Inhibition of Catalase by Antisense RNA Increases Susceptibility to Oxidative Stress and Chilling Injury in Transgenic Tomato Plants

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ABSTRACT. Transgenic tomatoes (*Lycopersicon esculentum* Mill. 'Ohio 8245') expressing an antisense catalase gene (ASTOMCAT1) were used to test the hypothesis that modification of the reactive oxygen species scavenging mechanism in plants can lead to changes in oxidative stress tolerance. A 2- to 8-fold reduction in total catalase activity was detected in the leaf extracts of transformants. A 2-fold increase in levels of H₂O₂ was observed in the transgenic plants with reduced catalase activity. Electrophoretic characterization of multiple catalase isoforms revealed the specific suppression of CAT1 in transgenic plants. Homozygous plants carrying the antisense catalase transgene were used to study the effect of alteration in the expression of catalase on stress tolerance. Transgenic plants treated with 3% H₂O₂ showed visible damage within 24 hours and subsequently died. In contrast, wild-type and azygous control plants recovered from the treatment. Transgenic plants did not survive 4 °C chilling stress compared to control wild-type and azygous lines. Physiological analysis of these plants indicated that suppression of catalase activity in transgenic tomato led to enhanced sensitivity to oxidative stress. Our data support a role for catalase in oxidative stress defense system in tomato.

All aerobic organisms produce reactive oxygen species (ROS) as a byproduct of the utilization and production of dioxygen (Bowler et al., 1992). These ROSs include superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH). ROSs can react with numerous cellular components leading to pigment bleaching, lesions in proteins and nucleic acids, and the peroxidation of membrane lipids (Scandalios, 1990). Extreme oxidative damage results in cell and tissue death.

Catalase is the primary H₂O₂ scavenger in peroxisomes and is also found to be associated with mitochondria in plants (Scandalios et al., 1980). Hydrogen peroxide is produced during many cellular processes. It is synthesized as a byproduct of photorespiration, β -oxidation of fatty acids, and also as a consequence of biotic and abiotic stresses. Chilling injury has been reported to be mediated, in part, by ROSs as agents causing secondary tissue injury (Raison and Lyons, 1986). Exposure of cucumber (*Cucumis sativus* L.) seedlings to chilling temperatures in the light induced production of lipid peroxidation and depletion of antioxidants including α -tocopherol, glutathione, and ascorbate (Wise and Naylor, 1987). Prasad et al. (1994) have also presented molecular and biochemical evidence to indicate that chilling imposes oxidative stress in dark grown seedlings during acclimation and chilling treatments. Maize (*Zea mays* L.) seedlings exposed to 4 °C accumulated higher H₂O₂ levels (Prasad et al., 1994). Because catalase is a major protectant against accumulation and toxicity of H₂O₂, several researchers have evaluated changes in catalase activities during chilling stress. Total catalase activity increased in maize seedlings in response to low temperature in the dark (Prasad et al.,

1994). However, a reduction in catalase activity in response to low temperature exposure in light has been observed in passion fruits (*Passiflora edulis* Sims., *P. ligularis* Juss., and *P. quadrangularis* L.), mung bean [*Vigna radiata* (L.) R. Wilcz], pea (*Pisum sativum* L. 'Greenfeast'), cucumber (MacRae and Ferguson, 1985), and spinach (*Spinacia oleracea* L.) (Schoner and Krause, 1990). These results support the suggestion that catalase plays an important role in low temperature stress.

Genetic engineering of plants to modify the activity of enzymes involved in scavenging ROSs has been accomplished in recent years. There have been a number of reports of transgenic plants expressing increased activities of enzymes in the oxidative defense system, including superoxide dismutase (Bowler et al., 1991; Mckersie et al., 1993; Perl et al., 1993; Sen Gupta et al., 1993), ascorbate peroxidase (Torsethaugen et al., 1997), glutathione reductase (Aono et al., 1991; Broadbent et al., 1995) and glutathione S-transferase/glutathione peroxidase (Roxas et al., 1997). Both sense and antisense technology have been used to generate transgenic plants that exhibited a deficiency in catalase activity (Brisson et al., 1998; Chamnongpol et al., 1996; Takahashi et al., 1997; Willekens et al., 1997). Transgenic tobacco (*Nicotiana tabacum* 'Petit Havana SR1') plants deficient in catalase exhibited enhanced sensitivity to oxidative stress. For example, these plants developed severe leaf necrosis upon exposure to high light intensities (Chamnongpol et al., 1996). In addition, catalase-deficient transgenic tobacco plants were more vulnerable to paraquat (1,1'-dimethyl-4,4'-bipyridylum), salt, and ozone stress as compared to wild-type plants (Willekens et al., 1997). Analysis of transgenic plants with changes in protective enzymes such as catalase should provide a better understanding of their relative contributions to oxidative stress tolerance.

We are interested in the role of catalase in oxidative stress tolerance in tomato. Previously, we reported identification of a cDNA clone encoding a subunit of tomato catalase gene (pTOMCAT1) (Drory and Woodson, 1992). To better understand the role of catalase in defense against oxidative stress, transgenic tomato plants expressing an antisense catalase gene (pTOMCAT1) were generated. Homozygous plants carrying two copies of the transgene were used to study the effect of reduced catalase activity

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and its role in oxidative stress. Our results show that transgenic tomato plants exhibited an 8-fold reduction in catalase activity and a concomitant increase in H_2O_2 levels. Further, we show that suppression of catalase activity in transgenic tomato leads to enhanced sensitivity to oxidative and low temperature stress.

Materials and Methods

PLANT MATERIAL. Plants of the *Lycopersicon esculentum* Mill. 'Ohio 8245' were maintained in pots under a 12-h photoperiod at 25/22 °C (12-h day/12-h night) Horticulture Greenhouse, Purdue University. Day/night greenhouse humidity were maintained at 60% and 50%, respectively.

CONSTRUCTION OF pTOMCAT1 ANTISENSE GENE. The cDNA pTOMCAT1, which encodes a subunit of tomato catalase (Drory and Woodson, 1992), was used to construct the antisense transgene. A partial pTOMCAT1 cDNA (1.2 kb) was obtained by digestion with XbaI and XhoI. This was ligated in the antisense orientation relative to the CaMV 35S promoter in the binary vector pKYLX71 (Scharl et al., 1987). The resulting plasmid pASTOMCAT1 (Fig. 1) contained the pTOMCAT1 gene between the cauliflower mosaic virus 35S promoter and the *rbcS* 3' terminator sequence, as well as a bacterial gene for neomycin phosphotransferase, which confers kanamycin resistance in plants.

TRANSFORMATION OF TOMATO. The resulting plasmid (pASTOMCAT1) was introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating. Tomato plants were transformed with the resulting construct by using a transformation protocol as described by Fillatti et al. (1987). Briefly, sterile tomato cotyledon pieces from 10-d-old seedlings were infected, and tobacco feeder cells were used in the cocultivation of the cotyledon pieces with the bacteria. After cocultivation with the bacteria for 48 h, regeneration of shoots was induced on regeneration medium containing 4.3 g·L⁻¹ MS salts (Murashige and Skooge, 1962), 20 g·L⁻¹ sucrose, 100 mg·L⁻¹ myo-inositol, 1 mL·L⁻¹ Nitsch's vitamins, 2 mg·L⁻¹ zeatin, 0.5 mg·L⁻¹ thiamine, 100 mg·L⁻¹ kanamycin, and 500 mg·L⁻¹ carbenicillin. Shoots were subsequently transferred to rooting medium containing half con-

centration of substances with 0.5 mg·L⁻¹ indolebutyric acid substituted for zeatin. The putative transgenic lines that exhibited kanamycin resistance were selected and planted in soil and grown in the greenhouse. Transformation was confirmed by Southern analysis.

DNA EXTRACTION AND HYBRIDIZATION. Tomato genomic DNA was isolated from leaves using the procedure of Dellaporta et al. (1983). Ten micrograms of genomic DNAs from wild type and putative transgenic tomato plants were digested with XbaI and separated by electrophoresis in a 1% agarose gel. DNA was then denatured and transferred to nylon membrane (Schleicher and Schuell, Keene, N.Y.). The nylon membrane was prehybridized 4 h at 42 °C in a solution containing 50% (v/v) formamide, 5× Denhardt's, 1% sodium dodecyl sulfate (SDS), 5× SSPE (1× SSPE is 15 mM NaCl, 10 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 1 mM EDTA), and 100 mg·mL⁻¹ denatured salmon sperm DNA. Hybridization was carried out overnight at 42 °C in a solution containing 50% (v/v) formamide, 1× Denhardt's, 1% SDS, 5× SSPE, and 100 mg·mL⁻¹ denatured salmon sperm DNA plus 1×10^6 cpm/mL denatured ³²P-labeled pTOMCAT1 insert DNA isolated from the recombinant plasmid. The membranes were first washed in 2× SSPE and 0.5% SDS at 25 °C. The second wash was with the identical solution at 68 °C. The third wash was carried out at 68 °C in 0.2× SSPE and 0.2% SDS. The membranes were exposed to Kodak XAR-5 film at -80 °C for 36 to 48 h.

MEASUREMENT OF CATALASE ACTIVITY. Leaf tissue from greenhouse-grown plants was analyzed for total catalase activity. Leaves (1 g fresh weight) were frozen in liquid nitrogen and ground to a powder in a mortar with a pestle and extracted with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mg·mL⁻¹ dithiothreitol (DTT), 1% polyvinyl pyrrolidone (PVP) (w/v), and 100 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were centrifuged at 10,000 g_n for 10 min, and the supernatants were strained through miracloth and used for the analysis of catalase activities. Protein content of all crude extracts was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard. Catalase activity was determined as the production of O_2 (mmol·min⁻¹·mg⁻¹ protein of crude extract) by a Clark-type oxygen electrode at 25 °C. The electrode was calibrated 10 mV at 100× (1.0 V range), and the strip chart paper was set at a rate of 1 cm·min⁻¹. The calibration was carried out by measuring fully oxygenated water in the cuvette before and after adding sodium dithionite, an oxygen-absorbing compound. The catalase assay was carried out in a stirred reaction cuvette containing 50 mM potassium phosphate buffer pH 7.0 and 13 mM H_2O_2 . The decomposition of H_2O_2 was initiated with 30 mL of the enzyme extract.

MEASUREMENT OF H_2O_2 LEVELS. Hydrogen peroxide was measured in leaf extracts by the method of Okuda et al. (1991) with some modifications. Extracts from leaves (0.2 g) were done in 2 mL 0.2 N HClO_4 with 0.1 g quartz sand at 0 °C. The homogenate was centrifuged at 12,000 g_n for 10 min. The supernatant was neutralized to pH 7.5 with 4 N KOH and the solution was centrifuged at 12,000 g_n for 1 min. A 200 mL sample of the supernatant was applied to a 1 mL mixed bed resin column and eluted with 1 mL of distilled water. The eluate (≈1 mL) was added to 400 mL of 12.5 mM dimethylaminobenzoic acid in 375 mM phosphate buffer (pH 6.5) and 80 mL of 100 mM methyl-2-benzothiazolinone hydrazone. The reaction was initiated by the addition of 0.25 units of peroxidase at 25 °C. The increase in absorbance at 590 nm was monitored in a dual-beam spectrophotometer.

RNA EXTRACTION AND GEL BLOT ANALYSIS OF TRANSFORMANTS.

Fig. 1. Construction of pASTOMCAT1. A partial pTOMCAT1 cDNA (1.2 kb) obtained by digesting pTOMCAT1 with XhoI and XbaI was ligated in the antisense orientation relative to the CaMV 35S promoter in the binary vector, pKYLX71.

Total RNA from tomato leaf tissue was extracted as previously described (Lawton et al., 1990). Ten micrograms of total RNA was fractionated by denaturing agarose gel electrophoresis and blotted to nitrocellulose (Schleicher and Schuell, Keene, N.Y.) which was subjected to hybridization with 32 P-dATP labeled probe prepared by random priming (Amersham, Arlington Heights, Ill.). Blots were washed twice for 15 min at 25 °C and at 55 °C in $1\times$ SSC ($1\times$ SSC is 15 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS. The membranes were exposed to XAR film (Kodak, Rochester, N.Y.) with an intensifying screen at -80 °C. Alternatively, DIG-labeled antisense and sense probes were prepared by in vitro transcription with T7 and SP6 RNA Polymerase by using an in vitro transcription kit-labeling with digoxigenin-UTP. Filters were prehybridized for 2 to 4 h at 68 °C in a solution at pH 7.5 containing $5\times$ SSC, 50% formamide, 0.02% SDS, 0.1% N-lauroylsarcosine, 20 mM sodium maleate, and 2% (w/v) blocking reagent for nucleic acid hybridization (The Genius System, Boehringer Mannheim, Indianapolis, Ind.). Hybridization was carried out overnight at 68 °C in an identical solution containing DIG-labeled antisense or sense probes. Filters were washed twice for 5 min per wash at 25 °C in $2\times$ SSC and 0.1% SDS and were washed twice for 15 min per wash at 68 °C in $0.5\times$ SSC and 0.1% SDS. The filters were detected by a chemiluminescent procedure using anti-DIG-alkaline phosphatase, diluted 1:5,000 in solution containing diluted 1:5 of 10% blocking reagent stock solution in maleate buffer. Membranes were incubated with anti-DIG-alkaline phosphatase for 30 min at 25 °C. Then, the membranes were washed twice for 15 min per wash at pH 7.5 with 100 mM maleic acid, and 150 mM NaCl at 25 °C. The membranes were placed between two sheets of acetate and Lumigen PPD, diluted 1:100 in

Genius buffer 3 containing 100 mM Tris HCl, and 100 mM NaCl. Then, 50 mM $MgCl_2$ were applied for 1 min to the membrane. The excess chemiluminescent alkaline phosphatase substrate was drained. The membranes were exposed to XAR film for 30 to 60 min.

CHARACTERIZATION OF MULTIPLE CATALASE ISOFORMS. Isozyme electrophoresis was performed to detect multiple catalase isoforms in extracts from leaves and flowers. Tissues from leaves and flowers were collected and ground to powder in liquid nitrogen, then homogenized at 4 °C and pH 8.0 in 100 mM Tris-Cl, 20% glycerol, 30 mM DTT, and centrifuged at $15,000 g_n$ at 4 °C for 30 min (Frugoli et al., 1996). Protein content of the extract was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard. Total protein (60 mg) was resolved on a 7.5% native polyacrylamide gel with a 3.5% stacking gel and stained for catalase activity, as previously described (Gregory and Fridovich, 1974).

PLANT MATERIALS FOR HYDROGEN PEROXIDE AND CHILLING TREATMENT. Homozygous transgenic tomato seedlings, azygous and wild-type plants, were germinated under mist during the weeks of 2, 9, and 16 Mar. 1998. Ten-day-old seedlings were transplanted into small pots and grown in the greenhouse under natural photoperiod and irradiance. Twenty-four seedlings per line were used in each experiment. Each experiment was repeated three times.

HYDROGEN PEROXIDE TREATMENT. Three-week-old homozygous transgenic, azygous, and wild-type tomato seedlings were sprayed to runoff with 3% H_2O_2 containing 0.05% Tween-20 at 1000 HR. Twenty-four hours after treatment, seedlings were assessed for survival.

CHILLING TREATMENT. Three-week-old homozygous transgenic, azygous and wild-type tomato seedlings were grown under greenhouse conditions. Seedlings were transferred to the 4 °C cooler at 1600 HR. The cooler was kept dark during the 96-h treatment period. Seedlings were then removed from chilling and placed in the greenhouse. Seedlings were assessed for survival after 48 h in the greenhouse.

Results

PRIMARY TRANSFORMANT ANALYSIS. Twenty-three putative transgenic lines exhibiting kanamycin resistance were obtained. Southern analysis of genomic DNAs digested with XbaI and XhoI confirmed a 1.2-kb band that represented the transgene in 11 independently transformed plants (data not shown). In general, catalase antisense lines exhibited lower catalase activities in the leaves as compared to wild-type plants (Fig. 2A). Reduced catalase activity was associated with reduced TOMCAT1 RNA and expression of antisense TOMCAT1 transcripts (Fig. 2B). Transgenic ASTOMCAT1 lines 17 and 23 died before RNA extraction was performed. Due to the significant effect of the antisense

Fig. 2. Suppression of catalase activity and mRNA level in antisense TOMCAT1 plant. (A) Catalase activity in wild-type and primary transgenic ASTOMCAT1 tomato plants. Recently mature leaves from wild-type tomato 'Ohio 8245' and primary transgenic lines were analyzed within 4 to 8 weeks of age. Catalase activity was measured by oxygen electrode as described in materials and methods. Average of three independent assays is reported with the standard error. (B) Antisense and endogenous catalase mRNA levels in primary transgenic tomato plants. Total RNA was extracted from leaf tissue of ASTOMCAT1-1, 2, 6, 9, 11, 13, 16, 22, and wild-type tomato. Ten micrograms of total RNA were separated by electrophoresis on a formaldehyde-agarose gel, blotted to a nylon membrane and hybridized with sense or antisense digoxigenin-labeled pTOMCAT1 RNA.

Fig. 3. Southern analysis of transgenic tomato lines transformed with p-ASTOMCAT1. Ten micrograms genomic DNA were digested with XbaI, separated on 1% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with ³²P-labeled pTOMCAT1 cDNA probe as indicated in materials and methods. Location of molecular marker is represented in kilobases. MW, molecular weight.

catalase gene on endogenous catalase activity in ASTOMCAT1-11, this plant was selected for further analysis.

INHERITANCE OF THE ANTISENSE GENE AND SEGREGATION ANALYSIS OF PROGENY FROM ASTOMCAT1-11 PLANT. The primary transgenic tomato plant ASTOMCAT1-11 contained four copies of the antisense gene (Fig. 3). The antisense pTOMCAT1 gene segregated in this seed population, yielding progeny with zero, one, two, three, and four copies of the inserted gene as detected by genomic DNA gel blot analysis (data not shown). The first two copies of the inserted gene were inherited together and presumably represented a single insertion or two closely linked transgenes. Based on the genetic analysis, each individual antisense transgene segregated 3:1 (data not shown). A homozygous population of T₂ plants (11-12) containing the first two copies of the transgene (number 1,2) and an azygous line (11-6) that had lost all transgenes during segregation were selected for further analysis.

CATALASE ACTIVITY AND HYDROGEN PEROXIDE LEVEL IN TRANSGENIC TOMATO (T₂). A 2-fold increase in the levels of H₂O₂ was observed in the transgenic 11-12 plants that showed limited catalase activity (Fig. 4A), while levels similar to wild-type were observed in azygous 11-6 plants, which expressed normal levels of catalase mRNA and activity (Fig. 4A). A limited change in absorbance was detected at 590 nm in catalase-treated samples and was considered to represent background values (data not shown).

Expression of the antisense catalase gene and its effectiveness in reducing expression of TOMCAT1 RNA was directly correlated with catalase activity (Fig. 4A and B). Azygous 11-6 and wild-type plants displayed high levels of endogenous catalase mRNA and enzyme activity, while transgenic 11-12 plants contained almost no detectable endogenous catalase mRNA and were severely inhibited in catalase activity (Fig. 4A and B). Analysis of catalase mRNA levels by RNA gel blot hybridization revealed a high level of expression of antisense TOMCAT1 RNA in transgenic tomato plants with no antisense RNA detected in azygous and wild-type plants of the same stage (Fig. 4B). Due to the significant effect of the antisense catalase gene on endogenous catalase activity in transgenic 11-12 plants and the lack of its effect in the azygous 11-6 plant, future studies focused on these lines.

CHARACTERIZATION OF MULTIPLE CATALASE ISOFORMS IN TRANSGENIC PLANTS. Staining of gels for catalase activity revealed three distinct isoforms in wild-type and azygous (11-6) tissue (Fig. 5).

This pattern varied between leaves and flowers. Expression of antisense TOMCAT1 RNA selectively reduced the level of one isoform found in both leaves and flowers and had no apparent effect on the other two isoforms present in tissue extracts.

HYDROGEN PEROXIDE AND CHILLING TREATMENT. By 24 h after H₂O₂ treatment, transgenic tomatoes exhibited severe damage and subsequently died. Wild-type and azygous plants exhibited necrotic lesions following H₂O₂ treatment, they recovered and survived.

Transgenic and control tomato seedlings exhibited no visible injury during the 96 h dark chilling. However, when the plants were removed from chilling and placed under greenhouse conditions for 48 h, all of the transgenic ASTOMCAT1 line 11-12 were severely damaged and subsequently died. In contrast, all of control seedlings survived the 96 h dark chilling.

Discussion

Catalase is the enzyme that converts H₂O₂ to water and dioxygen in peroxisomes and mitochondria. Catalase is thought to be a crucial component of the system for the detoxification of reactive oxygen species in plants. A loss of catalase activity could lead to

Fig. 4. Catalase activity and H₂O₂ levels in homozygous transgenic, azygous and wild-type (Ohio 8245) plants. (A) Catalase activity and H₂O₂ content in leaves of wild-type (Ohio 8245), azygous (ASTOMCAT1-11-6) and homozygous transgenic (ASTOMCAT1-11-12) tomato. Leaves from transgenic and control plants were analyzed within 4 to 8 weeks. Mean of three independent assays is reported with the standard error. (B) Antisense and endogenous catalase mRNA levels in wild-type (Ohio 8245), azygous (ASTOMCAT1-11-6) and transgenic (ASTOMCAT1-11-12). Total RNA was extracted from leaf tissue and 10 mg of total RNA were separated by electrophoresis on a formaldehyde-agarose gel, blotted to a nylon membrane and hybridized with sense or antisense digoxigenin-labeled pTOMCAT1 RNA probes.

Fig. 5. Characterization of catalase isoforms in leaf and flower tissue of 6-week-old wild-type (Ohio 8245), azygous (11-6) and homozygous ASTOMCAT1 (11-12) plants. Protein extracts were resolved in a 7.5% nondenaturing polyacrylamide gel and stained for catalase activity as described in materials and methods.

elevated H_2O_2 levels resulting in oxidative stress. We have generated transgenic tomato plants expressing antisense catalase mRNA in an effort to suppress catalase activity. A 2-fold increase in the levels of H_2O_2 was observed in these transgenic tomato plants. We have not seen any sign of damage caused by this elevated H_2O_2 in the transgenic ASTOMCAT1 plants under normal greenhouse conditions. Willekens et al. (1997) had observed variation in H_2O_2 levels among wild-type and Cat1-deficient tobacco leaf. However, they found no evidence for higher H_2O_2 levels in transgenic tobacco compared to wild-type. Endogenous H_2O_2 is generally toxic at cellular concentrations of $10 \mu M$ (Salin, 1987). Hydrogen peroxide content in the leaf extracts of transgenic 11-12 plant was estimated to be $2.3 \mu M$. The constitutively elevated levels of H_2O_2 in vivo could serve as a signal for the induction of defense-related genes. Low levels of H_2O_2 may be sufficient for the induction of defense-related genes, but higher levels are needed for induction of pathogen and cell death (Levine et al., 1994). Levine et al. (1994) also reported that in cell cultures, high concentrations of exogenous H_2O_2 (6 to 8 mM) were required for the induction of cell death, while concentrations of 1 to 2 mM were sufficient for induction of protectant genes such as glutathione s-transferase. Active oxygen species are involved in a variety of plant stresses. Once viewed as a toxic byproduct of redox reactions, H_2O_2 has now been directly linked with activation of gene expression. In animals, both NF- κB and AP-1 transcription factors respond to H_2O_2 (Abate et al., 1990; Schreck et al., 1991). Our development of transgenic tomato plants with reduced endogenous catalase activity and elevated H_2O_2 levels can provide a valuable tool for dissection of the processes thought to be mediated by reactive oxygen species.

In plants, several isoforms of catalase have been reported including two isozymes in castor bean (*Ricinus communis* L.) (Ota et al., 1992), three in tobacco (*Nicotiana sylvestris* L.) (Havir and McHale, 1987), five each in spinach (Galston, 1955), maize (Williamson and Scandalios, 1993), and cotton (*Gossypium*

hirsutum L.) (Ni et al., 1990), and six in Arabidopsis [*Arabidopsis thaliana* (L.) Heynh] (Frugoli et al., 1996), which suggest that catalases are present as a gene family (Scandalios, 1987). To date, only one catalase gene has been identified, encoding a subunit of tomato catalase (pTOMCAT1) (Drory and Woodson, 1992). The subcellular distribution of the pTOMCAT1 gene product in tomato has not been determined.

Three distinct isozymes were detected in wild-type and azygous plants. Two isoforms were present in leaves, and three in flowers. Expression of antisense TOMCAT1 RNA reduced the level of TOMCAT1 mRNA and selectively reduced one isoform found in both leaves and flowers, which presumably represented the CAT1 isozyme. The other catalase isoforms present in tomato tissue expressing antisense pTOMCAT1 may account for the low level of catalase activity in these plants. Transgenic antisense catalase plants grew normally under nonstressful conditions. This suggested that the low level of catalase activity was sufficient under nonstressful conditions. Similarly, under low light conditions, transgenic tobacco plants with decreased catalase activity showed no difference in phenotype from control plants indicating that only 10% of catalase activity is sufficient in the absence of stress (Chamnongpol et al., 1996). However, these transgenic tobacco plants developed leaf necrosis when exposed to higher light intensities. Transgenic tomato plants which were deficient in CAT1 activity were not capable of removing excess H_2O_2 as evidenced by tissue death following application. Similar results were obtained previously with Cat1-deficient tobacco plants (Willekens et al., 1997). Cat1-deficient tobacco leaf discs demonstrated severe bleaching when floated on 10 and 100 mM H_2O_2 solutions. Hydrogen peroxide can freely diffuse across membranes, and concentrations as low as 10 mmol inhibit many enzymes (Bowler et al., 1992). In addition to catalase, plants possess other mechanisms to remove H_2O_2 from cells. Enzymes capable of reducing peroxide levels in cytosol and chloroplast are guaiacol peroxidase and ascorbate peroxidase (Schmidt and Kunert, 1986). However, catalase activity of leaves is generally 100 to 1000 times higher than that of other peroxidases (Volk and Feierabend, 1989), making it unlikely that normal levels of ascorbate peroxidase activity or guaiacol peroxidase activity would compensate for the loss of catalase activity in these transgenic plants. It remains to be determined whether other enzymes capable of reducing peroxide levels, such as ascorbate peroxidase, are altered in these transgenic antisense catalase plants.

Environmental stress is the major factor that affects productivity of plants. Along with the tissue injury in response to various stresses, oxidative stress has been implicated as one of the underlying agents causing damage (Allen, 1995). Chilling injury has been reported to be mediated, in part, by reactive oxygen species as agents causing secondary tissue injuries (Raison and Lyons, 1986). Under conditions of temperature stress, plants require less energy, resulting in an excess of photons in the electron transport system in photosystem II (Somersalo and Krause, 1990). The excess electrons are transferred to oxygen molecules, thus causing the accumulation of toxic reactive oxygen species. An increase in production of the superoxide radicals and hydrogen peroxide induced by chilling stress can attack membrane lipids and inactivate many enzymes (Scandalios, 1990). Furthermore, catalase was photoinactivated at low temperature in chilling-sensitive cucumber and maize (Feierabend et al., 1992). This leads to accumulation of H_2O_2 where it diffuses to other sites in the chloroplast or cytosol, leading to harmful oxidation reactions. Prasad et al. (1994) have presented molecular and biochemical evidence to indicate that chilling also imposes oxidative

stress in dark-grown seedlings during acclimation and chilling treatments. Hydrogen peroxide also accumulates in plants chilled in the dark but the source of H_2O_2 is unknown (Purvis and Skewfelf, 1993). Cytosolic Cu/Zn superoxide dismutase increases in response to dark chilling; this suggests that the cytosol may be the site of H_2O_2 production under this condition (Tsang et al., 1991). Activation of oxygen in electron transport systems in mitochondria or plasma membrane may also contribute to the increase in H_2O_2 caused by low temperature stress. In addition, we have previously reported that acclimation to low temperature stress in tomato seedlings was associated with elevated levels of catalase (Kerdnaimongkol et al., 1997). The combination of elevated levels of catalase before and during dark chilling provided a defense against accumulation of H_2O_2 produced during chilling stress (Kerdnaimongkol et al., 1997). Results reported herein indicate that transgenic plants inhibited in CAT1 activity were more susceptible to low temperature stress than control plants. Willekens et al. (1997) demonstrated that catalase-deficient transgenic tobacco plants were more vulnerable to paraquat, salt, and ozone stress as compared to wild-type plants. However, chilling stress treatment at 4 °C under low light did not cause any visible sign of injury in either Cat1-deficient tobacco or control lines (Willekens et al., 1997). In addition, Willekens et al. (1997) detected no evidence for higher H_2O_2 levels in leaf tissue of Cat1-deficient tobacco and control plants. In maize seedlings, H_2O_2 levels were elevated during acclimation (Prasad et al., 1994). Prasad et al. (1994) suggested that during acclimation of maize, H_2O_2 signals induction of particular antioxidant enzymes, whereas in nonacclimated seedlings, H_2O_2 can increase to toxic levels in response to low temperature stress. The transgenic tomato plants generated in our laboratory exhibited increased levels of H_2O_2 . However, the reduced activity of catalase in these plants likely leads to a failure to scavenge the ROSs that accumulate during stress.

Literature Cited

- Abate, C., L. Patel, F.J. Rauscher, and T. Curran. 1990. Redox regulation of Fos and Jun DNA binding activity in vitro. *Science* 249:1157–1161.
- Allen, R.D. 1995. Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol.* 107:1049–1054.
- Aono, M., A. Kubo, H. Saji, T. Natori, K. Tanaka, and N. Kondo. 1991. Resistance to active oxygen toxicity of transgenic *Nicotiana tabacum* that expresses the gene for glutathione reductase from *Escherichia coli*. *Plant Cell Physiol.* 32:691–697.
- Bowler, C., L. Slooten, S. Vandenbranden, R. De Rycke, J. Botterman, C. Sybesma, M. Van Montagu, and D. Inze. 1991. Manganese SOD can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J.* 10:1723–1732.
- Bowler, C., M. Van Montagu, and Inze D. 1992. Superoxide dismutase and stress tolerance. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 43:83–116.
- Brisson, L.F., I. Zelitch, and E.A. Havir. 1998. Manipulation of catalase levels produces altered photosynthesis in transgenic tobacco plants. *Plant Physiol.* 116:259–269.
- Broadbent, P., G.P. Creissen, B. Kular, A.R. Wellburn, and P.M. Mullineaux. 1995. Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. *Plant J.* 8:247–255.
- Chamnonpol, S., H. Willekens, C. Langebartels, M. van Montagu, D. Inze, and W. van Camp. 1996. Transgenic tobacco with a reduced catalase activity develops necrotic lesions and induces pathogenesis-related expression under high light. *Plant J.* 10:491–503.
- Dellaporta, S.L., J. Wood, and J.B. Hicks. 1983. A plant DNA miniprep: Version II. *Plant Mol. Biol. Rpt.* 1:19–21.
- Drory, A. and W.R. Woodson. 1992. Molecular cloning and nucleotide sequence of a cDNA encoding catalase from tomato. *Plant Physiol.* 100:1605–1606.
- Feierabend, J., C. Schaan, and B. Hertwig. 1992. Photoinactivation of catalase occurs under both high- and low-temperature stress conditions and accompanies photoinhibition of photosystem II. *Plant Physiol.* 100:1554–1561.
- Fillatti, J.J., J. Kiser, B. Rose, and L. Comai. 1987. Efficient transformation of tomato and the introduction and expression of a gene for herbicides tolerance, p 199–210. In: D.J. Nevins and R.A. Jones (eds.). *Tomato biotechnology*. Alan R Liss, New York.
- Frugoli, J.A., H.H. Zhong, M.L. Nuccio, P. McCourt, M.M. McPeck, T.L. Thomas, and C.R. McClung. 1996. Catalase is encoded by a multigene family in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol.* 112:327–336.
- Galston, A.W. 1955. Plant catalase. *Methods Enzymol.* 2:789–791.
- Gregory, E.M. and I. Fridovich. 1974. Visualization of catalase on acrylamide gels. *Anal. Biochem.* 58:57–62.
- Havir, E.A. and N.A. McHale. 1987. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiol.* 84:450–455.
- Kerdnaimongkol, K., A. Bhatia, R.J. Joly, and W.R. Woodson. 1997. Oxidative stress and diurnal variation in chilling sensitivity of tomato seedlings. *J. Amer. Soc. Hort. Sci.* 122:485–490.
- Lawton, K.A., K.G. Raghothama, P.B. Goldsbrough, and W.R. Woodson. 1990. Regulation of senescence-related gene expression in carnation flower petals by ethylene. *Plant Physiol.* 93:1370–1375.
- Levine, A., R. Tenhaken, R. Dixon, and C. Lamb. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79:583–593.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
- MacRae, E.A. and I.B. Ferguson. 1985. Changes in catalase activity and hydrogen peroxide concentration in plants in response to low temperature. *Physiol. Plant.* 65:51–56.
- Mckersie, B.D., Y. Chen, M. de Beus, S.R. Bowley, C. Bowler, D. Inze, K. D'Halluin, and J. Botterman. 1993. Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.). *Plant Physiol.* 103:1155–1163.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.
- Ni, W., R.B. Turley, and R.N. Trelease. 1990. Characterization of a cDNA encoding cottonseed catalase. *Biochem. Biophys. Acta.* 1049:219–222.
- Okuda, T., Y. Matsuda, A. Yamanaka, and S. Sagisaka. 1991. Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold treatment. *Plant Physiol.* 97:1265–1267.
- Ota, Y., T. Ario, K. Hayashi, T. Nakagawa, T. Hahori, M. Maeshima, and T. Asahi. 1992. Tissue specific isoforms of catalase subunits in castor bean seedlings. *Plant Cell Physiol.* 33:225–232.
- Perl, A., R. Perl-Treves, S. Galili, D. Aviv, E. Shalgi, S. Malkin, and E. Gualn. 1993. Enhanced oxidative-stress defense in transgenic potato expression tomato Cu, Zn superoxide dismutases. *Theor. Appl. Genet.* 85:568–576.
- 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.
- Purvis, A.C. and R.L. Shewfelt. 1993. Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiol. Plant.* 88:712–718.
- Raison, J.K. and J.M. Lyons. 1986. Chilling injury: A plea for uniform terminology. *Plant Cell Environ.* 9:685.
- Roxas, V.P., R.K. Smith Jr., E.R. Allen, and R.D. Allen. 1997. Overproduction of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nature Biotech.* 15:988–991.
- Salin, M.L. 1987. Toxic oxygen species and protective systems of the chloroplast. *Physiol. Plant.* 72:681–689.
- Scandalios, J.G. 1987. The antioxidant enzyme genes Cat and SOD of

- maize: Regulation, functional significance and molecular biology, p. 19–43 In: M.C. Rattazi, J.G. Scandalios, and G.S. Whitt (eds.). *Isozymes: Current topics in biological and medical research*. Alan R. Liss, New York.
- Scandalios, J.G. 1990. Response of plant antioxidant defense genes to environmental stress. *Adv. Genet.* 28:1–41.
- Scandalios, J.G., W.F. Tong, and D.G. Roupakias. 1980. Cat3, a third gene locus coding for a tissue-specific catalase in maize: Genetics, intracellular location, and some biochemical properties. *Mol. Gen. Genet.* 179:33–41.
- Schardl, C.L., A.D. Byrd, G. Benzion, M.A. Altschuler, D.F. Hildebrand, and A.G. Hunt. 1987. Design and construction of a versatile system for the expression of foreign genes in plants. *Gene* 61:1–11.
- Schmidt, A. and K.J. Kunert. 1986. Lipid peroxidation in higher plant—The role of glutathione reductase. *Plant Physiol.* 82:700–702.
- Schoner, S. and G.H. Krause. 1990. Protective systems against active oxygen species in spinach: Response to cold acclimation in excess light. *Planta* 180:383–389.
- Schreck, R., P. Rieber, and P.A. Baeuerle. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF- κ B transcription factor and HIV-1. *EMBO J.* 10:2247–2258.
- Sen Gupta, A., J.L. Heinen, A.S. Holaday, J.J. Burke, and R.D. Allen. 1993. Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. USA* 90:1629–1633.
- Somersalo, S. and G.H. Krause. 1990. Reversible photoinhibition of unhardened and cold acclimated spinach leaves at chilling temperatures. *Planta* 180:181–187.
- Takahashi, H., Z. Chen, H. Du, Y. Liu, and D.F. Klessig. 1997. Development of necrosis and activation of disease resistance in transgenic tobacco plants with severely reduced catalase levels. *Plant J.* 11:993–1005.
- Torsethaugen, G., L.H. Pitcher, B.A. Zilinskas, and E.J. Pell. 1997. Overproduction of ascorbate peroxidase in the tobacco chloroplast does not provide protection against ozone. *Plant Physiol.* 114:529–537.
- Tsang, E.W.T., C. Bowler, D. Herouart, W. Van Camp, R. Villarroel, C. Genetello, M. Van Montagu, and D. Inze. 1991. Differential regulation of superoxide dismutase in plants exposed to environmental stress. *Plant Cell* 3:783–792.
- Volk, S. and J. Feierabend. 1989. Photoinactivation of catalase at low temperature and its relevance to photosynthetic and peroxide metabolism in leaves. *Plant Cell and Environ.* 12:702–712.
- Willekens, H., S. Chamnongpol, M. Davey, M. Schraudner, C. Langebartels, M. Van Montagu, D. Inze, and W. Van Camp. 1997. Catalase is a sink for H₂O₂ and is indispensable for stress defence in C₃ plants. *EMBO J.* 16:4806–4816.
- Williamson, J.D. and J.G. Scandalios. 1993. Response of the maize catalases and superoxide dismutases to cercosporin-containing fungal extracts; the pattern of catalase response in scutella is stage specific. *Physiol. Plant.* 88:159–166.
- Wise, R.R. and A.W. Naylor. 1987. Chilling-enhanced photooxidation evidence for the role of singlet oxygen and superoxide in the breakdown of pigments and endogenous antioxidants. *Plant Physiol.* 83:278–282.