and disease, a rise in several antioxidant enzymes activities, such as SOD, APX, and CAT is often observed (Allen, 1995; Alscher et al; 2002; Foyer et al., 1994). These antioxidants enzymes are not able to remove the most abundant ROS under severe stress conditions, though, they are able to alleviate oxidative damage to some degree. The effects of some environmental stresses in relationship to levels of ROS and levels of antioxidants are introduced next.

Heat, UV-B, and Sunscald

Heat stress is one of the most important constraints on crop production in tropical and subtropical regions, and the situation is growing worse due to global warming (Egawa et al., 2002). Heat can damage cellular structures and impair membrane functions. It also causes a decrease in normal protein synthesis (Scharf et al., 1998). Tomato plants have a well documented but not well understood sensitivity to high temperature (Picken, 1984). Heat, like many other environmental stresses, appears to elicit an oxidative stress in tomatoes. ROS can induce the accumulation of small heat shock proteins during heat stress, which may enhance thermotolerance in tomato cells (Banzet et al., 1998). Rainwater et al. (1996) found a strong correlation between antioxidant activity and tolerance to heat stress in tomatoes. They found that the more heat-tolerant tomato cultivars had an enhanced capacity for scavenging active oxygen species, and a more active

ascorbate-glutathione cycle. Heat shock in rice can elevate APX gene expression and cause cross-tolerance to chilling injury (Sato et al., 2001). Oxidative and antioxidant enzymes play a major role in heat-induced cell death (Davidson et al., 1996). Yeast mutants, from where the antioxidant genes catalase and superoxide dismutase were removed, were more sensitive to heat shock. In contrast, cells overexpressing antioxidant genes were more resistant to lethal heat shock (Davidson et al., 1996).

UV-B (280-320 nm) is high-energy solar radiation. Its potential for causing biological damage is exceptionally high. UV exposure resulted in the generation of intracellular ROS and lead to oxidative stress (A-H-Mackerness, 2000). Research on cyanobacteria revealed that moderate UV-B radiation resulted in an oxidative stress, which resulted in lipid peroxidation, DNA strand breaks, and chlorophyll bleaching (He et al., 2002). UV-B radiation enhances the activated oxygen species by increasing membrane-localized NADPH-oxidase activity and decreasing catalase activity (Rao et al., 1996). Studies on barley showed that solar UV-B caused measurable DNA damage, growth inhibition, and yield loss (Mazza et al., 1999). Lipid peroxidation in the leaves of UV-B-treated tomato plants was 32% higher when compared to controls (Balakumar et al., 1997). UV-B treatment can induce the accumulation of free radical-scavenging enzymes, such as SOD and APX, indicating that the plants responded to UV-B by expressing an oxidative stress response (Foyer et al., 1994). Mazza et al. (1999) reported that UV-B increases CAT and APX activity in barley plants. Tomato plants grown under field conditions with supplemental UV-B exposure showed SOD and CAT

activities that were elevated by 126% and 50%, respectively (Balakumar et al., 1997). Other studies, carried out under laboratory or greenhouse conditions, detected changes in antioxidant enzymes in UV-B-treated plants at the mRNA and protein levels (Rao et al., 1996; Willekens et al., 1994). UV-B treatment of *Arabidopsis thaliana* induced new isoforms of POX, APX, and Cu/Zn-SOD (Rao et al., 1996).

The interaction of high temperatures and high light intensity (UV-B radiation) can induce sunscald in the fruit of many horticulture crops (Rabinowitch et al., 1983, Renquist et al., 1989). Sunscald of fruits results from injury to the affected cells. In most cases, there is tissue browning or desiccation (Renquist et al., 1989). Tolerance to heat and UV-B stresses correlates with an increased capacity to scavenge or detoxify activated oxygen species (Chaitanya et al., 2002; Davidson et al., 1996; Mazza et al., 1999; Sairam et al., 2000) suggesting that increased antioxidant enzyme activity might protect against sunscald.

Artificial induction of tolerance to sunscald damage in tomato fruit by controlled heat treatment was accompanied by an increase in SOD activity (Rabinowitch and Sklan, 1980; Rabinowitch et al., 1982). Rabinowitch and Sklan (1980) reported that SOD activity levels were high in immature green tomato fruits and declined to a minimum in the mature-green and breaker stages, which are known to be most susceptible to sunscald damage (Retig and Kedar, 1967). Research on tomato ripening finds that oxidative processes, such as lipid peroxidation, protein oxidation, and H_2O_2 content, increase at the breaker stage. Activity of antioxidant enzymes, such as SOD, CAT, APX and GR, was found to

decrease at the breaker stage, which is more susceptible to sunscald (Jimenez et al., 2002).

In apple (*Malus domestica* Borkh. cv. Fuji), the hypothesis is that photooxidative injury to the peel leads to the production of free radicals. These damage cellular membranes and cause associated metabolic processes that result in symptoms of sunscald injury (Johnson and Andrew, 1997). Gong et al. (2001) reported that apple browning disorder was related to lower activity of SOD and CAT. Andrew and Johnson (1997) found the activity of SOD, and resulting concentrations of ascorbic acid and glutathione, were 64%, 13%, and 33% lower, respectively, in the peels of sunscald-damaged fruits than in undamaged peels. Transgenic apple plants that overexpressed cAPX have improved resistant to heat stress. Leaf disks from wild-type plants exhibited 100% electrolyte leakage, compared with 40% to 75% leakage in transgenic plants, at 40 °C for 14 hours (Wisniewski et al., 2002). Transgenic apple also showed tolerance to UV-B and freezing stress (Wisniewski et al., 2002). Overexpression of cytosolic SOD in potato increases tolerance to heat and reduces electrolyte leakage (Gusta et al., 2002). Chen and Pan (1998) reported that overexpression of Cu/Zn-SOD can enhance the tolerance to heat stress or UV-B radiation in *Arabidopsis thaliana.* These results suggest that ROS is involved in sunscald injury, and that tolerance might be acquired through increases in antioxidant enzyme activities.

Chilling and Freezing

Chilling injury is commonly observed in many species of tropical and subtropical origin, e.g. tomato, bell pepper (*Capsicum annuum*), and avocado (*Persea americana*) (Karpinski et al., 2002; Wang, 1994). Chilling injury symptoms frequently coincide with peroxidation of fatty acids (Parkin et al., 1989). Lipid peroxidation alters the physical properties of membrane lipids, and inhibits membrane-bound protein function (Shewfelt and Erickson, 1991). Chilling-induced oxidative stress occurs generally when photosynthesis is inhibited at low temperatures. At low temperatures, the energy supplied by light is in excess of that needed for photosynthesis and can lead to the production of ROS (Karpinski et al., 2002; Schoner and Krause, 1990). Free radical accumulation and lipid peroxidation are widely considered to be major contributors to chilling injury (Prasad, 1994a, b, 1996). Chilling tolerance is partly a result of an enhanced antioxidant defense system (El-Saht et al., 1998; Iannelli et al., 1999; Payton et al., 2001; Posmyk et al., 2001; Sato et al., 2001).

The activities of SOD, APX, and GR increased in chilling-treated bean seedlings. The activities of CAT and POX decreased in young chilling-treated plants and slightly increased in older ones (El-Saht, 1998). When plants are chilled, APX activity is much higher than in plants grown at higher temperatures. This suggests a requirement for greater rates of H_2O_2 detoxification at the lower temperatures (Pastori et al., 2000).

Tomato plants are very sensitive to the chilling stress (Karpinski et al., 2002). The main symptoms in tomato fruit are abnormal ripening, pitting, water-soaked

areas, and increased susceptibility to fungal infection (Morris, 1982). GR activity is greater in chilling-tolerant than in chilling-sensitive genotypes (Bruggemann et al., 1999). When exposed to cold, chilling-tolerant maize showed higher SOD and APX activity than chilling-sensitive maize (Pinhero et al., 1997). Schoner and Krause (1990) reported that chilling-acclimated spinach leaves exhibited considerably higher AA content and significantly increased activities of SOD, APX, and MDA in the chloroplast. This suggests that antioxidant enzyme activities are related to photoinhibition at cold temperatures. Overexpression of the *Arabidopsis C-repeat/dehydration response element binding factor 1* (*CBF1*) gene increases tolerance to chilling and oxidative stresses in transgenic tomato (Hsieh et al., 2002). Results suggest that heterologous *CBF1* expression in transgenic tomato plants may induce several oxidative stress-responsive genes to protect them from chilling stress (Hsieh et al., 2002). Inhibition of CAT by antisense RNA increases susceptibility to oxidative stress and chilling injury in transgenic tomato plants. This suggests that antioxidant genes play important roles in chilling tolerance (Kerdnaimongkol and Woodson, 1999).

Both freezing and paraquat treatment of winter wheat crown caused similar injury symptoms in cellular membranes, including increased formation of gel phase domains, degradation of phospholipids, and accumulation of free fatty acids (Kendall and McKersie, 1989). Martinez et al. (2001) reported a significant increase (approximately 350%) in total SOD and Fe-SOD activity in leaves of freezing-tolerant potato tissues when exposed to paraquat.

Overexpression of Mn-SOD in transgenic alfalfa increased tolerance to herbicide and increased regrowth after freezing stress (Mckersie et al., 1993). Overexpression also resulted in higher survival rates of transgenic plants (some at 100%) than in controls after one winter field trial (Mckersie et al., 1999). Wisniewski et al. (2002) reported that overexpression of *APX* in transgenic apple resulted in tolerance to freezing stress. These studies indicate that antioxidant enzyme might be involved in freezing stress resistance.

Salt Stress

Salt stress is a common environmental problem worldwide. High soil salinity can cause membrane disorganization and nutrient imbalances, resulting in the accumulation of elements toxic to plants (Zhu, 2000). As a result, much research is being done to improve salt tolerance. Zhang and Blumwald (2001) genetically modified tomato plants to overexpress the *Arabidopsis thaliana AtNHX1* antiport, which allowed plants to grow in the presence of 200 mM NaCl. Transgenic tomato plants grew, flowered, and produced seeds. Transgenic salt-tolerant tomato plants accumulate salt in the foliage but not in the fruit (Zhang and Blumwald, 2001). Plants subjected to salt stress undergo increased exposure to activated forms of oxygen and accumulation of free radicals in chloroplasts and mitochondria. Both of these conditions are associated with damage to membranes and the buildup of lipid peroxidation. All of these factors contributed to salt deleterious effects in plants (Dionisio-Sese and Tobita, 1998; Gueta-Dahan et al., 1997; Hernández et al., 1993, 1995).

Salt-dependent oxidative stress response, such as lipid peroxidation and H_2O_2 accumulation, was evident in tomato mitochondria (Mittova et al., 2003). Exposure of salt-tolerant pea plants to NaCl results in the formation of O_2^- and H_2O_2 and increases in the activity of SOD and other antioxidant enzymes, such as APX. Transcript levels for Mn-SOD, Cu/Zn-SOD, and APX were strongly induced in salt-tolerant but not in salt-sensitive pea plants (Hernández et al., 2000).

Salt-tolerant plants have higher antioxidant enzyme activity, such as SOD and APX, than salt-sensitive plants. This suggests that increased antioxidant enzyme activity could alleviate salt stress (Mittova et al., 2000; Sairam and Srivastava, 2002; Sreenivasulu et al., 2000). Researchers have found that antioxidant systems are related with salt stress resistance in rice (Dionisio-Sese and Satoshi, 1998), cotton (Rajguru et al., 1999), pea (Hernandez et al., 1999, 2001), wheat (Sairam and Srivastava, 2002), and tomato (Mittova et al., 2000).

The increase in tomato tolerance to salt-stress was associated with the antioxidant activity of AA, and a partial inhibition of salt-induced increases in lipid peroxidation by active oxygen species (Shalata and Neumann, 2001a). Shalata et al. (2001b) reported an increase in SOD activity in salt-tolerant tomato roots under salt stress. Salt stress pretreatment of tomato plants increased resistance to paraquat-induced oxidative stress (Mittova et al., 2002). Increased activities of SOD, CAT, APX and MDHAR, and the reduced form of ascorbate, were found in salt-tolerant tomato plant when treated with NaCl (100 mM), but

not in salt-sensitive tomato. Also, membrane lipid peroxidation remained unchanged (Shalata et al., 2001).

In salt-sensitive rice, roots treated with a NaCl solution could induce an increase in the total peroxide content, lipid peroxidation, and membrane damage, along with decreases in CAT and SOD activities (Khan and Panda, 2002). When rice shoot cultures were treated with NaCl (350 mM), Mn-SOD, Cu/Zn-SOD and GR activity were elevated, preventing oxidative stress (Fadzilla et al., 1997). Research indicates increased SOD and APX activity under salt stress (Kennedy and Filippis, 1999; Mittova et al., 2002) and decreased enzyme activity (Hernandez et al., 1993). Tanaka et al. (1999) confirmed that overexpression of yeast Mn-SOD in rice confers tolerance to salt stress.

Drought and Flooding Stress

Drought stress may be regarded as an oxidative stress (Burke et al., 1985). In response to dehydration, the stomata close and CO_2 fixation becomes low, while photosynthetic electron transport still operates at normal rates. Abscisic acid (ABA) causes stomatal closure and the impairment of the electron transport chain in chloroplasts, which is thought to be caused by leakage of electrons and the subsequent formation of ROS. Lin et al. (1999) demonstrated that applying ABA resulted in drought-stress-like effects, including the promotion of senescence and decreasing activities of POX, CAT, SOD, and APX. A study using tomato showed that drought stress significantly decreased yield, yield components, and dry matter production. SOD activity increased significantly in tomato exposed to

water stress treatment (Rahman et al., 2002). Osmotic stress induced changes in lipid composition and peroxidation in leaf discs of rape (*Brassica napus L.*). SOD and APX activity was found to be enhanced in osmotically stressed leaf discs compared with the control (Aziz and Larher, 1998). Drought stress resulted in an increase in cAPX and Cu/Zn-SOD gene expression and an increase in CAT activity in pea (Mittler and Zilinskas, 1994). Plants recovering from drought showed a dramatic increase in cAPX and Cu/Zn-SOD steady-state transcript level. These results suggest that oxidative stress might be involved in stomatal closure during drought stress (Mittler and Zillinskas, 1994). Harjanto (1993) reported that transgenic alfalfa overexpressing Mn-SOD showed drought tolerance. These studies suggest that antioxidant enzymes might play an important role in drought-induced oxidative stress.

Flooding stress causes the depletion of oxygen below optimal levels. There is little information about the changes in the activity of antioxidant enzymes in plants under anoxic or hypoxic conditions. In rice, after three days of anoxia, SOD activity was 65% higher than in control rice roots. In the more anoxia-tolerant rice, anoxia did not affect SOD activity (Chirkova et al., 1998). The activities of enzymes involved in the antioxidative system were lower in rice seedlings that germinated under water, but expression commenced when the seedlings were exposed to air. Activity was lower for SOD, DHAR, and GR, and higher for APX, in seedlings germinated under water in darkness than in seedlings germinated not under water in darkness. CAT activity increased in aerobically-grown rice seedlings when submerged (Ushimaru et al., 1997, 1999, 2001).

Herbicides

Herbicides are widely used on many agricultural crops. They can directly affect chloroplast activity and stimulate production of oxygen free radicals (Bowler et al., 1992). Herbicides can block photosynthetic electron transport and the light energy may be transferred to oxygen, generating ROS that can cause lipid damage.

Paraquat, a bipyridylium herbicide, is a redox-active compound that is photoreduced by PSI. It is subsequently reoxidized, by transfer of its electrons to oxygen, forming O_2^- in a chain reaction (Donahue et al., 1997). Paraquat is used to study superoxide-mediated damage to plants that might be detected at PSII site (Slooten et al., 1995). Paraquat is often used to study oxidative stress. Paraquat treatment of bean leaves induced O_2^- production and membrane deterioration (Chia et al., 1982). Herbicide resistance was correlated with increased activity of SOD and other free radical scavenging enzymes in various plants (Donahue et al., 1997; Perl et al., 1993). Paraquat tolerance in maize was related to an increase in SOD and APX activities (Iannelli et al., 1999). Donahue et al. (1997) showed that resistance to paraquat was correlated with leaf age, photosynthetic rate, and antioxidant enzyme activities. In response to paraquat, small increases in the activities of GR and APX were observed in the more paraquat-resistant pea leaves. Decrease in the activity of GR enhances paraquat sensitivity in transgenic tobacco (*Nicotiana tabacum*) (Aono et al., 1995). A cyanobacterium lacking Fe-SOD has been sensitized to paraquat, indicating a specific role for the Fe-SOD in protection against superoxide generation in the cytosol (Thomas et al., 1998).

Also, overexpression of SOD has resulted in paraquat resistance in many plants (Bowler et al., 1991; Perl, et al., 1993; Sen Gupta et al., 1993; Slooten et al., 1995; Van Camp et al., 1996).

1.6 Increasing Oxidative Stress Tolerance by Genetic Engineering in Plants

Plants can adapt to elevated ROS levels by increasing endogenous levels of antioxidant enzymes such as SOD, APX, and GR (Allen, 1995; Mittler and Zilinskas, 1994). It is thought that overexpression of one antioxidant enzyme in plants could cause an increase in one or more of the other antioxidant enzymes, thus increasing oxidative stress tolerance (Allen, 1995; Kingston-Smith and Foyer, 2000). Based on these findings, genetic engineering is being used to improve stress tolerance in plants by increasing the activity of enzymes involved in scavenging ROS (Alscher et al., 2002; Foyer et al., 1994).

Various SOD genes have been successfully transformed into plants (Allen, 1995; Foyer et al., 1994). Overexpression of Cu/Zn-SOD, Fe-SOD, or Mn-SOD in potato, tobacco, and alfafa has resulted in increased protection against oxidative stresses (Bowler et al., 1992; Mckersie et al., 1993, 1999; Perl et al., 1993; Van Camp et al., 1994, 1996). Transgenic tobacco plants overexpressing a gene for SOD from *Nicotiana plumbaginifolia* showed increased tolerance to paraquat and ozone (Bowler et al., 1991; Perl et al., 1993; Van Camp et al., 1994). McKersie et al. (1999) reported that transgenic alfalfa plants overexpressing Mn-SOD had increased vigor after freezing stress and increased winter survival under field conditions. Tanaka et al. (1999) reported that overexpression of yeast Mn-

SOD in rice confers tolerance to salt stress. Increasing the activities of SOD, APX, or GR using transformation improved the recovery of photosynthesis following chilling (Foyer et al., 1995; Payton et al., 2001; Sen Gupta et al., 1993). Van Breusegem et al. (1999, 2002) concluded that overproduction of Mn-SOD in chloroplasts increases the antioxidant capacity and tolerance to cold in maize leaves. Transgenic tobacco plants overproducing APX showed increased tolerance to either paraquat (Yabuta et al., 2002) or aminotriazole treatments (Wang et al., 1999). Wheat CAT expressed in transgenic rice was shown to improve tolerance to low temperature (Matsumura et al., 2002). Transgenic apple plants that overexpressed pea cytosolic *APX* gene showed tolerance to UV-B, heat, and freezing stress (Wisniewski et al., 2002). These studies suggest that overexpression of antioxidant enzymes are important in ROS scavenging, and that the modification of ROS scavenging systems may help increase oxidative stress tolerance (Allen, 1995; Foyer et al., 1994).

However, some studies showed that overexpression of antioxidant genes in plants did not provide substantial protection against oxidative stresses (Tepperman and Dunsmuir, 1990; Payton et al., 1997; Pitcher et al., 1991). These inconsistent, though limited, results may have resulted from differences in the ability of the antioxidant gene products to function properly, the physiological target studied, the severity of the stresses imposed, and/or the plant systems used (Allen, 1995).

Transgenic plants also showed increases in biomass production (Samis et al., 2002; Yan et al., 2003) and vigor (McKersie et al., 1999, 2000). Antioxidant gene

expression increased the size of the root and crown systems, which resulted in a larger available quantity of carbohydrate and protein reserves and improved persistence (McKersie et al., 1999, 2000). Allen (1995) predicted that overexpression of antioxidant genes might enhance yield. In field trials, yield and survival of transgenic plants were significantly improved, supporting the hypothesis that tolerance to oxidative stress is important in adaptation to field environments (Mckersie et al., 1996, 1999, 2000).). These researches have raised hopes that in the future, overexpression of antioxidants genes can be used to improve the stress tolerance of economically important plants (Allen, 1995).

1.7 Objectives

The primary goal of this project is to evaluate the response of tomato plants overexpressing the ROS-scavenging enzymes Mn-SOD and cAPX to abiotic stresses. Studied was the commercially important tomato variety (*L. esculentum* cv. Zhongshu 5). This species was studied because: a) it has a short life cycle (two to three months from seed to production of mature fruit); b) it has been used by other researchers to study plant responses to oxidative stresses; c) it is amenable to *Agrobacterium*. The overall objectives of this study were to: a) determine whether the overexpression of the antioxidant enzymes can increase the stress resistance of the transformed tomato plant to, salt, heat, chilling, and UV-B stresses; and b) determine if there is a relationship between resistance and the activities of SOD and APX in transgenic plants grown under varying degrees of abiotic stress.

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Chapter 2

Overexpression of Cytosolic Ascorbate Peroxidase in Tomato (*Lycopersicon esculentum*) Confers Tolerance to Chilling and Salt Stresses

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2.1 Abstract

Ascorbate peroxidase (APX) plays an important role in the metabolism of hydrogen peroxide (H_2O_2) in higher plants, affording them protection against oxidative stress. We studied the effect of overexpressing a cytosolic ascorbate peroxidase (cAPX) gene–derived from pea (*Pisum sativum*)–in transgenic tomato plants (*Lycopersicon esculentum*). Transformants were selected *in vitro* using kanamycin resistance, and confirmed by PCR and northern analyses. The APX native gel assay indicated that, in the absence of stress, APX activity in transgenic plants was several times greater than that measured in wild-type (WT) plants. Several independently transformed lines were propagated and evaluated for resistance to chilling and salt stress. At 9 °C after five weeks, percent germination was greater for transgenic plant seeds (26% to 37%) compared with the WT (3%). Transgenic plants also had lower electrolyte leakage (20% to 23%) than WT (44%) in response to cold temperature (4 °C). Transgenic seedlings also showed enhanced tolerance to NaCl stress (200 or 250 mM). Moreover, APX activity was nearly 25-fold and 10-fold higher in the leaves of transgenic plants in response to these chilling and salt stresses, respectively. Therefore, our results substantiate that increased levels of APX may play an important role in plant defense mechanisms.

Key words: Ascorbate peroxidase, chilling, oxidative stress, salt, transgenic plants.

Abbreviations: SOD, superoxide dismutase; APX, ascorbate peroxidase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; WT, wild-type.

2.2 Introduction

As with many other environmental stresses, exposing plants to high salt levels or chilling results in oxidative damage from reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Bruggemann et al., 1999; Prasad et al., 1994a, b). ROS also may be generated in living cells during normal metabolism (Allen, 1995). Oxidative damage can affect plant membrane lipids as well as the levels of proteins and nucleic acids (Becana et al., 1998; Posmyk et al., 2001). Plants have developed both enzymatic, e.g., superoxide dismutase (SOD, EC 1.15.1.1) and ascorbate peroxidase (APX, EC 1.11.1.11) and non-enzymatic (ascorbate or glutathione) systems to counter the effects of oxidative stress. SOD scavenges O_2^- radicals and converts them to H_2O_2. APX catalyse the conversion of H_2O_2 to water, with ascorbate serving as the electron donor (Asada, 1992). In general, APX activities increase in plants exposed to various environmental stresses. This elevated activity is correlated with a rise in the levels of other antioxidant enzymes, such as catalase (CAT), SOD, and glutathione reductase (GR), which suggests that the components of ROS-scavenging systems are co-regulated (Allen, 1995; Pastori and Trippi, 1992; Sen Gupta et al., 1993; Shigeoka et al., 2002).

The injury manifested by low-temperature and salt stresses is believed to be lipid peroxidation caused by an increase in oxygen radical generation (Prasad et

al., 1994a, b; Shalata et al., 2001). After exposure to low temperatures, chilling-tolerant maize (*Zea mays*) exhibited higher SOD and APX activities compared with chilling-sensitive maize (Pinhero et al., 1997). In tomato, higher chilling tolerance prevents ROS formation and allows for better conversion of light to photochemical energy at suboptimal temperatures (Bruggemann et al., 1999).

The relationship between salt stress and antioxidant enzyme activity has been investigated in various plants (Dionisio-Sese and Tobita, 1998; Hernández et al., 1995, 1999, 2001; Shalata et al., 2001). Gueta-Dahan et al. (1997) have found that cAPX and Cu/Zn-SOD in citrus (*Citrus sinensis*) cells were similarly induced by salt and oxidative stresses. The APX has been proposed as a key enzyme for imparting salt tolerance in citrus because its steady-state activity in salt-sensitive citrus callus is far below that observed in salt-tolerant citrus callus. Mittova et al. (2003) reported that the salt-induced oxidative stress increase in the antioxidant enzyme activities in salt-tolerant tomato species conferred cross-tolerance towards enhanced mitochondrial and peroxisomal ROS production. Shalata et al. (2001) found that, under salt stress, membrane lipid peroxidation gradually increased in salt-sensitive tomato roots. This response was accompanied by decreased activities of SOD, APX, and CAT. Their activities increase in salt-tolerant tomato roots, in which the membrane lipid peroxidation levels remain unchanged.

Increased expression of antioxidant enzyme activity is effective in overcoming chilling and salt stresses (Payton et al., 2001; Roxas et al., 2000; Tanaka et al., 1999; Van Breusegem, et al., 1999). The objective of this research

was to quantify changes in tolerance to chilling and salt stresses resulting from the overexpression of pea cytosolic APX in tomato.

2.3 Materials and Methods

Production and Propagation of Transgenic Plants

Tomato plants (*Lycopersicon esculentum* cv. Zhongshu No. 5) were transformed with a binary vector containing pea cAPX cDNA (Mittler and Zilinskas, 1992) under the control of a dual cauliflower mosaic virus 35S promoter and 35S terminator (Payton et al., 2001). The T-DNA contained the nopaline synthase (*NOS*) promoter fused to a neomycin phosphotransferase II gene (*NPT II*). This construct, pCGN1578, was transferred to the tomato cells via *Agrobacterium tumefaciens* strain EHA105. Kanamycin-resistant plants were then regenerated using a technique described by Frary and Earle (1996), with modifications (Wang, 2003). Cotyledonary and hypocotyl explants were excised from 8- to 10-d-old seedlings. These were incubated for 10 to 15 min in an *Agrobacterium* suspension diluted to an OD_{600} of 0.2 in a liquid Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) that had been supplemented with 30g.L^{-1} sucrose (MSO; pH 5.8). Afterward, they were blotted on sterile paper towels, plated on a co-cultivation medium (MS + 40 mg.L^{-1} acetosyringone + 1 mg.L^{-1} zeatin + 7 g.L^{-1} agar), and placed in the dark for 2 to 4 d. The explants were then transferred to a selection medium (MS + 1 mg.L^{-1} zeatin + 50 mg.L^{-1} kanamycin + 400 mg.L^{-1} cefotaxime + 7 g.L^{-1} agar), and the resultant shoots were

placed on a rooting medium (MS + 50 $mg.L^{-1}$ kanamycin + 0.2 $mg.L^{-1}$ NAA + 400 $mg.L^{-1}$ cefotaxime + 7 $g.L^{-1}$ agar). Culture was at 23 °C /21 °C (± 2 °C, day/night temperature) under cool white fluorescent and Gro-Lux lights (100 to 150 $\mu mol.m^{-2}.s^{-1}$) with a 16-hr photoperiod. Cuttings from the regenerated plants were rooted in a potting mix containing peat moss (Lakeland Peat Moss, Ltd., Edmonton, Alberta, Canada), and grown under standard greenhouse conditions [approximately 23 °C /21 °C (± 2 °C, day/night temperature)] with natural lighting supplemented with sodium vapor lights (1000W, Philips, Inc., Eindhoven, Netherlands) from 6:00 AM to 10:00 PM. These original transgenic lines (T_0 generation) were self-pollinated to produce T_1 progeny. The T_1 plants were then self-pollinated to yield the T_2 generation. All the transgenic plants were resistant to kanamycin.

Analysis of Transgenic Plants

Putative transformants were screened for T-DNA insertion using PCR primers to amplify the 35S::*APX* fusions. Genomic DNA was isolated from wild-type (WT) and *APX*-overexpressing plants (T_1), according to procedures described by Doyle and Doyle (1987). The sequences for the forward and reverse primers for 35S::*APX* included 5'–CACGTCTTCAAAGCAAGTGG–3' and 5'–GACTGCAGCTTCAGCAAATCC–3', respectively. About 20 ng DNA was used for PCR reaction, under the following conditions: 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C for 30 cycles. The amplified products were then separated electrophoretically on a 0.8% agarose gel, stained with ethidium bromide, and

visualized and photographed under UV light. For the segregation analysis, about 50 seeds from WT and each independent line (T_0) were germinated on a solid MS medium containing kanamycin. After 7 to 10 d, the cutting shoots were transferred to a selective rooting medium. Seedlings were scored for kanamycin resistance, and the ratio of resistant to sensitive plants was used to estimate the number of independent T-DNA insertions. All transformants were grown in potting mix to maturity in the greenhouse. These plants were then self-fertilized, and the resulting seed was collected from individual lines.

Northern Blots

Total RNA was prepared from the leaves of the WT and transgenic plants, using Tri-reagent as specified by the manufacturer (Molecular Research, Inc., Cincinnati, OH). Briefly, 30 μg aliquots of total RNA were separated on a 1% (w/v) denatured agarose gel and transferred to a nylon membrane. This procedure was followed by a Super Hyb Kit northern blot system procedure (Molecular Research, Inc., Cincinnati, OH). The membrane was probed with a ^{32}P-labeled *APX*-PCR fragment, and hybridizations were detected by a Phosphor-Imager SI (Molecular Dynamics, Inc., Sunnyvale, CA).

Enzyme Activity Assays

For the APX gel activity assays, about 100 mg of the leaf tissue was ground to a fine powder in liquid nitrogen, and homogenized in 200 μl of grinding buffer [100 mM $NaPO_4$ (pH 7.0), 5 mM ascorbate, 1 mM EDTA (pH 8.0), 10% glycerol,

and 0.001% bromophenol blue]. The supernatant was collected and protein concentration was determined using a Protein Assay System (Bio-Rad, Hercules, CA). Approximately 70 μg of total protein was loaded on a non-denaturing, 10% polyacrylamide gel. It was then electrophoretically separated (via PAGE) for 5 h at 4 °C in a 1X Tris-glycine buffer (24 mM Tris and 192 mM glycine), followed by staining for APX activity, as described by Mittler and Zilinskas (1993).

For the SOD gel activity assays, about 100 mg of the leaf tissue was finely ground in liquid nitrogen and homogenized in 200 μl of grinding buffer [50 mM Tris (pH 6.8), 10% glycerol, and 0.001% bromophenol blue]. About 70 μg of soluble protein was then loaded on a non-denaturing, 10% polyacrylamide gel and electrophoretically separated for no more than 6 h at 4 °C, as described by Van Camp et al. (1996). After staining, the gels were scanned with a Densitometer SI (Molecular Dynamics Inc., Sunnyvale, CA). ImageQuant 5.2 was used for image analysis (Molecular Dynamics Inc., Sunnyvale, CA).

Evaluation of Tolerance to Chilling Stress

Seeds (n≈50) from WT and T_1 generation were surface-sterilized and germinated at 9 °C in the dark on a solid MS medium. They were considered viable if the radicles were fully extended from the seed coats. After five and eight weeks, percent seed germination was determined. Means ± SE for four replicates were determined. Differences between means were evaluated by Duncan's multiple range test (NCSS-PASS software, NCSS Inc., Williamsport, PA). Arcsine square root transformation was performed before data analysis.

Four-week-old transgenic T_2 and the WT plants grown in plastic pots (15 cm diameter, 14.5 cm height) in the greenhouse were transferred to 4 °C in the dark for chilling treatment. Two discs (1.02 cm in diameter) from the fourth or fifth leave from the apex were excised after 2, 6, 8, or 10 d and ion leakage was determined with a conductivity meter (Markson Science, Inc., Del Mar, CA) according to the method described by Wisniewski et al. (1997). The percentage of electrolyte leakage due to the chilling treatment was calculated from these values. Means ± SE for three replicates were determined. Differences between means were evaluated by Duncan's multiple range test (NCSS-PASS software, NCSS Inc., Williamsport, PA). Arcsine square root transformation was performed before data analysis. For enzyme activity assay, leaf discs (under 4 d of the chilling stress) were taken from the WT and transgenic plants, frozen in liquid nitrogen, and stored at –80 °C.

Evaluation of Tolerance to Salt Stress

Cuttings from WT and transgenic T_2 were grown in rooting medium for two weeks. Transgenic cuttings were screened on media containing 50 $mg.L^{-1}$ kanamycin. Healthy seedlings were transferred in 5.8 × 5.8 × 8-cm plastic pots filled with peat moss soil and watered with tomato fertilizer (9N-4.4P-12.5K; Schultz, Inc.). Plants were grown under standard greenhouse conditions [approximately 23 °C /21 °C (± 2 °C, day/night temperature)] with natural lighting supplemented with sodium vapor lights (1000W, Philips, Inc., Eindhoven, Netherlands) from 6:00 AM to 10:00 PM. After one week, plants to be

stressed were watered with tomato fertilizer (Tomato Food, Schultz Inc., Bridgeton, MO) and NaCl (200 or 250 mM) every 3 d, and evaluated after 10, 20, and 30 d. Non-stressed controls were watered only with tomato fertilizer. Growth (from apex to soil) was measured after 10 d of the stress period. The extent of injury to leaves and whole seedlings was visually scored, with a scale of 0 to 5 used to estimate damage: 0 = no damage symptoms; 1 = slow growth, but no obvious damage and <20% leaf area exhibiting injury; 2 = yellowing leaves, 21-40% of the leaf area injured; 3 = plants wilted, 41%-60% leaf area injured; 4 = seriously damaged, the plant becoming soft and not remaining upright, 61%-80% leaf area injured; 5= 81%-100% of the leaf area injured or else the plant had died. WT and individual transgenic lines set as one block represented the experimental unit. The experimental design was a randomized complete block with five blocks. Means ± SE of five replicates were determined. The non-parametric Kruskal-Wallis test was used for analysis of ranks. The leaf discs from WT and transgenic plants under 10-d NaCl (200 mM) stress were then frozen in liquid nitrogen and stored at –80 °C for further enzyme activity gel analyses.

2.4 Results

Overexpression of the *cAPX* Gene

Transformed tomato explants and shoots growing in the presence of kanamycin are shown in Fig. 2.1A and B. No PCR product was obtained from WT plants with 35S promoter- and *cAPX*-specific primers, whereas an expected 0.9 kb fragment was amplified from DNAs from all transgenic lines (Fig. 2.2). In

10 transformants, seven overexpressed *APX*, segregated in a 3:1 ratio for APX+ to APX- plants (Table 2.1).

RNA northern blot analyses showed that when total RNA from the WT and transgenic tomato leaves was hybridized with a full-length *cAPX* probe,a message was detected only in the transgenic lines (Fig. 2.3). Five of 14 lines exhibited relatively high levels of expression. Moreover, APX activity for two selected lines, A9 and A16, was 3- to 6-fold higher than from the WT plants (Fig. 2.4A).

Both SOD and APX are key enzymes in the oxygen-scavenging system. To determine if the expression of pea *cAPX* in tomato plants affected SOD activity, we also examined levels of leaf SOD via gel assay, and found that activity in the APX+ lines was no difference compared to WT plants (Fig. 2.4B).

Effects of Chilling

Hypocotyls and cotyledons developed normally from the T_1 seeds from T_0 generation. However, hypocotyl elongation from the WT seed was very slow, and no cotyledon expansion was apparent even after eight weeks (Fig. 2.5). At 9 °C, germination was significantly greater among T_1 seeds compared to WT seeds ($P<0.05$). For example, after five weeks, 37% and 26% of the T_1 seeds germinated in lines A9 and A16, respectively, compared to 3% of the WT seeds. After eight weeks, the percent germination of transgenic seeds was 58% and 35% for lines A9 and A16, respectively, compared to 7% for WT seeds (Fig. 2.6).

To study the effect of *cAPX* expression on chilling tolerance, cellular injury was assessed by measuring solute leakage (Bowler et al., 1991). Significant differences ($P<0.05$) were found between WT and *cAPX* plants after 8 d of the

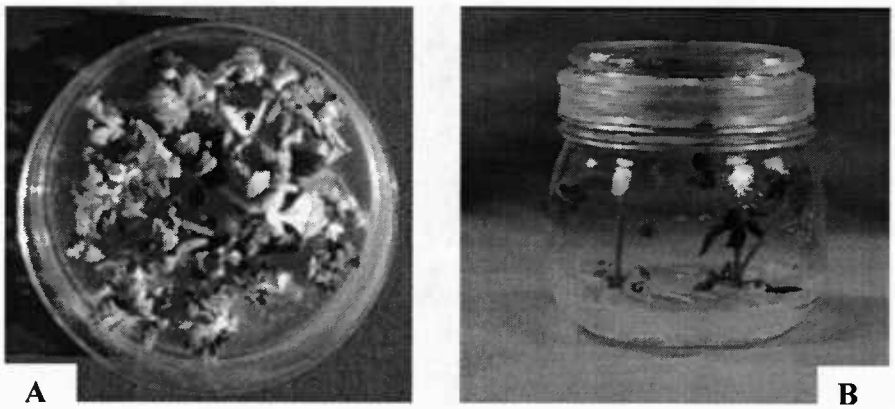

Fig. 2.1. Tomato explants were transformed by *Agrobacterium tumefaciens* (A) and the transformed shoots growing in the presence of kanamycin (50 $mg.L^{-1}$) selective medium (B).

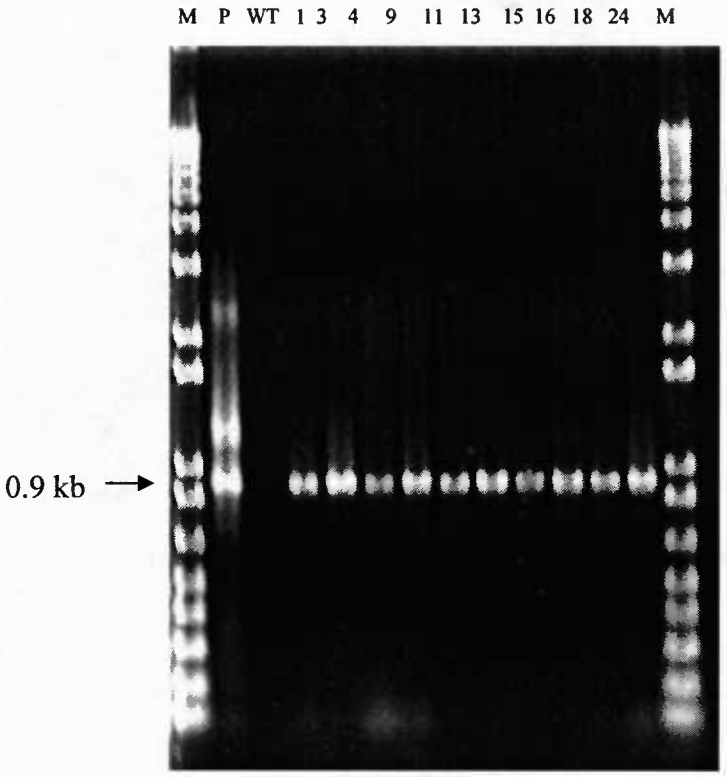

Fig. 2.2. PCR analysis of wild-type and transgenic tomato plants used confirm the presence of the *cAPX* transgene. DNAs isolated from wild-type and independently transformed lines was used in PCR reactions template; 35S promoter- and *cAPX*-specific sequences were used for the forward and reverse primers, respectively. M, 1 kb DNA marker; P, positive plasmid control; WT, wild-type plant; 1 to 24, independent transgenic lines.

Lines tested	Kan+/Kan-	Ratio	$\chi 2$	P -value
Wild-type	0/89	---	---	
A1	36/0	ND	ND	ND
A3	39/14	3:1	0.06	0.19
A4	19/31	ND	ND	ND
A9	39/12	3:1	0.06	0.19
A13	36/12	3:1	0	0.00
A15	43/12	3:1	0.30	0.41
A16	44/13	3:1	0.15	0.28
A18	41/18	3:1	0.95	0.67
A25	1/39	Untransformed	---	---
A30	27/9	3:1	0	0

Table 2.1. Expression of kanamycin resistance in the T_1 generation of transgenic tomato plants.
($\chi 2$ tests indicated good agreement with the segregation ratio indicated.)
Kan+: roots produced in kanamycin (50 $mg.L^{-1}$) medium.
Kan-: no roots developed in kanamycin (50 $mg.L^{-1}$) medium.
ND: Not determined.
A1 to A30: transgenic seeds from independent lines.

Fig. 2.3. Northern-blot analysis of RNA isolated from T_1 tomato plants. Approximately 30 μg of total RNA was loaded in each lane. The blot was probed with a full-length, ^{32}P-labeled *cAPX* PCR product. WT, wild-type plants; A1 to A30, independent transgenic lines.

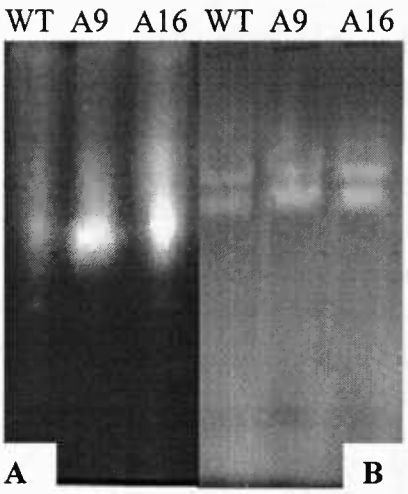

Fig. 2.4. Gel assays for detecting APX (A) and SOD (B) activities in wild-type and transgenic tomato plants under non-stressed condition. Approximately 70 μg of protein was loaded in each lane of a non-denaturing polyacrylamide gel. WT, wild-type plants; A9 and A16, independent transgenic lines.

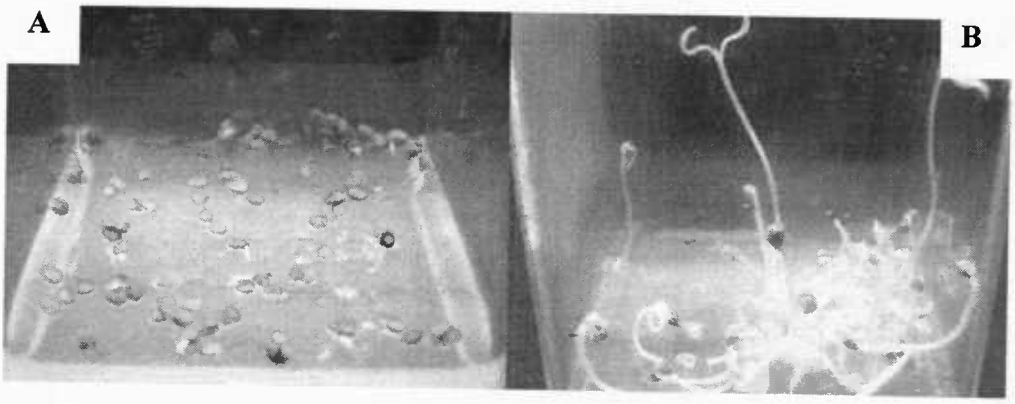

Fig. 2.5. Germination of wild-type (A) and T_1 (B) tomato seeds in the dark at 9 °C after 8 weeks.

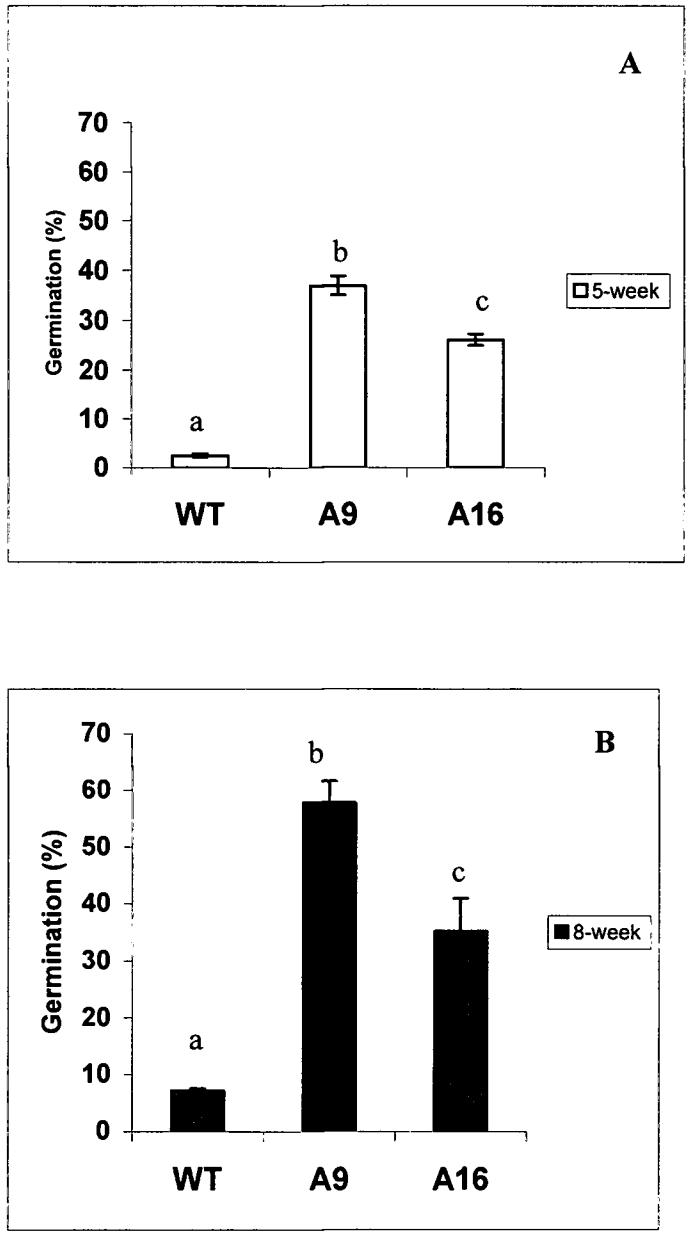

Fig. 2.6. Germination of transgenic (Lines A9 and A16) and wild-type (WT) tomato plants after 5 (A) and 8 (B) weeks in the dark at 9 °C. Values are means ± SE for four replicates. WT, wild-type; A9 and A16, independent transgenic lines. Different letters indicate significant differences ($P<0.05$) between lines at either 5 or 8 weeks by Duncan's multiple range test.

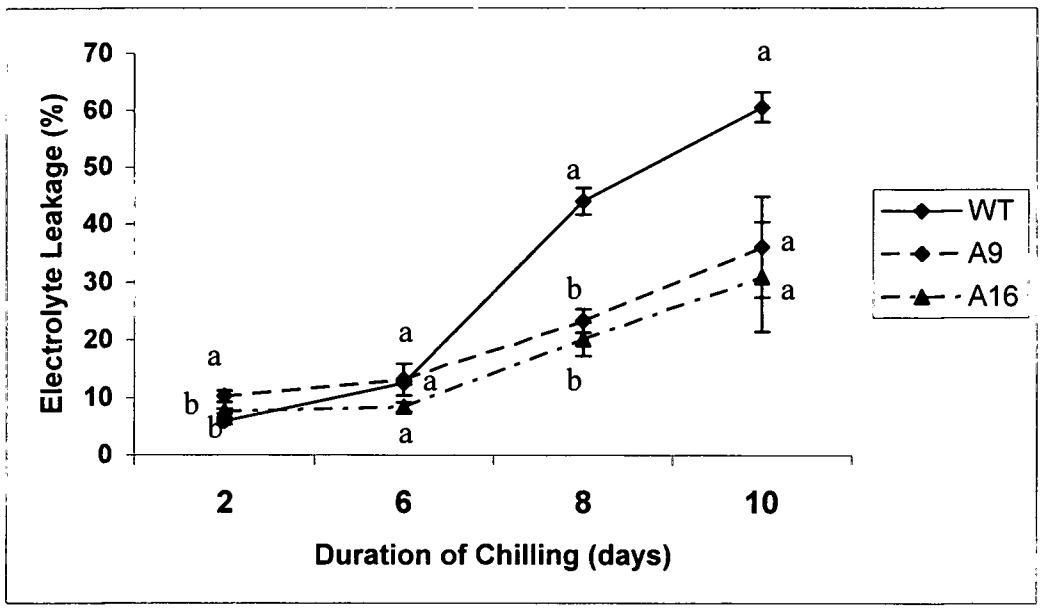

Fig. 2.7. Electrolyte leakage from wild-type (WT) and transgenic tomato plants (Lines A9, A16) following chilling stress (4 °C) for 2, 6, 8, and 10 d. Data for three measurements were averaged, and error bars represent SE. Different letters indicate significant differences ($P<0.05$) between means within each sampling time (Duncan's multiple range test).

low-temperature (4 °C) treatment, with the transgenics showing lower leakage (23% for A9 and 20% for A16) than from the WT (44%) (Fig. 2.7). Nevertheless, after 10 d, electrolyte leakage did not differ between WT and transgenic plants. APX and SOD enzyme activities were analyzed by gel assays after treatment for 4 d at 4 °C (Fig. 2.8). APX+ plants had nearly 25-fold greater APX activity than the WT plants (Fig. 2.8A). SOD activity decreased in both the transgenic and the WT plants in comparision to non-stressed condition (Fig. 2.4 B, 8B).

Effects of Salt Stress

Compared with the control plants, height growth of both WT and transgenic plants was severely inhibited by salt (Fig. 2.9). However, the transgenic plants were visibly less affected by higher salinity (Fig. 2.10). Wilting was obvious in the leaves and stems of WT plants after 10 d, and leaf-area injury, leaf and stem chlorosis, and, occasionally, necrosis and stem collapse had occurred. The average injury is 2.2 for WT plants receiving 200 mM NaCl (injury scale is 2.2, Fig. 2.9A) and 4 for receiving 250 mM NaCl (injury scale is 4, Fig. 2.9B). Plants from both the A9 and A16 lines began showing symptoms after 10 d of salt treatment, but had significantly less severe injuries ($P<0.05$) – injury scale is 1 at 200 mM NaCl (Fig. 2.11A) and 2.2 at 250 mM NaCl (Fig. 2.11B). After 20 d, the WT plants irrigated with 200 mM NaCl showed injury scale 3.6; none that had received 250 mM NaCl survived (Fig. 2.11A, B). Transgenic lines A9 and A16 were less affected ($P<0.05$), with injury scale 2.4 and 1.6, respectively, under the 200 mM treatment. Significant difference was found between those lines when

treated at 250 mM. By day 30, WT plants showed injury scale 5, transgenic plants (A9) showed less ($P<0.05$) damage, with injury scale 4.

APX activity was also induced by salt stress, with levels in the transgenic leaves being about 10-fold higher (compared with WT plants) after 10 d of treatment with 200 mM NaCl (Fig. 2.12A). In contrast, salt stress decreased SOD activity in both the transgenic and the WT plants compared to normal condition (Fig. 2.4B, 12B).

2.5 Discussion

Overexpression of antioxidant genes in plants has been previously shown to provide enhanced tolerance to oxidative stress (Allen, 1995; Gupta-Dahan et al., 1993; Payton et al., 2001; Wang et al., 1999). The results show that ectopic expression of the pea *cAPX* gene in tomato conferred protection against the oxidative damage caused by exposure to chilling or high salt levels.

Seed germination is characterized by the rapid generation of superoxide and H_2O_2 following imbibition (Gidrol et al., 1994; Puntarulo, 1994). Under chilling conditions, the activities of antioxidant enzymes, including SOD, peroxidase, and glutathione reductase, significantly increase to limit the damage caused by such oxidative stress (Posmyk et al., 2001). We demonstrated that, at a low temperature (9 °C), germination rates were better for seeds which were from transgenic tomato plants that overexpressed cAPX compared with WT seeds (Figs. 2.5 and 2.6). These results suggest that the enhanced level of APX may

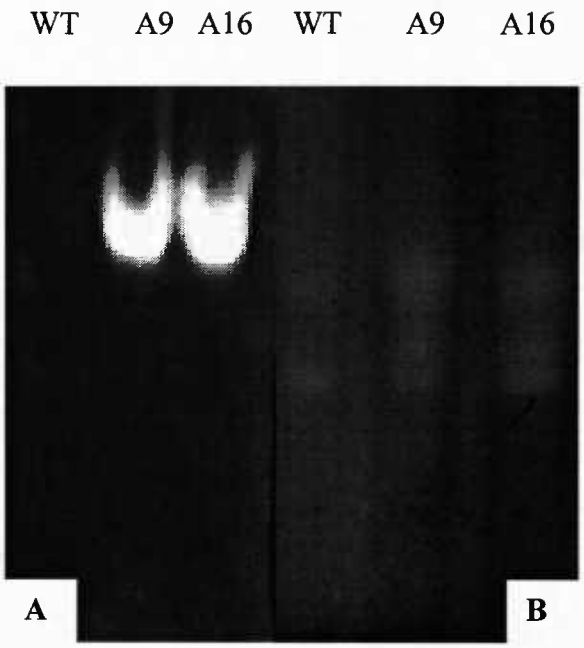

Fig. 2.8. APX (A) and SOD (B) activities in wild-type (WT) and transgenic plants (Lines A9, A16) after chilling for 4 d at 4 °C. About 70 μg of protein was loaded in each lane of the non-denaturing polyacrylamide gel.

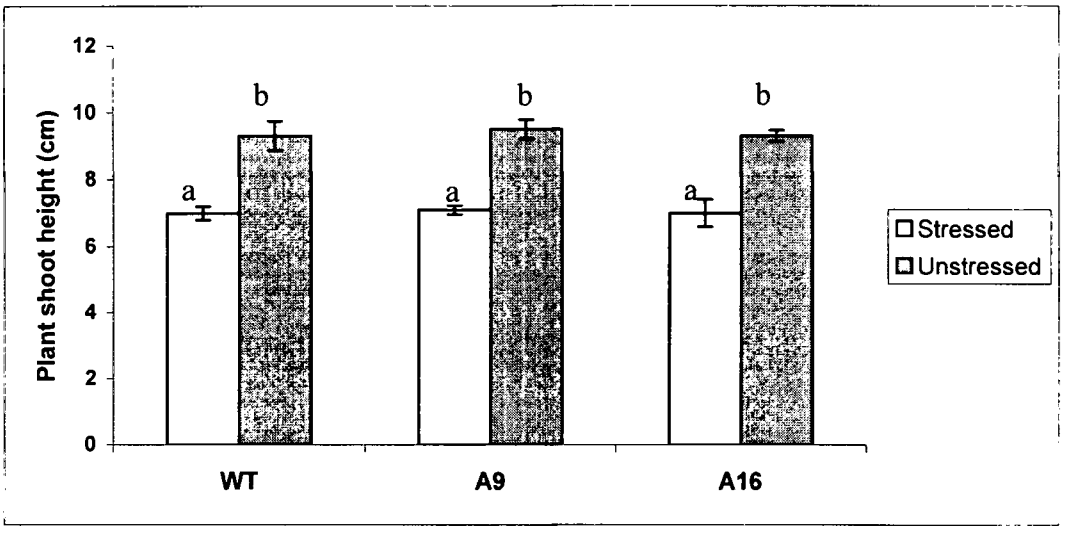

Fig. 2.9. Effects of salt stress on height growth of seedlings of wild-type (WT) and transgenic tomato plants (Lines A9, A16). Plant shoot height (highest point of the apex reaches relative to the soil) was measured after 10 d. Unstressed, 0 mM NaCl; Stressed, 200 mM, NaCl. Data are means ± SE with three replicates. Different letters indicate significant differences ($P<0.05$) between means (t test).
* Data for WT are also cited in the next chapter.

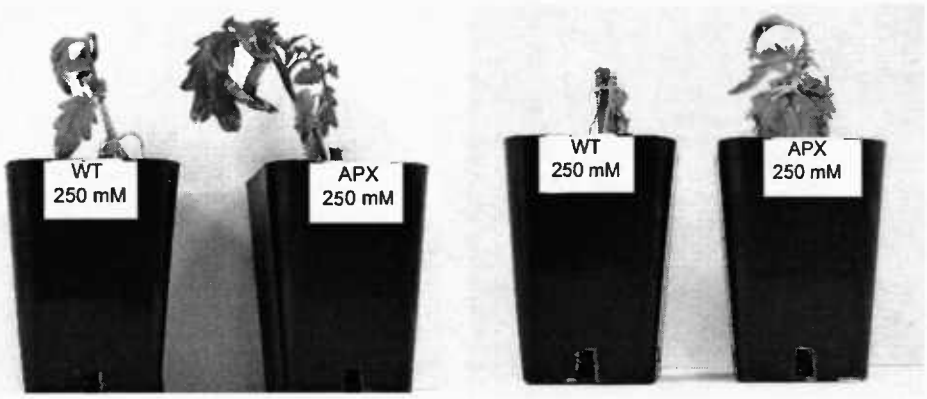

Fig. 2.10. Effects of salt stress on wild-type (WT) and transgenic tomato (APX) plants. Plants were watered with 250 mM NaCl for 10 d (A) and 18 d (B), respectively. WT died after 18 d treatment. Transgenic leaves had less curl and wilting, an indication of greater tolerance.

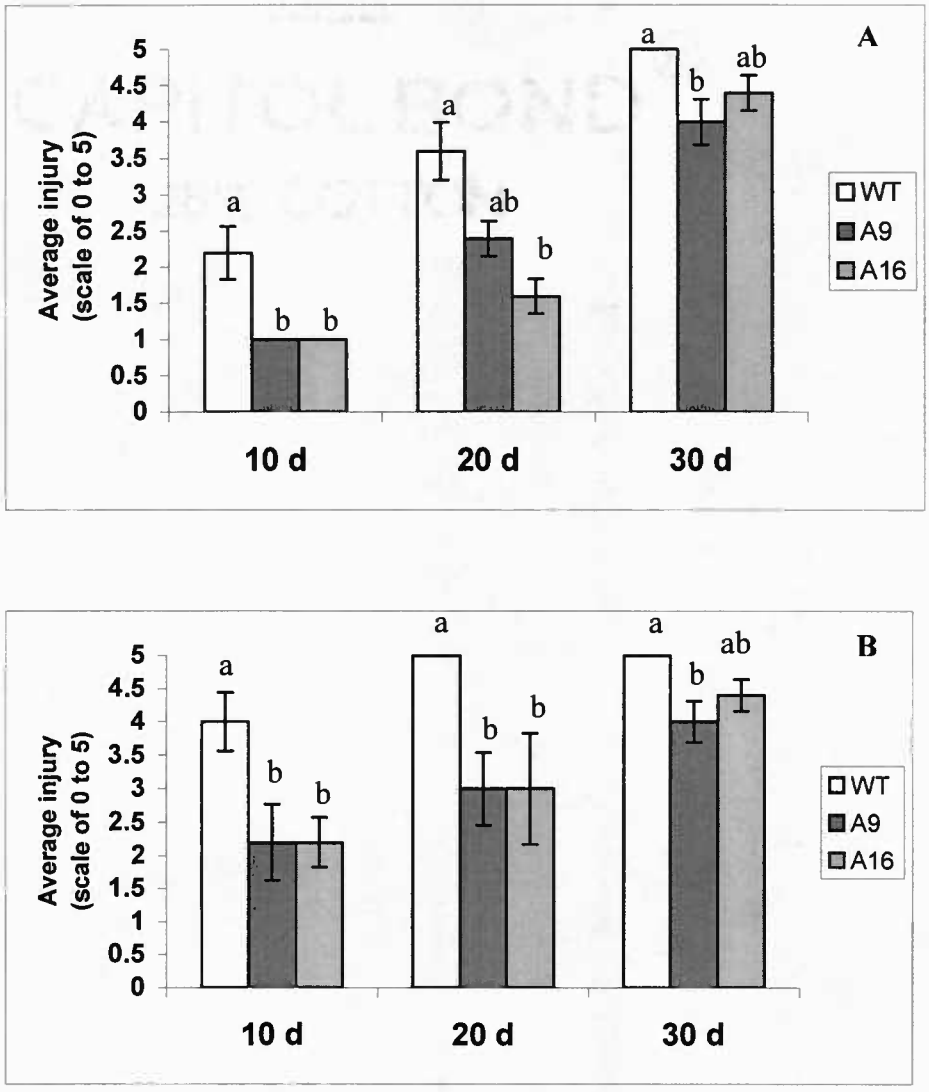

Fig. 2.11. Effects of 200 mM (A) or 250 mM (B) NaCl treatments on injury to wild-type (WT) and transgenic (Lines A9, A16) tomato plants after 10, 20, and 30 d. No visible injury = 0; slow growth, and <20% visible injury = 1; yellowing leaves and 21-40% of leaf area with visible injury = 2; wilted plants and 41-60% visible injury = 3; seriously damaged plant unable to remain upright with 61-80% visible injury = 4; dead plant or >80% visible injury = 5. Values are means ± SE (n=5). Different letters indicate significant differences (P<0.05) between means within a sampling time (Kruskal-Wallis test).
*Data for WT are also cited in next chapter.

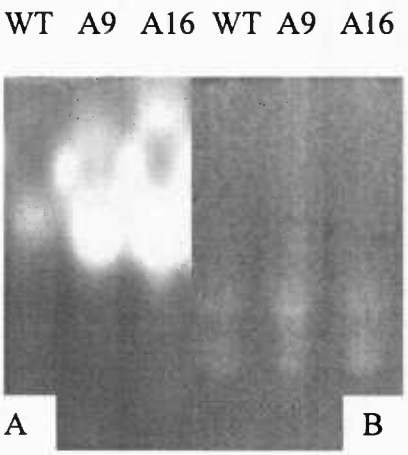

Fig. 2.12. APX (A) and SOD (B) activities in WT and transgenic (Lines A9, A16) tomato plants treated with 200 mM NaCl for 10 d. Approximately 70 μg of protein was loaded in each lane of a non-denaturing polyacrylamide gel.

detoxify H_2O_2 to H_2O, thereby alleviating oxidative stress and increasing germination. In addition, the leakage of electrolytes was lower in transgenic plants in response to chilling at 4 °C (Fig. 2.7). We propose that the cell membranes, which normally are more likely to lose solutes during chilling, may be less altered in transgenic plants that undergo such stress. At 4 °C, APX activity in the transgenic tomatoes was 25-fold higher than in the WT plants (Fig. 2.8A). Therefore, we suspect that this elevated activity is a requirement for higher rates of H_2O_2 detoxification at lower temperatures (Pastori et al., 2000), thereby protecting membrane integrity.

Mittova et al. (2002) have demonstrated that salt stress induces up-regulation of an efficient chloroplast antioxidant system in salt-tolerant wild tomatoes but not in the cultivated species. This response by the former is characterized by increased activities of the ROS-scavenging enzymes SOD, APX, and GR. In our study as well, the level of APX was about 10 times higher in the transgenic cAPX tomatoes than in the WT plants watered with 200 mM NaCl (Fig. 2.12A). This overexpression apparently enabled the transformed plants to better tolerate the salt stress.

In both chilling- and salt-stress tests, APX activities were higher level in the transgenic plants, effectively detoxifying the increased level of ROS (Bruggemann et al., 1999; Shalata et al., 2001). Higher levels of APX activity was associated with increased tolerance to oxidative stress. However, both transgenic and WT plants showed decreased SOD activity, perhaps as a result of long-term exposure to those stresses.

Acknowledgments

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Chapter 3

Transformation of *Lycopersicon esculentum* with Mn-SOD: Enhanced Tolerance to Salt and Oxidative Stress

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3.1 Abstract

Production of reactive oxygen species (ROS) is associated with a number of physiological disorders in plants. Superoxide dismutase (SOD) catalyzes the breakdown of superoxide (O_2^-) into O_2 and H_2O_2 and provides the first line of defense against the toxic effects of elevated levels of ROS. The effect of increased Mn superoxide dismutase (Mn-SOD) on salt stress tolerance was studied using transformed tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) plants. Northern blots confirmed expression of the heterologous Mn-SOD in transgenic plants. Strong Mn-SOD enzyme activity was detected by native PAGE in transformed plants. Transgenic plants showed resistance to the superoxide-generating herbicide methyl viologen (MV, 10^{-4} M). The total SOD activity was one and one half- to two-fold higher, and APX activity was six- to seven-fold higher in transgenic, than in wild-type (WT) plant under MV stress. Germination of transgenic tomato seeds at NaCl concentration of 150 mM was greater than of WT seeds. When exposed to salt stress, roots of transgenic plants were less stunted and leaf injury was lower than that observed in WT plants. The total APX activity of transgenic plants was 4- to 5-fold higher than that of WT under NaCl (200 mM) stress.

Key words: Superoxide dismutase, oxidative stress, salt, transgenic, tomato.

Abbreviations: SOD, superoxide dismutase; APX, ascorbate peroxidase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; WT, wild-type; MV, methyl viologen (paraquat).

3.2 Introduction

Salt stress is a common abiotic stress that limits the production of agricultural plants around the world (Zhu, 2000). Plants subjected to salt stress also show elevated levels of activated forms of oxygen and free radicals. These elements often are associated with damage to membranes and essential macro-molecules such as protein, DNA, and lipids (Fadilla et al., 1997; Gueta-Dahan et al., 1997; Hernández et al., 1995).

Superoxide dismutase (SOD) is an important enzyme in a plant's defense against oxidative stress. It catalyzes the conversion of two superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and O_2 and alleviates oxidative stress (Bowler et al., 1992). SODs are a group of metal-containing enzymes and are classified into three types according to their metal cofactor requirements: iron SOD (Fe-SOD) is localized in the chloroplast; copper-zinc SOD (Cu/Zn-SOD) is localized in the chloroplast, cytosol, and possibly the extracellular space; and manganese SOD (Mn-SOD) is found mainly in mitochondria and peroxisomes (Alscher et al., 2002).

Antioxidant enzyme activity found in plants responding to various environmental and chemical stresses (Allen 1995), such as freezing (Martinez et al., 2001), chilling (El-Saht, 1998; Iannelli et al., 1999), salt (Gueta-Dahan et al., 1997; Hernández et al., 1995; Rajguru et al., 1999), and methyl viologen (MV; Bowler et al., 1991; Donahue et al., 1997).

The role of SOD during salt stress has received much attention. Exposure of salt-tolerant pea plants to NaCl resulted in the formation of O_2^- and H_2O_2 and

increases in the activity of SOD and other antioxidant enzymes, such as ascorbate peroxides (APX). Transcripts levels for Mn-SOD, Cu/Zn-SOD, and APX were strongly induced in the salt-tolerant variety but not in the salt-sensitive one (Hernández et al., 2000). Reports dealing with rice (Dionisio-Sese and Tobita, 1998) and tomato plant (Shalata et al., 2001), have also reported increased SOD activity in salt-tolerant cultivars when exposed to salt stress. Additionally, Tanaka et al. (1999) confirmed that overexpression of a yeast *Mn-SOD* gene in rice confers tolerance to salt stress.

Expression of SODs by gene transfer technology has demonstrated that enhanced SOD gene expression and enzyme activity confers greater tolerance to MV (Allen, 1995; Perl et al., 1993), freezing (McKersie et al., 1999), and salt (Tanaka et al., 1999). In order to further understand the role of APX and SOD in response to oxidative stress induced by abiotic stresses, transgenic tomato plants were produced that overexpress either cAPX or Mn-SOD and evaluated their response to several abiotic stresses. The purpose of this paper was to evaluate the tolerance of transgenic *Mn-SOD* tomato plants to salt (NaCl) and oxidative (MV) stress.

3.3 Materials and Methods

Generation and Analysis of Transgenic Tomato Plants

Mn-SOD cDNA cloned from the rubber tree (Miao and Gaynor, 1993) was mobilized into the binary vector pDU96.2144 between the cauliflower mosaic

virus 35S promoter and terminator regions. The construct contained the β-glucuronidase (GUS) reporter gene and the *NPTII* selectable marker, and was transferred into the tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) genome via the *Agrobacterium tumefaciens*-mediated transformation method described by Frary and Earle (1996) with modifications by Wang (2003). The kanamycin-resistant transformants were screened by GUS histochemical staining assay according to method developed by Jefferson et al. (1987). Genomic DNA was isolated from wild-type (WT) and *Mn-SOD*-overexpressing plants (T_1), according to the procedure of Doyle and Doyle (1987).

PCR reaction used the 35S promoter forward primer and rubber *Mn-SOD*-specific reverse primer (Miao et al., 1993). The sequences for the forward and reverse primers for 35S::*Mn-SOD* included 5'–CACGTCTTCAAAGCAAGT GG–3' and 5'–CTAAGAAGAAGGGCATTCTTTGGCAT –3', respectively. About 20 ng DNA was used for the PCR reaction, under the following conditions: 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C for 30 cycles.

Mn-SOD expression was assayed by northern analysis and SOD activity gel assay. Wild-type (WT) and transgenic plants were grown in a greenhouse under natural lighting supplemented with sodium vapor lamps (1000W, Philips, Inc., Eindhoven, Netherlands) for a 16-h photoperiod at approximately 23 °C /21 °C (± 2 °C, day/night temperature). Seedlings were grown in a potting mix containing peat moss (Lakeland Peat Moss, Ltd., Edmonton, Alberta, Canada). T_1 seeds were obtained by self-pollination of each of T_0 plants and then screened for resistance to kanamycin. T_2 seeds were obtained from individual T_1 plants by self-

pollination and were used to generate T_2 transgenic progeny. All the transgenic plants were resistant to the kanamycin.

Northern Blot Assay

Total RNA was extracted from leaves of transgenic and WT plants using Tri-reagent (Molecular Research, Inc.). 30 μg of total RNA from each sample was used for northern blot assay. The hybridization procedure was as described by Super Hyb kit (Molecular Research, Inc., Cincinnati, OH). The hybridization probe was a ^{32}P-labeled Mn-SOD PCR fragment. RNA hybridization was detected using a PhosphorImager SI (Molecular Dynamics Inc., Sunnyvale, CA).

APX and SOD Gel Activity Assay

About 100 mg of leaf tissue was ground to a fine powder in liquid nitrogen and homogenized in 200 μl of APX activity gel grinding buffer [100 mM $NaPO_4$ (pH 7.0), 5 mM ascorbate, 1 mM EDTA (pH 8.0), 10% glycerol, and 0.001% bromophenol blue] and SOD activity gel grinding buffer [50 mM Tris (pH 6.8), 10% glycerol, and 0.001% bromophenol blue], respectively. The supernatant was collected and protein concentration was determined using a Protein Assay system (Bio-Rad, Hercules, CA). 70 μg of total protein was loaded into each lane of a native PAGE gel. APX gel activity analysis was conducted as described by Mittler and Zilinska (1993). The SOD gel activity assay was described by Payton et al. (1997). After staining, the gels were scanned with a Densitometer Scanner I

(Molecular Dynamics, Inc., Cincinnati, OH). The bands were analyzed using ImageQuant 5.2 software (Molecular Dynamics, Inc., Cincinnati, OH).

Application of Methyl Viologen

Shoots were treated with methyl viologen (MV, Sigma) following the procedure described by Perl et al. (1993). Shoot cuttings (with three to four leaves) from WT and T_0 transgenic plants were obtained from containerized mature plants grown in the greenhouse. The cut end of the shoots was placed in 10^{-4} M MV. After 16 h, the cut ends were transferred to tap water for an additional two days. Two leaf discs (1.02 cm in diameter) of the third leaf from the apex were excised after the MV treatment, and MV-induced oxidative damage was evaluated using the leaf electrolyte leakage (Wisniewski et al., 1997) with a conductivity meter (Markson Science, Inc., Del Mar, CA). Means for all values were an average of two subsamples in each plant with three replications. The data were subjected to Duncan's multiple range test (NCSS-PASS software, NCSS Inc., Williamsport, PA). Arcsine square root transformations were performed before data analysis; nontransformed means are presented. MV treated leaf discs were also sampled and stored at –80 °C prior to APX and SOD analyses.

Salt Stress

To evaluate to salt stress, seeds from WT and T_1 plants were placed in petri dishes on filter paper (Whatman 3MM) saturated with 150 mM NaCl solution. They were germinated at 23 °C /21 °C (± 2 °C, day/night temperature) under cool

white fluorescent and lights (100-150 μmol m^{-2} sec^{-1}) using a 16-h photoperiod. At the end of two weeks, germination was measured. Germination was considered successful when the radicle protruded through the seed coat.

To evaluate the tolerance of developing roots to salt stress, shoot cuttings from 10-day-old WT and T_2 seedlings were grown in sterile, solidified Murashige-Skoog (MS) (Murashige and Skoog, 1962) medium (Sigma) amended with either 200 mM or 250 mM NaCl. After 5 weeks at 23 °C /21 °C (± 2 °C, day/night temperature), the fresh roots were excised, blotted on filter paper, and weighed. Values are means ± SE (six replicates).The data were subjected to Duncan's multiple range test.

WT and T_2 shoot cuttings were grown in rooting medium for 2 weeks. Healthy seedlings were transferred to 5.8 × 5.8 × 8-cm plastic pots with peat moss soil and watered with tomato fertilizer ((9N-4.4P-12.5K, Schultz, Inc., Bridgeton, MO) for one week. Stress was imposed by watering plants with tomato fertilizer (Tomato Food, Schultz, Inc., Bridgeton, MO) and NaCl (200 mM or 250 mM) solution every three days. Controls received only fertilizer. The plants were maintained in a greenhouse with natural lighting supplemented with sodium vapor lamps (1000W, Philips, Inc., Eindhoven, Netherlands) for 16-h photoperiod at approximately 23 °C /21 °C (± 2 °C, day/night temperature). Height (from the apex to soil) was measured after 10 d. The extent of injury was evaluated visually at 10, 20, and 30 d following treatment. The scale was as follows: 0, no injury; 1, slow growth but no obvious damage, ≤20% leaf area exhibited injury; 2, leaves turned yellow, 21 to 40% of the leaf area injured; 3, plants wilted, 41% to 60%

leaf area injured; 4, seriously damage, the plant became soft and could not remain upright, 61% to 80% leaf area injured; 5, 81% to100% leaf area injured or plant died. Mean values of 5 replicates were calculated and the ranks were subjected to the non-parametric Kruskal-Wallis test. The leaf discs from WT and transgenic plants grown for 10-d under NaCl (200 mM) stress were then frozen in liquid nitrogen and stored at –80 °C for further enzyme activity gel analyses.

3.4 Results

Overexpression of the Mn-SOD Gene

No detectable GUS activity was seen in WT leaves (Fig. 3.1A). Transgenic leaves stained intensely blue, indicating high levels of GUS activity (Fig.3.1B). No PCR products could be amplified from WT plants, whereas products of the expected size for *Mn-SOD* (≈0.8 bp) were amplified from all transgenic lines (Fig. 3.2). Northern-blot analysis was performed to access the mRNA levels in transgenic plants. All the transgenic plants contained transcript but levels varied. WT plants did not exhibit transcript that hybridized to the probe (Fig 3.3). Two transgenic lines, S4 and S20, were selected for further study because they showed higher expression level.

The selected transgenic lines were screened by gel assay for the presence of Mn-SOD activity. Two isoforms were observed in all plants, and may represent chloroplastic (chl) and cytosolic (cyt) Cu/Zn-SOD (Perl et al., 1993). Transgenic plants displayed an additional lower mobility band corresponding to the Mn-SOD

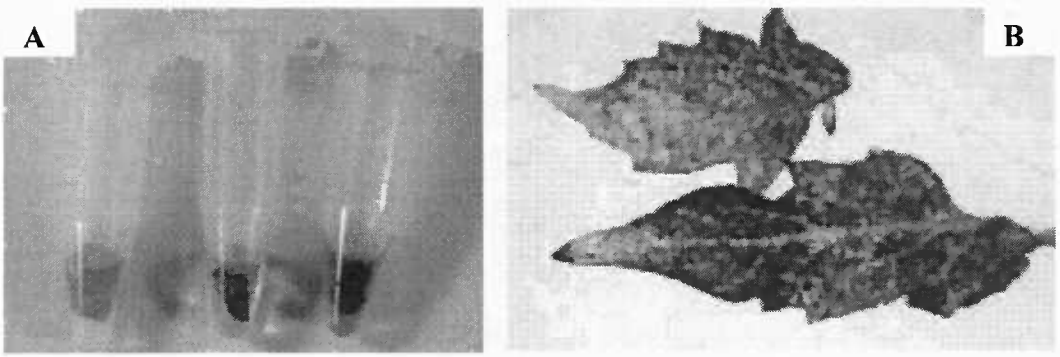

Fig. 3.1. Leaf discs from wild-type (WT) and transgenic (Lines S1, S2) tomato plants stained for GUS activity (A). Staining of entire leaves (B).

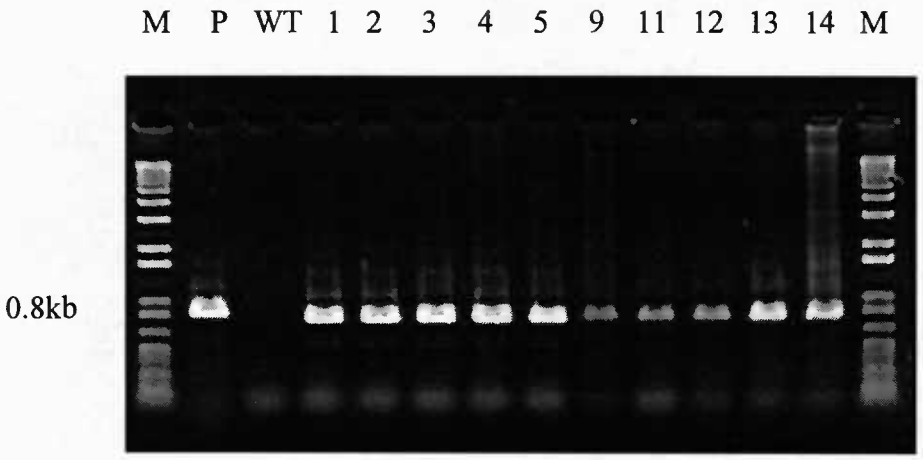

Fig. 3.2. PCR analysis of wild-type and transgenic tomato plants used to confirm presence of the *Mn-SOD* transgene. Genomic DNA isolated from WT and independently transformed lines was used in PCR reactions with a 35S promoter- and *Mn-SOD*-specific primers, respectively. M, 1 kb DNA marker; P, positive plasmid control; WT, wild-type plant; 1 to 14, independent transgenic lines.

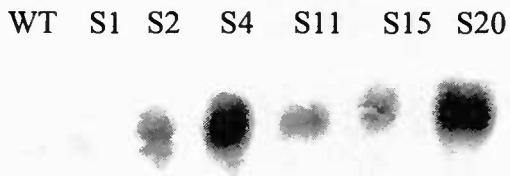

Figure. 3.3. Northern blot analysis of RNA isolated from the T_1 plants. 30 µg of total RNA was used per lane for each blot. Blots were probed with ^{32}P-labeled *Mn-SOD* PCR products. WT, wild-type plant; S1 to S20, independent transgenic lines.

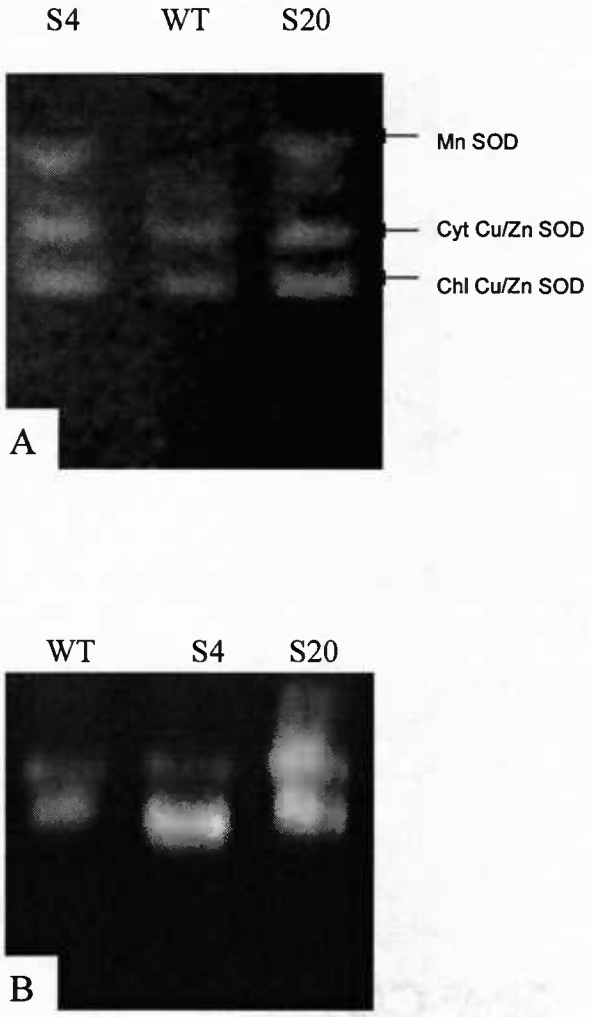

Figure. 3.4. Detection of (A) SOD and (B) APX activity in leaves of wild-type (WT) and transgenic *Mn-SOD* (Lines S4, S20) tomato plants. About 70 μg protein was loaded on the non-denaturing polyacrylamide gel.

enzyme activity (Fig. 3.4A). Total APX activity was also measured. Lines S4 and S20 exhibited 2- to 3-fold higher APX activity than in WT plants (Fig. 3.4B).

Effects of MV Stress

Methyl viologen-induced electrolyte leakage to transgenic plants was significantly less than to WT ($P<0.05$, Fig. 3.5). Mean electrolyte leakage in WT leaves was about 47%. In comparison, Mn-SOD-expressing lines (S4, S20) had significantly lower electrolyte leakage (approximately, 30%). After two days under MV treatment, total SOD activity was 1.5- to 2-fold higher in transgenic plants (Fig. 3.6A). APX activity of transgenic plants was 6- to 7-fold higher than WT plants following MV treatment (Fig. 3.6B).

Effects of NaCl Stress

The effect of 150 mM NaCl on the percent seed germination is shown on Fig. 3.7. 50% to 70% germination was recorded for transgenic and 30% for WT seeds after 13 days of treatment.

After 5 weeks of treatment, the fresh root weight of transgenic plants was significantly ($P<0.05$) greater than that of WT tomato plants at 200 mM NaCl and NaCl (250 mM) respectively (Fig. 3.8).

Irrigation of transgenic and WT tomato plants with NaCl solution severely inhibited their shoot height growth (Fig. 3.9). The margins of leaflets in WT plants became necrotic following 10 d of salt treatment (Fig. 3.10). Visible injury to WT was rated as 2.2. By contrast, injury to transgenic plants was significantly

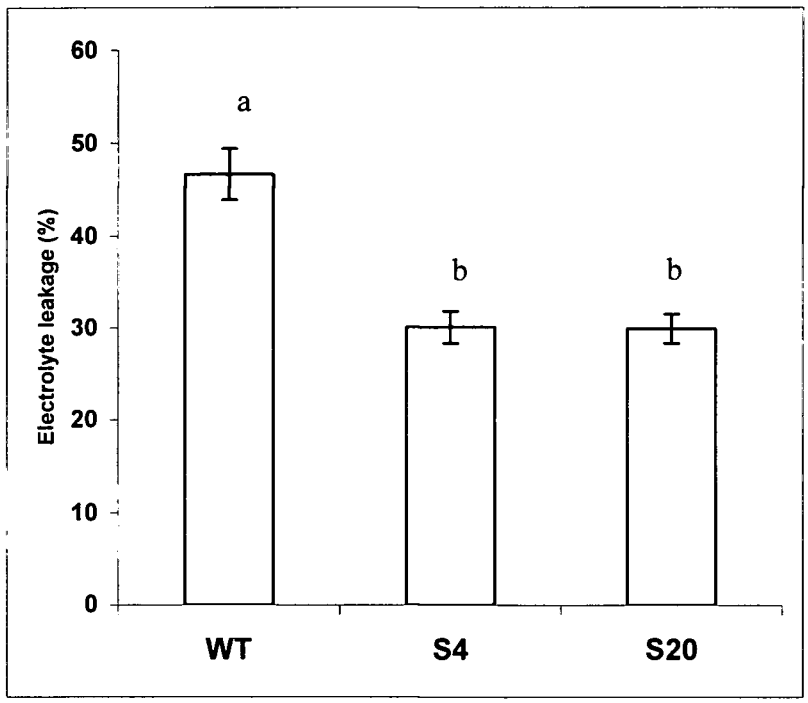

Fig. 3.5. Effects of methyl viologen (10^{-4} M) stress on electrolyte leakage from transgenic *Mn-SOD* (Lines S4, S20) and wild-type (WT) tomato plants. Bars represent SE for three replicates. Different letters indicate significant differences ($P<0.05$) between means (Duncan's multiple range test).

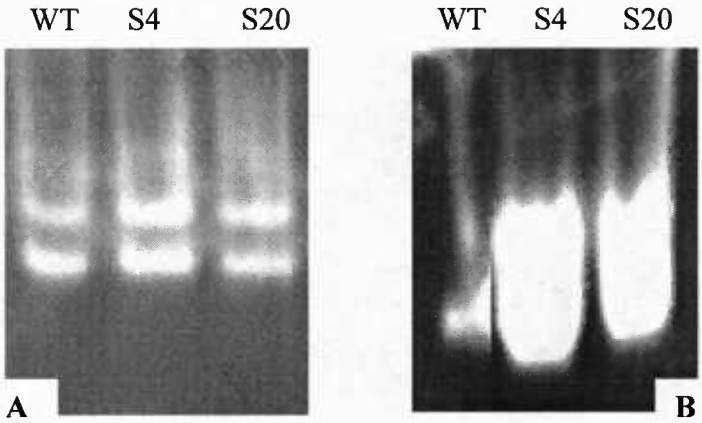

Fig. 3.6. Detection of (A) SOD, and (B) APX activity in wild-type (WT) and transgenic *Mn-SOD* (Lines S4, S20) tomato plants under MV (10^{-4} M) stress. Approximately 70 μg protein was loaded on the native PAGE gel.

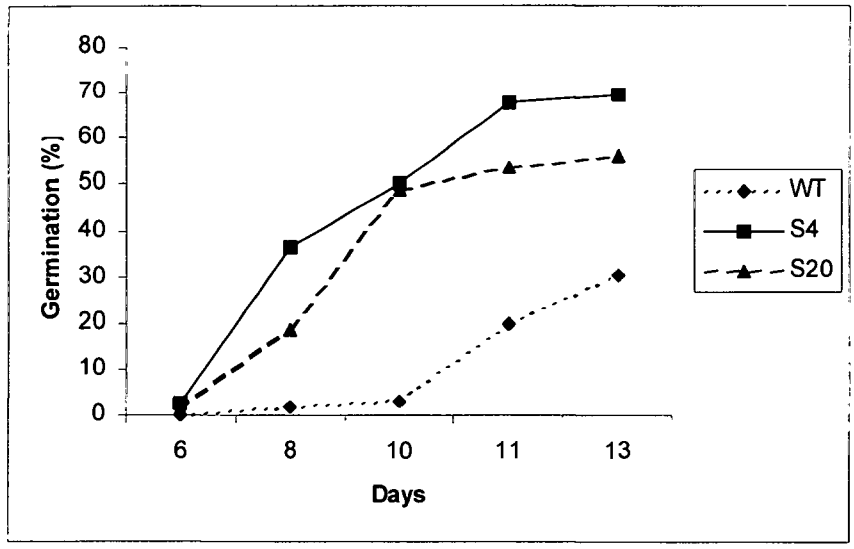

Fig. 3.7. Germination in T_2 transgenic *Mn-SOD* (Lines S4, S20) and wild-type tomato seeds (n≈80) treated with NaCl (150 mM) for various periods of time.

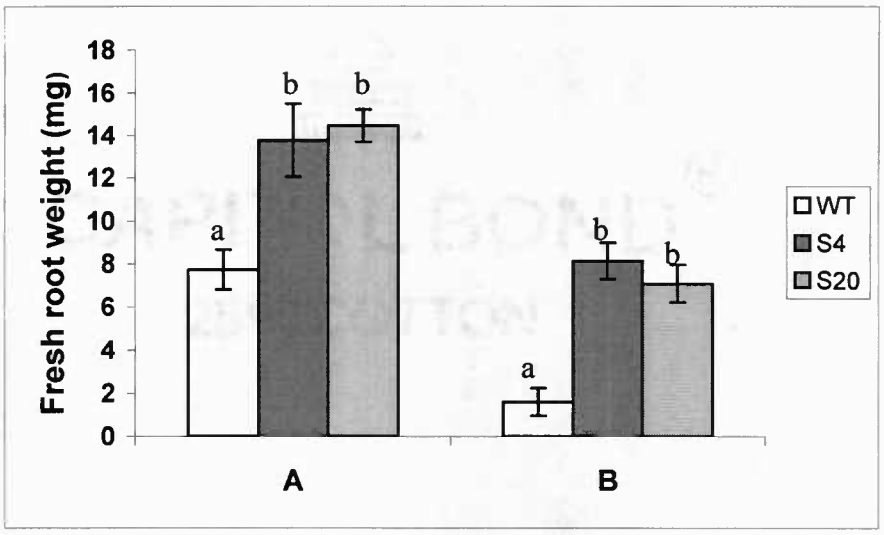

Fig. 3.8. Effects of 200 mM (A) or 250 mM (B) NaCl treatments on root development of wild-type (WT) and transgenic *Mn-SOD* (Lines S4, S20) tomato plants. Fresh weight was determined 5 weeks after the treatment. Values are means ± SE (n=6). Different letters indicate significant differences ($P<0.05$) between means within each NaCl treatment level (Duncan's multiple range test).

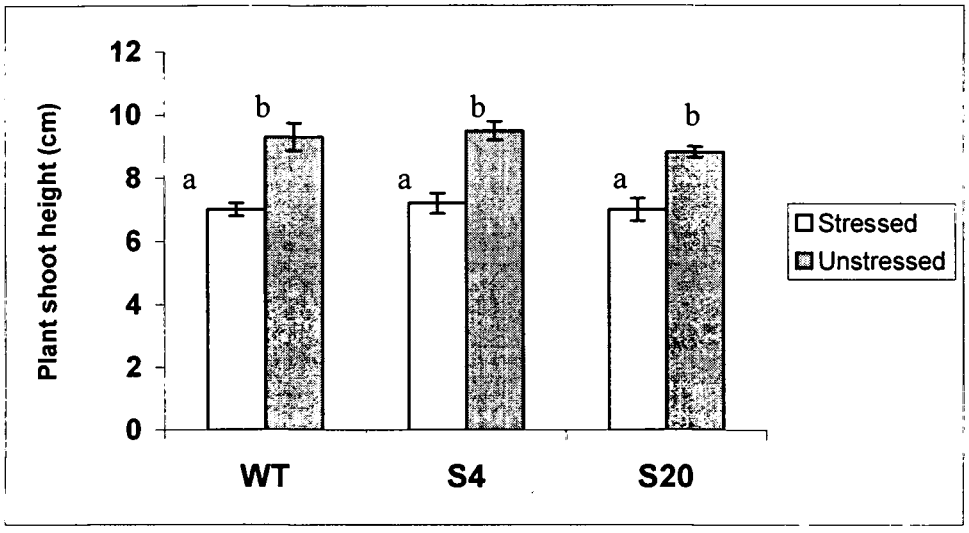

Fig. 3.9. Effects of salt stress on the height growth of seedlings of wild-type (WT) and transgenic *Mn-SOD* (Lines S4, S20) tomato plants. Plant shoot height (highest point of the apex reaches relative to the soil) was measured after 10 d treatment. Unstressed = 0 mM NaCl; Stressed = 200 mM NaCl. Data are means ± SE with three replicates. Different letters indicate significant differences ($P<0.05$) between means (*t* test).

* Data for WT was also cited in the previous chapter.

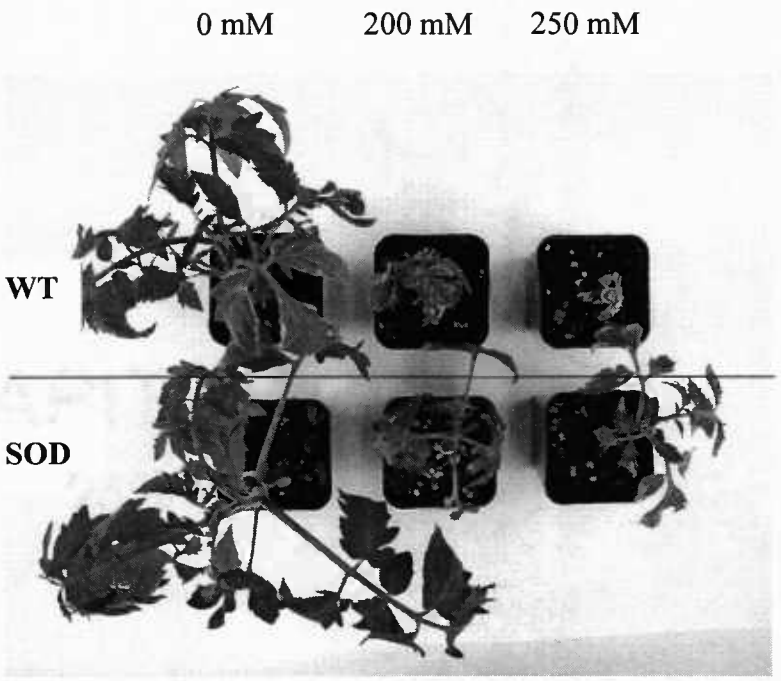

Fig.3.10 Effects of salt stress on wild-type (WT) and transgenic *Mn-SOD* (Line S20) tomato plants. Plants were treated with 200 mM and 250 mM NaCl for 18 d in the greenhouse.

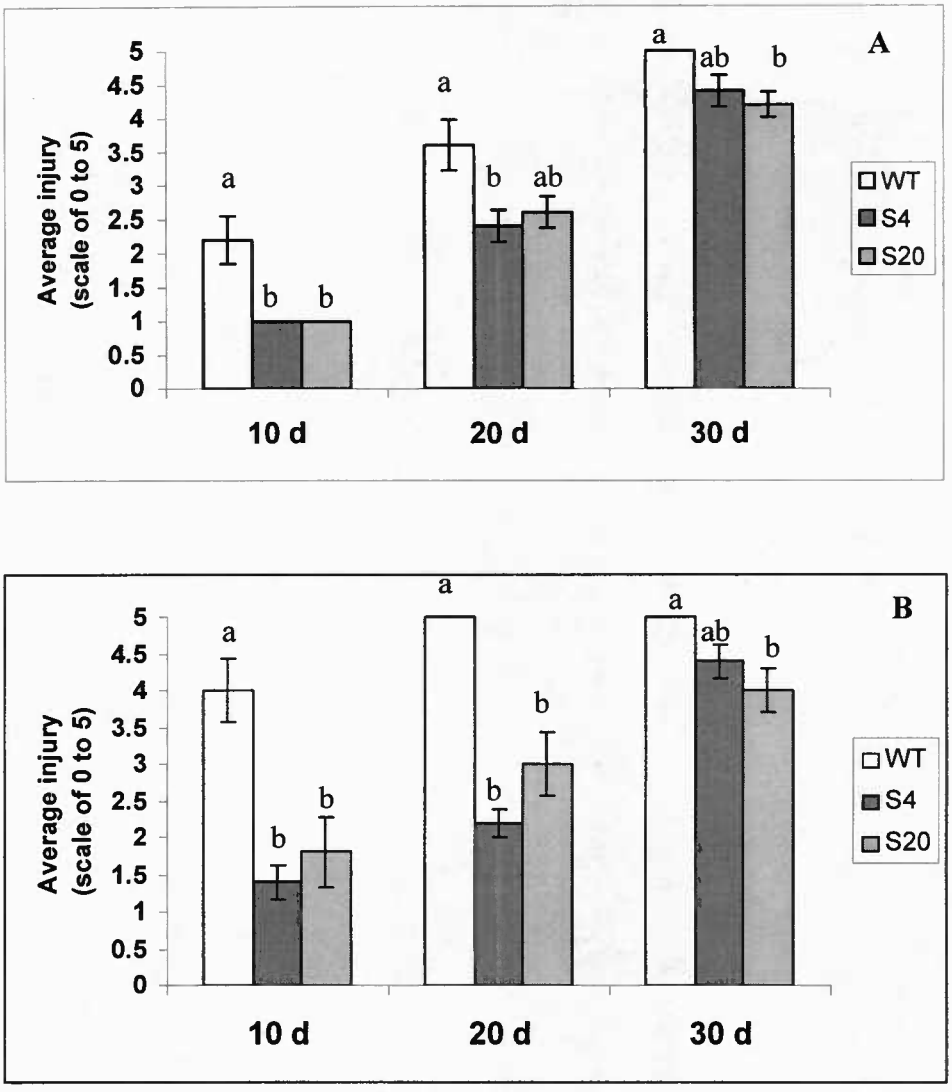

Fig. 3.11. Injury to wild-type (WT) and transgenic *Mn-SOD* (Lines S4, S20) tomato plants under stress from (A) 200 mM or (B) 250 mM NaCl treatments after 10, 20, and 30 d. No visible injury = 0; slow growth, and <20% visible injury = 1; yellowing leaves and 21-40% of leaf area with visible injury = 2; wilted plants and 41-60% visible injury = 3; seriously damaged plant unable to remain upright with 61-80% visible injury = 4; dead plant or >80% visible injury = 5. Values are means ± SE (n=5). Different letters indicate significant differences ($P< 0.05$) between means within each sampling time (Kruskal-Wallis test).

lower ($P<0.05$; Fig. 11), showing only slight leaf area injury (scale 1). The effect of the salt treatment was still apparent but severe after 20 d. WT plants displayed visible necrotic injury (scale 3.6) after 20 d. In contrast, the transgenic seedlings showed less injury (Fig. 3.11A). At 250 mM NaCl, WT seedlings showed injury scale 5 after 20 d, whereas the transgenic plants exhibited less wilting injury (average scale 2.2 for S4 and 3 for S20). The differences in visible injury between transgenic and WT plants were statistically significant ($P<0.05$, Fig. 3.11B). The leaf APX activity in transgenic plants was about 4- to 5-fold higher than that in WT plants after 10 d of NaCl (200 mM) treatment (Fig. 3.12).

3.5 Discussion

Numerous studies have indicated that oxidative stress enhances SOD activity (Donahue et al., 1997; El-Saht, 1998). Abiotic stresses, such as chilling, drought, and salt stress have been correlated with increased in SOD activity (El-Saht, 1998; Dionisio-Sese and Tobita, 1998; Fadzilla et al., 1997; Scandalios, 1993).

Our study indicated that transgenic tomato plants expressing the rubber *Mn-SOD* gene had enhanced tolerance to both MV- and salt-induced oxidative stress. Transgenic plants had less electrolyte leakage than WT plants (Fig. 3.5), suggesting that overexpression of Mn-SOD in the transgenic plants reduced cellular damage caused by ROS (Bowler et al., 1991). After MV treatment, APX and SOD activity was higher in transgenic plants and related to a plant's resistance to ROS damage (Allen, 1995; Van Camp et al., 1994).

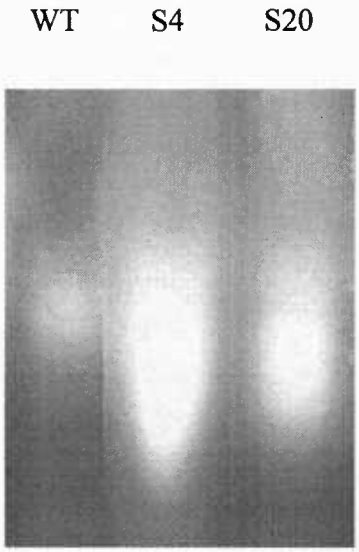

Fig. 3.12. Detection of APX activity in wild-type (WT) and transgenic *Mn-SOD* (Lines S4, S20) tomato plants treated with 200 mM NaCl for 10 d. Approximately 70 μg of total protein was loaded in each lane of a non-denaturing polyacrylamide gel.

An increase in ROS scavenging capacity is required to enable rapid removal of ROS produced during early seed imbibition (Gidrol et al., 1994). In this study, transgenic seeds were more tolerant of NaCl than WT seeds (Fig. 3.7). The transgenic plants had higher SOD and APX activity, which could prevent the accumulation of O_2^- and H_2O_2 concentration during the early stages of seed germination.

In our study, we found that shoots of transgenic tomato plants produced more roots biomass than WT plants under salinity stress (Fig. 3.8), indicating that overexpression of Mn-SOD enabled the transgenic plants to better deal with oxidative stress. Also, seedlings of transgenic *Mn-SOD* tomato plants showed less injury (Fig. 3.11) following NaCl (200 mM and 250 mM) stress than WT plants. This is consistent with the theory that increased antioxidant enzyme activity can prevent NaCl-induced oxidative stress (Fadzilla et al., 1997; Tanaka et al., 1999).

Our study showed that APX activity increased due to NaCl treatment (Fig. 3.10), and is consistent with other reports (Hernández et al., 1999; Mittova et al., 2002; Sairam and Sreenivasulu et al., 2000; Srivastava, 2002). This confirms earlier reports that APX plays an important role in scavenging H_2O_2 induced by NaCl stress. However, SOD activity decreased after 10 d of NaCl treatment (data not shown). The reason for this decrease in activity is not known but may be related to the long exposure to NaCl.

In this study, the overexpression of Mn-SOD in transgenic tomato plants enhanced seed germination, root development and seedling tolerance to NaCl stress. We conclude that increased antioxidant levels may play an important role

in scavenging ROS when plants are exposed to salt stress.

Acknowledgments

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Chapter 4

Transgenic Tomato (*Lycopersicon esculentum*) that Overexpress Mn-SOD or cAPX Exhibit Enhanced Tolerance to UV-B and Heat Stress

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4.1 Abstract

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and hydroxyl radicals, are by-products of biological redox reactions. ROS can denature enzymes and damage important cellular components. Plants have developed antioxidant enzymes, such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) to scavenge ROS and detoxify them. The effect of increased Mn-superoxide dismutase (Mn-SOD) and cytosolic ascorbate peroxidase (cAPX) on heat and UV-B stress tolerance was studied using transformed tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) plants. This research demonstrates, in either laboratory or field tests, the potential to enhance tolerance to heat, UV-B, and sunscald stress by gene transfer. Overexpression of Mn-SOD or cAPX in transgenic tomato enhanced resistance to heat (40 °C) and UV-B stress compared to wild-type (WT) plants. When leaf disks were placed at 40 °C for 13 h, the electrolyte leakage of disks from WT were 93%, whereas transgenic lines exhibited 24% to 52% leakage, depending on the specific lines. When fruits of WT and transgenic plants were exposed to UV-B (2.5mW/cm^2) for five days, the extent of browning was 95% and 33-85%, respectively. In field tests, the detached fruits from field-grown transgenic plants showed more resistance to exposure to direct sunlight than fruits from WT plants. APX activity in leaves of *cAPX* transgenic plants was several folds higher than in leaves of WT plants when exposed to heat, UV-B, and drought stresses.

Key words: *Lycopersicon esculentum*, Mn-SOD, cAPX; heat, UV-B, sunscald.

Abbreviations: SOD, superoxide dismutase; APX, ascorbate peroxidase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; WT, wild-type.

4.2 Introduction

Reactive oxygen species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), can have deleterious effects such as lipid peroxidation, DNA mutation and protein denaturation on cells (Bowler et al., 1992; Foyer et al., 1994; Scandalios, 1993). ROS may be produced in response to abiotic and biotic stresses, and are associated with a number of physiological disorders in plants (Allen, 1995). These stresses, to varying degrees, affect crop growth, resulting in huge losses of plants and plant products annually throughout the world (Scandalios, 1993). Plants have evolved efficient nonenzymatic and enzymatic systems to cope with ROS. Nonenzymatic systems, such as ascorbate, glutathione, α-tocopherol, and carotenoids, can react directly with ROS (Allen 1995; Foyer et al., 1994). Enzymatic systems like SOD scavenge the superoxide anion. APX removes H_2O_2. Glutathione reductase (GR) also can remove H_2O_2 via the ascorbate-glutathione cycle (Foyer et al., 1994; Noctor and Foyer, 1998; Scandalios, 1993). Scavenging of ROS is important for maintenance of normal plant growth (Allen, 1995).

Heat and ultraviolet B (UV-B, 280-320 nm) stimulates the production and accumulation of toxic ROS, which results in lipid peroxidation and membrane injury (A-H-Mackerness, 2000; Davidson et al., 1996; He et al., 2002; Jiang and Huang, 2001a, b; Sairam et al., 2000). An interaction of high temperatures and

light intensity (UV-B) can induce sunscald in fruits of many horticulture crops (Rabinowitch et al., 1983; Renquist et al., 1989). Sunscald of fruits is manifested as either tissue browning or desiccation (Renquist et al., 1989). Tolerance of tomato fruits to sunscald damage by controlled heat treatment was accompanied by an increase in superoxide dismutase (SOD) activity (Rabinowitch et al., 1982; Rabinowitch and Sklan 1980). Rabinowitch and Sklan (1980) reported that SOD activity levels were high in immature green fruits and declined to a minimum in the mature-green and breaker (early ripening) stages, which are known to be most susceptible to sunscald (Retig and Kedar, 1967). During tomato ripening, oxidative processes such as lipid peroxidation, protein oxidation, and hydrogen peroxide content, increase at the tomato color breaker stage. In contrast, antioxidant enzyme activities of SOD, and APX decreased at the breaker stage of ripening (Jimenez et al., 2002).

Tolerance to heat and UV-B correlates with an increased capacity of the plants to scavenge or detoxify activated oxygen species (Chaitanya et al., 2002; Davidson et al., 1996; Mazza et al., 1999; Sairam et al., 2000), suggesting that increased antioxidant enzyme activity might protect plant tissues against sunscald. Wisniewski et al. (2002) reported that transgenic apple plants that overexpressed cAPX had improved resistance to heat stress. Leaf disks from wild-type (WT) apple plants exhibited 100% electrolyte leakage, whereas transgenic lines exhibited 40% to 75% leakage. Transgenic apple plants also showed tolerance to UV-B and freezing stress. Chen and Pan (1998) reported that overexpression of

Cu/Zn-SOD can enhance the tolerance to heat stress or UV-B radiation in *Arabidopsis thaliana*.

The overexpression of enzymes involved in scavenging ROS in plants by gene transfer technology may be able to increase tolerance of plants to oxidative stresses and improve plant performance under these conditions (Foyer et al., 1994; Sen Gupta et al., 1993a, b; Kubo et al., 1995; McKersie et al., 1999; Wang et al., 1999;Yoshimura et al., 2000).

The objective of this study was to determine the tolerance of tomato plants and fruits to heat and UV-B stresses when *Mn-SOD* and *cAPX* genes are introduced into the plants.

4.3 Materials and Methods

Plant Material and Growth Conditions

Tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) was previously transformed by *Agrobacterium tumefaciens* with a binary vector containing pea cAPX cDNA (Mittler and Zilinskas, 1991) and rubber Mn-SOD cDNA (Miao and Gaynor, 1993), respectively. Independently-transformed *cAPX* and *Mn-SOD* lines of T_2 generation and wild-type (WT) plants were used in the experiments. Cuttings from regenerated transgenic T_2 plants were rooted in rooting medium (MS + 0.2 $mg.L^{-1}$ NAA + 400 $mg.L^{-1}$ cefotaxime + 7 $g.L^{-1}$ agar) and transplanted in peat moss soil (Lakeland Peat Moss, Inc., Edmonton, Alberta, Canada). They were grown in a greenhouse with natural lighting supplemented with sodium

vapor lamps (1000W, Philips, Inc., Eindhoven, Netherlands) to provide a 16-h photoperiod at approximately 23 °C /21 °C (± 2 °C, day/night temperature).

Heat, UV-B, Sunscald, and Drought Stress Tests

Heat Stress Tests

Leaf discs from WT and T_2 plants were heat stressed and the disruption of membrane integrity was estimated by electrolyte leakage. Two leaf discs (0.62 cm in diameter) were punched out with a cork borer from the youngest fully expanded leaves of T_2 transgenic and WT plants of the same age. The leaf discs were inserted in a test tube containing 10 mL of deionized, distilled water. The base of the tube was submerged in a water bath at 40 °C and removed after 0.5, 2, 3, 5, 7, 9, 11, and 13 h. Following heat treatment, electrolyte leakage was measured using a conductivity meter (ElectroMark Analyzer, Markson Science, Inc., Del Mar, CA). Determining the percent electrolyte leakage was done based on the method of Wisniewski et al. (1997). Means for all values are an average of two subsamples in each plant with three replications. The significance of differences ($P \leq 0.05$) between means was estimated by Duncan's multiple range test (NCSS-PASS software, NCSS Inc., Williamsport, PA). Arcsine square root transformation was performed before data analysis. Nontransformed means are presented.

Discs (0.62 cm) from fourth or fifth leaves of WT and T_2 transgenic *cAPX* and *Mn-SOD* lines were put into an eppendorf tube (1.5 mL) and were incubated

in a water bath (42 °C) for 4 h. The samples were then frozen in liquid nitrogen and stored at –80 °C for further enzyme activity gel analysis.

UV-B Stress Tests

Uniform mature green tomato fruits, randomly detached from WT and T_2 plants grown in the field (Lewis-Brown Farm at Corvallis, Oregon), were exposed to UV-B radiation provided by UV-B fluorescent lamps (Blak-Ray lamp, UVP, Inc., San Gabriel, CA, USA) in a room with no other source of light. The fruits were placed 20-cm away from the light source (2.5 mW/cm^2) and exposed 10 h per day for 3, 4, or 5 days. The extent of injury was based on colors from a white to yellow color of the epidermis, followed by browning when the injury was more severe. UV-B fruit injury was assessed by estimating the percent of exposure area that exhibited browning. The means of injury values are an average of three replicates. The significance of differences ($P<0.05$) were estimated by Duncan's multiple range test. Arcsine square root transformation was performed before data analysis; nontransformed means are presented.

For UV-B stress enzyme activity gel analysis, shoots with three to four leaves from greenhouse-grown WT and T_2 plants were placed in tubes containing 50 mL of distilled water. The shoots were placed 20 cm below the UV light source for 4 h. After treatment, leaf discs (0.62 cm) were excised and frozen in liquid nitrogen before being stored at –80 °C.

Sunscald Tests

In a field test, mature green tomato fruits, randomly detached from WT and T_2 plants were exposed to field conditions for 15 d (from September 8 to 23, Lewis-Brown Farm, Corvallis, Oregon). Fruit injury was visually recorded as described by Rabinowitch et al. (1986). Sunscald injury was characterized by the bleaching (with a brown or yellow halo around the bleached area) and necrosis of the pericarp. The injured area eventually became sunken and dry. The percent of injured fruit area was estimated visually. Mean values are an average of five replicates. The significance of differences ($P<0.05$) were estimated by Duncan's multiple range test. Arcsine square root transformation was performed before data analysis; nontransformed means are presented.

Drought Stress Test

For drought stress test, 15 d-old rooted WT and transgenic cuttings were grown in plastic pots (15 cm diameter; 14.5 cm height) on peat moss (Lakeland Peat Moss, Inc., Edmonton, Alberta, Canada). After four weeks in a greenhouse, the plants were subjected to drought stress by withholding water for 7 d. Leaf discs (1.02 cm in diameter) were excised from the fourth or fifth leaf of the treated plants, frozen in liquid nitrogen, and stored at –80 °C for later use in enzyme gel activity experiments.

APX Enzyme Gel Activity Assay

About 100 mg stored leaf tissue was ground to fine powder in the liquid nitrogen and homogenized in 200 µl of grinding buffer (100 mM $NaPO_4$, pH7.0; 5mM ascorbate; 1 mM EDTA, pH 8.0; 10% glycerol; and 0.001% bromophenol blue), and centrifuged at 13,000g for 6 min at 4 °C. The supernatant was collected and protein concentration was determined using a Protein Assay System (Bio-Rad; Hercules, CA). Approximately 70 µg of total protein was loaded into each lane of a non-denaturing, 10% polyacrylamide gel and electrophoretically (PAGE) separated for 5 h at 4 °C in a 1X Tris-glycine buffer (24 mM Tris, 192 mM glycine), with subsequent staining for APX activity as described by Mittler and Zilinska (1993).

4.4 Results

Heat Stress Tests

The electrolyte leakage of the leaf discs are presented in Fig. 4.1. The amount of leakage increased with time of exposure. The differences in heat stress resistance between the WT and *Mn-SOD*- (S15) and *cAPX*- (A9) expressing plants were statistically significant after 2 h treatment, and all transgenic plants were different after 3 h treatment (P <0.05). After 13 h treatment, the electrolyte leakage in WT plants was 93%, whereas that in transgenic tomato lines A9, A16, S2, and S15 was 24%, 52%, 42%, and 32%, respectively.

UV-B Stress Tests

The first visible sign of damage was seen on day 3, with transgenic and WT fruits showing about 10-35% and 61% injury, respectively. After 5 d, WT and transgenic fruits showed 95% and 33-85% injury, respectively, as evidenced by a brown discoloration of the exocarp tissue (Table 4.1). Compared to WT fruits, transgenic fruits suffered significantly (P <0.05) less UV-B browning injury.

Sunscald Tests

Exposure of detached fruits to natural sunlight under field conditions resulted in sunscald injury to all fruits after 15 d. However, the fruits of the transgenic tomatoes were less injured (Fig. 4.2). Sunscald injury in WT fruit averaged about 21%. In contrast the *cAPX*- and *Mn-SOD*-transgenic tomatoes exhibited 1% to 17% sunscald injury. Significant differences in sunscald injury between transgenic APX lines (A9, A13, A16, A24), SOD lines (S4, S11, S15), and WT fruit (P<0.05) were found (Fig 4.2).

APX Enzyme Activity

The APX enzyme activity gel assay revealed higher APX activity in transgenic plant lines (A9, A16) than in WT plants after UV-B (9- to 10-fold), heat (3- to 3.2-fold), and drought stress (5- to 6-fold) treatment (Fig. 4.3). We also tested APX activity of Mn-SOD transgenic plants (data not shown). APX enzyme activity was not different between transgenic *Mn-SOD* and WT plants.

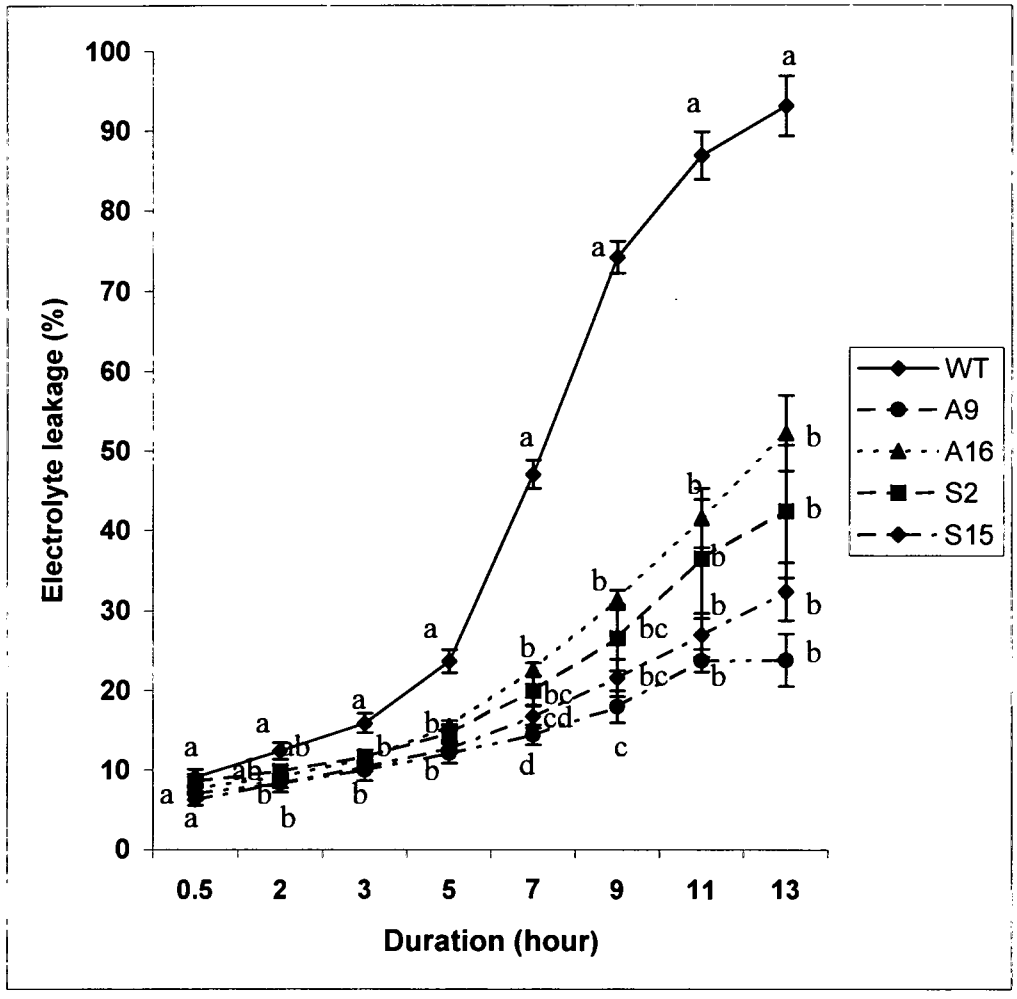

Fig. 4.1. Electrolyte leakage of leaf discs of wild-type (WT), and transgenic *cAPX* (Lines A9, A16) and *Mn-SOD* (Lines S2, S15) tomato plants following heat stress (40 °C) of varying duration (0.5, 3, 5, 7, 9, 11, and 13 d). Values are means ± SE (n=3). Different letters indicate significant differences ($P<0.05$) between means within each sampling time (Duncan's multiple range test).

Genotype	No. days UV-B treatment 3	4	5
WT	61.4 ± 10.6 a	92.8 ± 3.8 a	94.6 ± 3.0 a
A9	20.4 ± 5.8 b	30.1 ± 2.8 b	33.4 ± 4.4 c
A16	34.3 ± 0.8ab	36.9 ± 0.9 b	37.4 ± 1.0 c
A18	19.1 ± 6.0 b	49.6 ± 11.0 b	52.0 ± 11.2 bc
S1	34.0 ± 9.5ab	58.8 ± 15.8 b	84.7 ± 7.0 ab
S2	35.5 ± 2.4 b	59.1 ± 7.7 b	69.4 ± 3.2 abc
S11	9.0 ± 1.5 b	26.0 ± 7.8 b	44.7 ± 17.3 bc
S15	23.1 ± 10.6 b	29.2 ± 9.3 b	60.1 ± 14.1 bc

Table 4.1. Percent injury (exposed area exhibiting browing) in fruit of wild-type (WT) and transgenic *cAPX* (Lines A9, A16, A18) and *Mn-SOD* (Lines S1, S2, S11, S15) tomato plants after 3, 4, and 5 days UV-B (2.5 mW/cm^2) treatment. Values are means ± SE (n=3). Different letters indicate significant differences ($P \leq 0.05$) between means within a sample time (Duncan's multiple range tests).

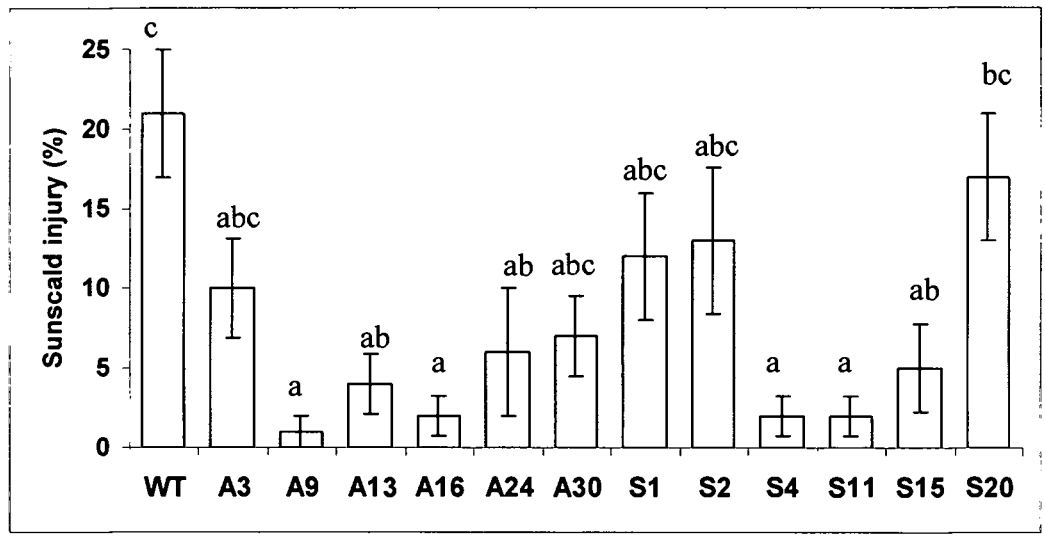

Fig. 4.2. Percent sunscald injury to detached fruit from wild-type (WT) and transgenic *cAPX* (Lines A3-A30) and *Mn-SOD* (Lines S1-S20) tomato plants under field conditions for 15 d. Values are means ± SE (n=5). Different letters indicate significant differences ($P<0.05$) by Duncan's multiple range tests.

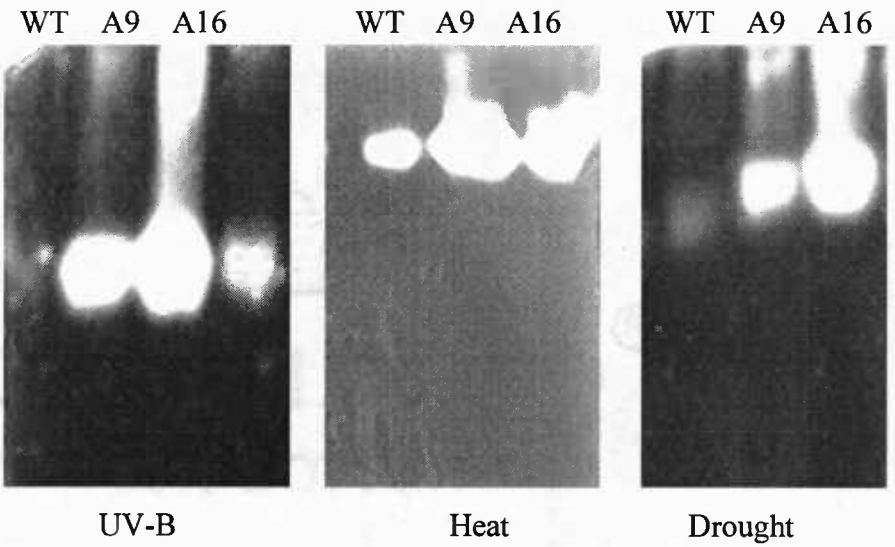

Fig. 4.3. APX enzyme activity gel assay. UV-B (2.5 mW/cm^2) for 4 h; heat (42 °C) for 4 h; and drought stress (withholding water for 7 d). About 70 µg protein was loaded in each lane of the native gel.

4.5 Discussion

The overexpression of the Mn-SOD and cAPX enzymes in tomato plants increased the resistance of their leaves and fruit tissues to heat and UV-B stresses, supporting the results reported by others (Wisniewski et al., 2002; Chen and Pan, 1998).

Electrolyte leakage in stressed transgenic plants was much less than in WT plants (Fig. 4.1) providing strong evidence that overexpression of cAPX and Mn-SOD resulted in enhanced protection of membrane lipid peroxidation caused by ROS during heat stress (Huang et al., 2001). After UV-B stress, WT fruits showed more browning damage than did transgenic fruits (Table 4.1), indicating that overexpression of antioxidants might play an important role in detoxifying heavy loads of ROS during UV-B stress to provide protection (Balakumar et al., 1997; Mazza et al., 1999).

Several transgenic fruit lines showed significantly less ($P<0.05$) sunscald damage than did WT fruits (Fig. 4.2), suggesting that overexpression of antioxidant genes might increase tolerance to sunscald. Transgenic lines A18, S2, and S15 were more tolerant of heat and UV-B stress in the laboratory tests but not in the field test. It is possible that plants utilize different antioxidant mechanisms against ROS during various physiological stages and exposure to various degrees of stress (Allen, 1995).

APX enzyme activity was higher in *cAPX*-expressing plants after heat, UV-B, and drought stress (Fig. 4.3), which suggests that the overexpression of antioxidant enzymes may play a role in protecting fruits from sunscald injury. We

also tested SOD enzyme activity by gel assay (data not shown) and found that after heat and UV-B stresses, the SOD bands were very faint, indicating that SOD enzyme activity was very low (data not shown). The reason for this low enzyme activity is not known but may be related to the loss of SOD activity following long exposure to the stress treatment. It is also possible that the effect of SOD is indirect, because the product of SOD activity is hydrogen peroxide, which has been implicated as an elicitor of genes related to stress tolerance (Sen Gupta et al., 1993b; McKersie et al., 1996; Prasad et al., 1994).

The results obtained with transgenic tomato plants overexpressing either SOD or APX provides better protection again heat, UV-B and sunscald. This elevated activity of several antioxidant enzymes might play an important role in increasing stress tolerance against fruit sunscald. Given these results, along with those of other studies in which heat and UV-B effects on SOD or APX in transgenic plants (Chen and Pan, 1998; Wisniewski, et al., 2002), we believe that a similar approach might be applicable to other important fruits, such as apple, to improve tolerance against sunscald.

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Chapter 5

Conclusions

Increased activity and over-expression of the enzymes ascobate peroxidase (APX) and superoxide dismutase (SOD) in transgenic tomato plants increased their resistance to abiotic stresses. Seeds from transgenic plants germinated and grew better at low temperatures (9 °C) than seeds from WT plants. When exposed to stresses from salt (200 mM and 250 mM NaCl), temperature extremes (4°C or 40°C), or paraquat (10^{-4} M, MV), transgenic plants suffered less injury than WT plants. Finally, compared to WT plants, fruit from transgenic plants were found to be more tolerant to UV-B stress and sunscald.

Increased resistance of the transgenic tomato plants to these abiotic stresses, suggests that free radicals are involved, to some degree, in response of plants to these stresses. When exposed to MV, the activity of both SOD and APX in transgenic plants was higher than that of WT plants. In addition, we found APX activity of transformed *cAPX* plants was higher than that of WT plants when exposed stress from salt, UV-B, or temperature extremes. Increased activity of SOD or APX may increase the rate of superoxide conversion to hydrogen peroxide (H_2O_2) and/or H_2O_2 scavenging, resulting in increased protection of cells against free radicals damage.

Although increased APX activity of transgenic *cAPX* plants was well correlated with increased stress tolerance, we found the SOD activity of

transgenic Mn-SOD plants was not consistently correlated with stress tolerance. The poor correlation between stress tolerance and SOD activity in the Mn-SOD transgenic plants suggests that induction of other antioxidant genes, e.g. glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase, and expression of these genes may be required to maintain some specific amount of ascorbate in the plants, and, hence, maintain APX-mediated conversion of H_2O_2 to water. Interestingly, when exposed to MV or salt stress, foliar APX activities were higher in Mn-SOD transgenic plants than in WT tomato plants. This suggests that foliar APX activity was sufficient to destroy the H_2O_2 produced in transformed plants. It is also possible that the role of SOD in stress tolerance is indirect. The product of SOD activity is H_2O_2, which has been implicated as an elicitor of genes related to stress tolerance.

Our work has shown that increasing the levels of stress resistance by reinforcing the plant's reactive oxygen species (ROS) scavenging defense system with a single antioxidant transgene is an attainable goal. Since different enzymes may play specific roles in a plants ROS scavenging system, it is possible that if a transgenic plant contained a combination of more than one antioxidant transgenes, the transgenes would confer greater or broader stress tolerance to a transgenic plant. Further work needs to address whether optimal combinations of different transgenes may result in an increase in the level or extent of tolerance to abiotic stresses. Also, the specificity of gene combinations to different abiotic stresses needs to be determined in order to develop appropriate transgenic plants with increased tolerance to specific abiotic stresses.

The *cAPX* and *Mn-SOD* transgenes used in our work were under the control of the cauliflower mosaic virus 35S promoter. This is generally considered to be a constitutive promoter, able to control the expression of genes in all parts of the plant. Increased enzyme production in all plant tissues may not be the optimal means of regulating plant response to stress. Further study evaluating how expression of native genes which regulate antioxidant enzymes are controlled, and where and when these genes are expressed in the plant when the plants are under oxidative stress and in keeping a well-balanced and coordinated expression may increase our ability to develop stress-resistant, agronomically important crops. We have had to limit our discussion to the two antioxidant enzymes (cAPX and Mn-SOD) studied in our program. However, many parts of the antioxidative network are largely unexplored. We must also consider that antioxidants are not always accessible to some of the sites (e.g. membranes) where they are most needed in times of stress. Molecular analyses of antioxidant gene structure and expression, particularly promoter analysis, along with quantitative expression mutant analyses, may help unravel the mechanisms regulating the expression of the individual antioxidant genes at the appropriate location and time in the plant.

Finally, it remains to be seen whether yield production will be increased in the transgenic plants. The degree of protection observed in this study at the membrane, young seedling, and fruit of tomato will provide a substantial knowledge that overexpression of antioxidant genes might increase the yield production, because during crop production the plants are exposed to frequent period of mild or moderate stress throughout a growing season. Further field tests

of transgenic plants that overexpress antioxidant genes will provide the answer to this question that overexpression of antioxidant genes in plants is important in adaptation to field environment and enhance yield production.

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Appendix

Transfromation Protocol for Tomato Plants

1. Tomato (*Lycopericon esculentum*) seeds are sterilized for 20 min in 20% (v/v) chlorox (1% Na-hypochloride) plus Tween 20 (2-3 drops/100 mL), rinsed 3 times with sterile, distilled water, and then germinated aseptically on ½ MSO, under cool white fluorescent and Gro-Lux lights (100 to 150 $\mu mol.m^{-2}.s^{-1}$) with a 16-h photoperiod at 23 °C /21 °C (± 2 °C, day/night).

2. Cotyledon and hypocotyl explants are excised from 8-10-day-old seedlings and placed on the pre-culture medium for 24 h, under cool white fluorescent lights (100 to150 $\mu mol.\ m^{-2}.\ s^{-1}$) a 16-h photoperiod.

3. *A* single colony of *Agrobacterium* carrying the appropriate construct from the freshly streaked plate was used to inoculate a 25-mL culture medium (LB + antibiotics) and incubated at 28 °C with shaking (approx. 250 rpm) for one day.

4. Explants were incubated in *Agrobacterum* suspension diluted to OD_{600} 0.2 in MSO (liquid) for about 10-15 min, blotted on a sterile paper towels, and placed on solid cocultivation media for 2-4 days of co-cultivation at 23 °C /21 °C (± 2 °C, day/night) in the dark.

5. Explants were then transferred to selection medium for every 2- to 3- weeks. Plates are sealed with parafilm tape. Shoots regeneration is induced on selection medium for several months.

6. Regenerated shoots are further screened for kanamycin resistance by rooting on root induction medium.

Tissue culture media:

LB: 10 $g.L^{-1}$ Bacto Tryptone, 10 $g.L^{-1}$ Bacto Yeast Extract, and 5 $g.L^{-1}$ NaCl, adjusted to pH 7.0.

MSO liquid: MS salt with vitamins (Sigma, Inc.) + 30 $g.L^{-1}$ sucrose; pH 5.8.

MSO solid: MS salts with vitamins (Sigma, Inc.) + 30 $g.L^{-1}$ sucrose + 7 $g.L^{-1}$ agar; pH 5.8.

½ MSO: half strength MS salts with half stength vitamins + 20 $g.L^{-1}$ sucrose + 7 $g.L^{-1}$ agar; pH 5.8.

Pre-culture and co-cultivation medium: MSO solid + 1 $mg.L^{-1}$ Zeatin + 40 $mg.L^{-1}$ acetosyringone

Selection medium: MSO solid + 1 $mg.L^{-1}$ Zeatin + 100 $mg.L^{-1}$ Kanamycin + 500 $mg.L^{-1}$ cefotaxime.

Rooting medium: MSO+ 0.2 $mg.L^{-1}$ indole-3-acetic acid (IAA) + 50$mg.L^{-1}$ + Kanamycin + 500 $mg.L^{-1}$ cefotaxime.