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Transgenic tomato (*Lycopersicon esculentum*) overexpressing *cAPX* exhibits enhanced tolerance to UV-B and heat stress

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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 develop antioxidant enzymes, such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) to scavenge ROS and detoxify them. The effect of increased 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 cAPX in transgenic tomato enhanced resistance to heat (40 °C) and UV-B stress compared to wild-type plants. When leaf disks were placed at 40 °C for 13 hours, the electrolyte leakage of disks from wild-type were 93%, whereas two tested transgenic lines (A9, A16) exhibited 24% and 52% leakage respectively. When fruits of wild-type and transgenic plants were exposed to UV-B (2.5mW cm^{-2}) for five days, the extent of browning was 95%, and 33%, and 37%, respectively. In field tests, the detached fruits from field-grown transgenic plants showed more resistance to exposure to direct sunlight than fruits from wild-type plants. APX activity in leaves of *cAPX* transgenic plants was several folds higher than in leaves of wild-type plants when exposed to heat, UV-B, and drought stresses.

Key words: *Lycopersicon esculentum*, overexpression, ascorbate peroxidase (APX), heat, UV-B, oxidative stress, sunscald.

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 in cells (Bowler *et al.*, 1992; Scandalios, 1993; Apel and Hirt, 2004). 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 non-enzymatic and enzymatic systems to cope with ROS. Non-enzymatic systems, such as ascorbate, glutathione, α-tocopherol, and carotenoids, can react directly with ROS (Allen, 1995). 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 (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) stimulate 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, 2001; 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. During tomato ripening, oxidative processes such as lipid peroxidation, protein oxidation, and hydrogen peroxide content, increase at the 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 (Sen Gupta *et al.*, 1993a, b; Kubo *et al.*, 1995; McKersie *et al.*, 1999; Wang *et al.*, 1999; Wang *et al.*, 2005; 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 *cAPX* gene is introduced into the plants.

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). Independently-transformed *cAPX* 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 complimented with antibiotic (MS + 50 mg L^{-1} kanamycin + 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 23/21°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 immerged 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 for testing. Following heat treatment, electrolyte leakage was measured using a conductivity meter (ElectroMark Analyzer, Markson Science, Inc., Del Mar, CA). Determinination of 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 between means were determined using Duncan's multiple range test at $P < 0.05$ level (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* lines were placed in 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 were estimated by Duncan's multiple range test at $P < 0.05$ level.

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, 2002, 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 between means were determined by Duncan's multiple range test at $P < 0.05$ level.

Drought stress test: For drought stress test, 15 d-old rooted WT and transgenic plants 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 4ºC 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).

Results

Heat stress tests: The electrolyte leakage of the leaf discs are presented in Fig. 1. The amount of leakage increased with time of exposure. The differences in heat stress resistance between the WT and cAPX- expressing plants (A9 and A16) were statistically significant after 2 h treatment (P <0.05). After 13 h treatment, the

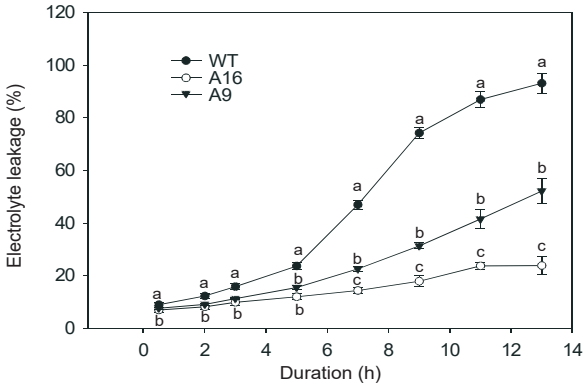

Fig. 1. Electrolyte leakage of leaf discs of wild-type (WT), and transgenic cAPX (Lines A9 and A16) tomato plants following heat stress (40 °C) of varying duration (0.5, 3, 5, 7, 9, 11, and 13 h). Values are means ±SE (n=3). Different letters indicate significant differences (P<0.05) between means within each sampling time (Duncan's multiple range test).

electrolyte leakage in WT plants was 93%, whereas that in transgenic tomato lines A9 and A16 was 24 and 52%, respectively.

UV-B stress tests: The first visible sign of damage was seen on day 3, with transgenic A9, A16 and WT fruits showing about 20, 34 and 61% injury, respectively. After 5 d, WT and transgenic A9, A16 fruits showed 95%, 33% and 37% injury, respectively, as evidenced by a brown discoloration of the exocarp tissue (Fig. 2). Compared to WT fruits, transgenic fruits suffered significantly less UV-B browning injury (P <0.05).

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. 3). Sunscald injury in WT fruit averaged about 21%. In contrast the cAPX- transgenic tomatoes (A3, A9, A13, A16, A24 and A30) exhibited 1% to 10% sunscald injury. Significant differences (P <0.05) in fruit sunscald injury between transgenic APX lines and WT fruit were observed (Fig. 3).

APX enzyme activity: The APX enzyme activity gel densitometric 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).

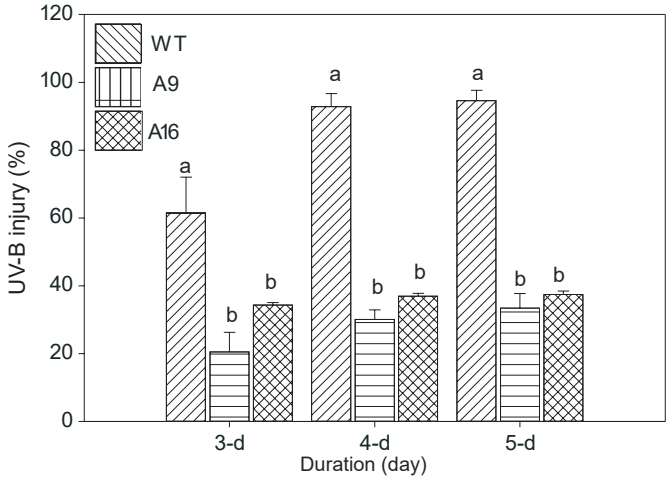

Fig. 2. Percent injury (exposed area exhibiting browning) in fruit of wild-type (WT) and transgenic cAPX (A9 and A16) tomato plants after UV-B (2.5 mW/cm2) treatment for 3, 4, and 5 days. Values are means ± SE (n=3). Different letters indicate significant differences (P<0.05) between means within a sample time (Duncan's multiple range tests).

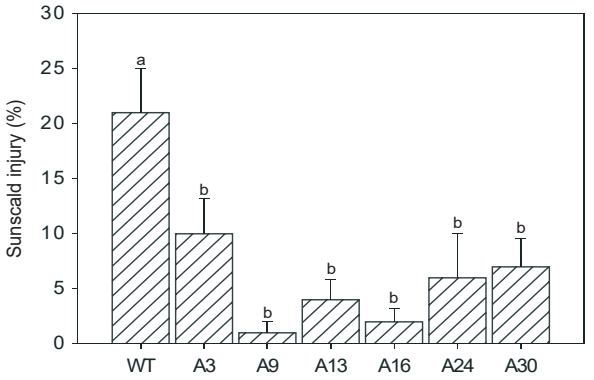

Fig. 3. Percent sunscald injury to detached fruit from wild-type (WT) and transgenic cAPX (A3-A30) tomato plants under field conditions for 15 d. Values are means ± SE (n=5). Different letters indicate significant differences (P<0.05) by Duncan's multiple range tests.

Discussion

The overexpression of the cAPX enzymes in tomato plants increased the resistance of their leaf and fruit tissues to heat and UV-B stresses, supporting the results reported by previous work in apple (Wisniewski *et al.*, 2002).

Electrolyte leakage in stressed transgenic plants was much less than in WT plants (Fig. 1) providing strong evidence that overexpression of cAPX 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 transgenic fruits (Fig. 2), 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 sunscald damage than WT fruits (P <0.05) (Fig. 3), suggesting that overexpression of antioxidant genes might increase tolerance to sunscald. APX enzyme activity was higher in *cAPX*-expressing plants after heat, UV-B, and drought stress (Fig. 4), which suggests that the overexpression of APX may play a role in protecting plant against ROS during various physiological stress (Allen, 1995).

We also tested SOD enzyme activity by gel assay 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

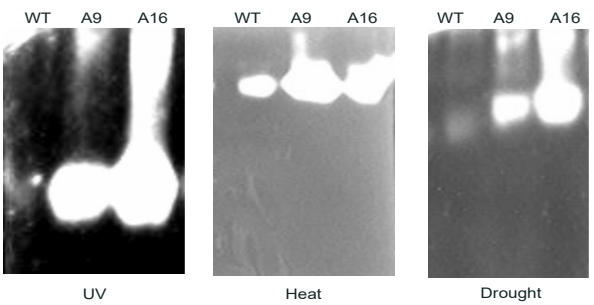

Fig. 4. APX enzyme activity gel assay. UV-B (2.5 mW cm^{-2}) for 4 h; heat (42 °C) for 4 h; and drought stress (withholding water for 7 d). About 70 µg protein was loaded in each lane of the native gel.

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).

Overexpression of APX in transgenic tomato plants provides better protection against heat, UV-B and sunscald. This elevated enzyme activity might play an important role in increasing stress tolerance against fruit sunscald. Given these results, along with those of other studies showing 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 that are sensitive to sunscald, such as apple, to improve their tolerance against this stress.

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References

A-H-Mackerness, S. 2000. Plant responses to ultraviolet-B (UV-B: 280-320 nm) stress: What are the key regulators? *Plant Growth Regul*., 32(1): 27-39.

Allen, R.D. 1995. Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol*., 107: 1049-1054.

Apel, K. and H. Hirt, 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol*., 55: 373-99.

Balakumar, T., B. Garathri and P.R. Anbudurai, 1997. Oxidative stress injury in tomato plants induced by supplemental UV-B radiation. *Bio. Plant*., 39(2): 215-221.

Bowler, C., M. Van Montagu and D. Inzé, 1992. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* 43: 83-116.

Chaitanya, K.V., D. Sundar, S. Masilamani and A. Ramachandra Reddy, 2002. Variation in heat stress-induced antioxidant enzyme activities among three mulberry cultivars. *Plant Growth Regul*., 36(2): 175-180.

Chen, M.K. and S.M. Pan, 1998. Constitutively elevated levels of Cu/Zn-superoxide dismutase can enhance the tolerance to heat stress or UV-B radiation in *Arabidopsis thaliana*. *Plant Biology Electronic Abstract Center*, No. 376 (Abstr.).

Davidson, J.F., B. Whyte, P.H. Bissinger and R.H. Schiest, 1996. Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci., USA,* 93: 5116-5121.

He, Y.Y., M. Klisch and D.P. Hader, 2002. Adaption of cyanobacteria to UV-B stress correlated with oxidative stress and oxidative damage. *Photochem. Photobiol*., 76(2): 188-196.

Huang, B., X. Liu and Q. Xu, 2001. Supraoptimal soil temperatures induced oxidative stress in leaves of creeping bentgrass cultivars differing in heat tolerance. *Crop Sci*., 41: 430-435.

Jiang, Y. and R. Huang, 2001. Drought and heat stress injury to two cool-season turfgrasses in relation to antioxidant metabolism and lipid peroxidation. *Crop Sci*., 41: 436-442.

Jimenez, A., G. Creissen, B. Kular, J. Firmin, S. Robinson, M. Verhoeyen and P. Mullineaux, 2002. Changes in oxidative processes and components of the antioxidant system during tomato fruit ripening. *Planta,* 214: 751-758.

Kubo, A., H. Saji, K. Tanaka and N. Kondo, 1995. Expression of *Arabidopsis* cytosolic ascorbate peroxidase gene in response to ozone or sulfur dioxide. *Plant Mol. Biol*., 29: 479-489.

Mazza, C.A., D. Battista, A.M. Zima, M. Szwarcberg-Bracchitta, C.V. Giordano, A. Acevedo, A.L. Scopel and C.L. Ballare, 1999. The effects of solar ultraviolet-B radiation on the growth and yield of barley are accompanied by increased DNA damage and antioxidant responses. *Plant Cell Environ*., 22(1): 61-70.

McKersie, B.D., S.R. Bowley, E. Harjanto and O. Leprince, 1996. Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol*., 111: 1177-1181.

McKersie, B.D., S.R. Bowley and K.S. Jones, 1999. Winter survival of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol*., 119: 839-847.

Mittler, R. and B.A. Zilinska, 1991. Molecular cloning and nucleotide sequence analysis of a cDNA encoding pea cytosolic ascorbate peroxidase. *FEBS Lett*., 2: 257-259.

Mittler, R. and B.A. Zilinskas, 1993. Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. *Anal. Biochem*., 212: 540-546.

Noctor, G. and C.H. Foyer, 1998. Ascorbate and glutathione: Keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 49: 249-279.

Prasad, T.K., M.D. Anderson, B.A. Martin and C.R. Stewart, 1994. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *Plant Cell.,* 6: 65-74.

Rabinowitch, H.D. and D. Sklan, 1980. Superoxide dismutase: A possible protective agent against sunscald in tomatoes (*Lycopersicon esculentum* Mill.). *Planta,* 148: 162-167.

Rabinowitch, H.D., D. Sklan and P. Budowski, 1982. Photo-oxidative damage in the ripening tomato fruit: Protective role of superoxide dismutase. *Plant Physiol*., 54: 369-372.

Rabinowitch, H.D., B. Ben-David and M. Friedmann, 1986. Light is essential for sunscald induction in cucumber and pepper fruits, whereas heat conditioning provides protection. *Sci. Hortic*., 29: 21-29.

Rabinowitch, H.D., M. Friedmann and B. Ben-David, 1983. Sunscald damage in attached and detached pepper and cucumber fruits at various stages of maturity. *Sci. Hortic*., 19: 9-18.

Renquist, R.R., H.G. Hughes and M.K. Rogoyski, 1989. Combined high temperature and ultraviolet radiation injury of red raspberry fruit. *HortScience,* 24(4): 597-599.

Sairam, R.K., G.C. Srivastava and D.C. Saxena, 2000. Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. *Bio. Plant*., 43(2): 245-251.

Scandalios, J.G. 1993. Oxygen stress and superoxide dismutase. *Plant Physiol*., 101: 7-12.

Sen Gupta, A.S., J.L. Heinen, A.S. Holaday, J.J. Burke and R.D. Allen, 1993a. Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. USA,* 90: 1629-1633.

Sen Gupta, A.S., R.B. Webb, A.S. Holaday and R.D. Allen, 1993b. Overexpression of superoxide dismutase protects plants from oxidative stress (induction of ascorbate peroxidase in superoxide dismutase-overexpressing plants). *Plant Physiol*., 103: 1067-1073.

Wang, J., H. Zhang and R.D. Allen, 1999. Overexpression of an Arabidopsis peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress. *Plant Cell Physiol*., 40(7): 725-732.

Wang, Y., M. Wisniewski, R. Meilan, M. Cui, R. Webb and L. Fuchigami, 2005. Overexpression of cytosolic ascorbate peroxidase in tomato (*Lycopersicon esculentum* L.) confers tolerance to chilling and salt stress. *J. ASHS,* 130(2): 167–173.

Wisniewski, M., J. Sauter, L. Fuchigami and V. Stepien, 1997. Effects of near-lethal heat stress on bud break, heat-shock proteins and ubiquitin in dormant poplar (*Populus nigra charkowiensis* x P. *nigra incrassata*). *Tree Physiol*., 17(7): 483-450.

Wisniewski, M., L. Fuchigami, Y. Wang, C. Srinivasan and J. Norilli, 2002. Overexpression of a cytosolic ascorbate peroxidase gene in apple improves resistance to heat stress. *XXXVI International Horticultural Congress & Exhibition*. p. 147 (*Abstr*.).

Yoshimura, K., Y. Yabuta, T. Ishikawa and S. Shigeoka, 2000. Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. *Plant Physiol*., 123: 223-234.