ANALYSIS OF TRANSGENIC TOBACCO THAT EXPRESS MAIZE CATALASE3

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A DISSERTATION

IN

AGRONOMY

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

DOCTOR OF PHILOSOPHY

Approved

Accepted

May, 1999

ACKNOWLEDGEMENTS

As with any endeavor, many people contributed throughout this process. First, I would like to thank my committee members (Drs. Randy Allen, Lou Densmore, Richard Durham, Dan Krieg, and Henry Nguyen) for their support and guidance during my graduate studies. I am especially indebted to Dr. Randy Allen for all of his expertise, mentoring, and belief in me throughout my years at Texas Tech. In addition, my fellow labmates were invaluable in providing guidance and support in science and in life. You guys made it fun. I also would like to acknowledge my home department, Plant and Soil Science, for financial and moral support and my other "home away from home" department, Biological Sciences, for making me feel at home.

I must also mention the support and guidance provided by my family that helped me see everything to the end. My friends whom I have almost ignored but I have not forgotten I cherish.

This work is dedicated to Jeanie Heinen whose strength and dedication I admire greatly.

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ABSTRACT

Catalases (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6; CAT) are heme-containing tetramers that are important in destroying H_2O_2 found in different cellular compartments. Maize *Cat3* has been shown to be capable of dismutating H_2O_2 via either a catalatic or peroxidatic reaction. In addition, increased maize CAT3 transcripts were detected during periods of chilling acclimation. In this study, a maize *Cat3* cDNA was isolated using reverse transcriptase polymerase chain reaction. To better understand the role of maize CAT3 in oxidative stress, we have introduced the transgene that expresses this enzyme into wild type Xanthi NN tobacco (*Nicotiana tabacum*). Total catalase activities were only slightly higher in transgenic plants as compared to Xanthi NN. While total peroxidatic activity of these transgenic plants was found to be 12-fold higher than in the wild-type tobacco. The transgenic Cat3 plants were exposed to various abiotic stresses such as, low temperatures, high temperatures, salinity, chemical treatments, and photooxidation. Increased seedling growth was evident in transgenic seedlings during treatments at low temperatures, high temperatures, and salinity which could implicate increased protection from oxidative damage. No significant protection was evident when transgenic seedlings were treated with methyl viologen or photooxidative stress. In addition, lower lipid peroxidation levels in transgenic plants correlated with increased peroxidatic activity in these plants. These data suggests that in wild-type tobacco that express maize CAT3 have increased protection against various forms of oxidative stress.

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ABA abscisic acid

ABTS 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)

APX ascorbate peroxidase

AT aminotriazol

BHT butylated hydroxytoleune

CAR chilling acclimation-responsive

CAT catalase

cDNA complementary DNA

CHL chloroplastic

CYT cytosolic

DEPC diethylpyrocarbonate

DPI days post imbibition

EDTA ethylene diamine tetraacetic acid

EP-CAT enhanced peroxidatic catalase

EXP expressor

GPX glutathione peroxidase

GST glutathione S-transferase

HR hypersensitive response

LP-CAT low peroxidatic catalase

MDA malondialdehyde

MOPS [3-(N-morpholino) propane sulfonic acid]

NE	non-expressor
PCR	polymerase chain reaction
PFD	photon flux density
PR	pathogenesis-related
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
Rp/c	ratio of peroxidatic to catalatic activity in catalase
SA	salicylic acid
SOD	superoxide dismutase
TCA	Trichloroacetic acid

CHAPTER I

INTRODUCTION

1.1 Oxidative Stress Overview

Oxidative stress occurs in all aerobic organisms and is a result of the production of reactive oxygen intermediates (ROI's) during photosynthesis, respiration, and other metabolic events. These toxic oxygen compounds are kept to minimal levels under non-stress conditions due to the metabolism of ROI's by both enzymatic and non-enzymatic mechanisms. Detoxification of ROI's can occur either at the site of production or after diffusion. During periods of environmental stresses such as extreme temperatures, water stress, or chemical treatments, oxidative stress can lead to severe damage in plant cells by damaging proteins, and causing lipid peroxidation resulting in impairment of photosynthetic components and deactivation of Calvin cycle enzymes. Environmental stresses limit the overall productivity of crop plants resulting in a 75% decrease in potential yield (Boyer, 1982).

ROI's produced during oxidative stress include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$). The ROI's, $O_2^{\cdot -}$ and H_2O_2, can be scavenged by the multiple isoforms of the enzymes, superoxide dismutase (SOD) and ascorbate peroxidase (APX), in the cytosol and various organelles. APX has a high affinity for H_2O_2 and requires ascorbate as a substrate (Willekens et al., 1997). In a non-enzymatic reaction ascorbate can also detoxify $^1O_2$ and $O_2^{\cdot -}$ (Asada and Takahasi, 1987). The primary line of

cellular defense against ROI's formed in the mitochondria includes the enzymes superoxide dismutase and catalase. In addition, glutathione peroxidases (GPX) are important in the mitochondria in maintaining low levels of H_2O_2. Some Glutathione S-transferases (GST) normally involved in the conjugation of reduced glutathione to xenobiotic compounds have also been shown to have GPX activity and are therefore able to detoxify H_2O_2.

In mitochondria, univalent reduction of molecular oxygen to form ROI's can occur (Figure 1.1; Scandalios et al., 1997). Since increased oxygen consumption in the mitochondria leads to greater flow of electrons through the electron transport chain, this ultimately leads to enhanced production of H_2O_2. Additionally, it is thought that $O_2^{\cdot-}$, produced from an electron-rich form of ubiquinone, serves as precursor to H_2O_2 in the mitochondria (Scandalios et al., 1997). H_2O_2 is capable of diffusing across membranes and therefore can be metabolized either within the mitochondria by enzymes such as glutathione peroxidase (GPX) or catalase, or it can diffuse out of the mitochondria and is scavenged by other means.

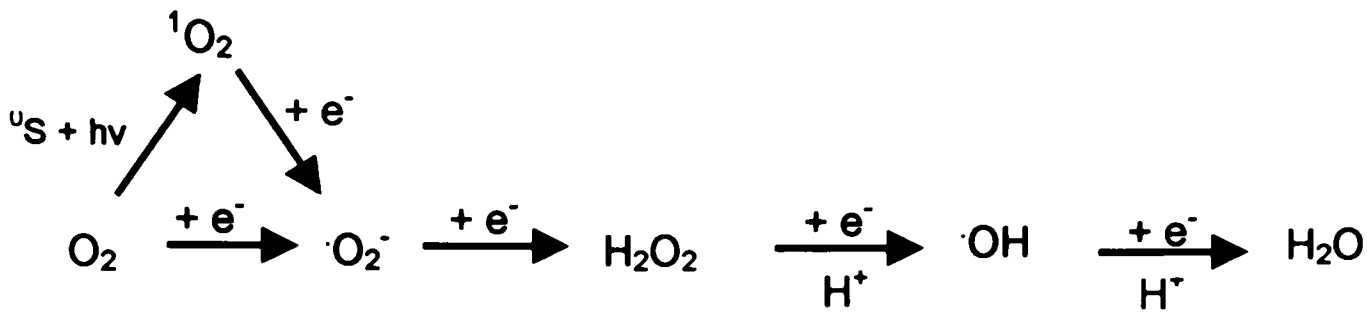


Figure 1.1 – Univalent reduction of oxygen that produces the ROI's superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen ($^1O_2$) in mitochondria. (Scandalios et. al, 1997)

Removal of H_2O_2 is imperative to maintain normal cellular function. The damaging effects of toxic H_2O_2 can lead to the inactivation of SH-enzymes, DNA damage, attack of lipids and the inactivation of Cu/Zn SODs and other metalloproteins. Without the removal of H_2O_2, the formation of the highly toxic $\cdot OH$ can alter proteins that can become targeted for proteolytic cleavage (Alscher, 1997).

1.2 Characterization of Catalases

Catalases (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6; CAT) are heme-containing tetramers that are important in destroying H_2O_2 in different cellular compartments without the requirement of reducing equivalents (Scandalios et al., 1997). The catalytic center of the catalase decomposes H_2O_2 at an extremely rapid rate of 10^7 min^{-1}. Multiple isoforms of plant catalases have been documented in a number of different species. During germinating of oil-storing seeds, lipids are converted to carbohydrates via the glyoxylate cycle in order to

provide sucrose to developing shoots and roots (Figure 1.2). H_2O_2 is produced by acetyl CoA oxidase in the glyoxylate cycle. Glyoxysomal catalase destroys this H_2O_2 resulting in the production of oxygen and water (Havir and MacHale, 1987). Specifically, upon the start of germination in oil seeds, triglycerides stored in spherosomes are hydrolyzed to free fatty acids by lipases. In the glyoxysome, a "specialized peroxisome," the fatty acids are broken down to acyl CoA in a step-wise fashion by the cyclic β-oxidation pathway. During this sequential breakdown of C_n fatty acids to n/1 acetyl CoA molecules during β-oxidation H_2O_2 is produced as a result of the reaction by acetyl CoA oxidase. Other products include NADH and acetyl CoA that are metabolized by the glyoxylate cycle to produce succinate. This product is then imported into the mitochondria and converted to malate that is exported to eventually provide the developing seedling with a carbohydrate source.

As seedlings begin to convert from a heterotrophic metabolism that depends on the breakdown of stored lipids to an autotrophic metabolism including photosynthesis, the glyoxysome microbodies are replaced with leaf-type peroxisomes. This microbody is a major source of O_2 consumption that is comparable to the mitochondria. Peroxisomes are responsible for the conversion of harmful metabolic by-products into water and other harmless products.

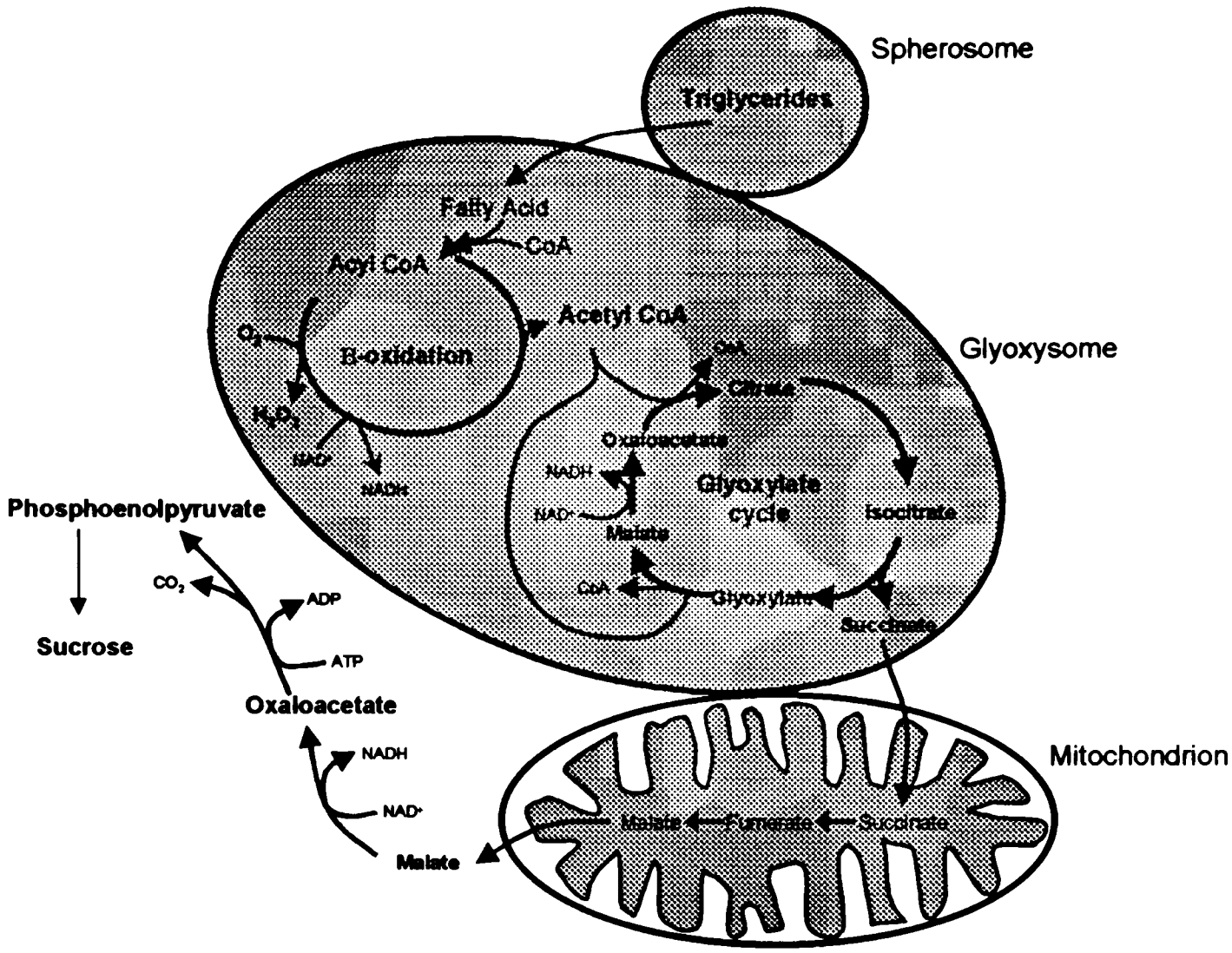


Figure 1.2 – Conversion of lipids to carbohydrates. Triglycerides are broken down to fatty acids that are sequentially oxidized by the β-oxidation cycle to produce acyl CoA, acetyl CoA, NADH, and H_2O_2. In the glyoxylate cycle the acetyl CoA is used in the production of succinate that is transported to the mitochondrion for conversion to malate. The malate is transported to the cytosol and oxidized to oxaloacetate that is converted to phosphoenolpyruvate (PEP). Through gluconeogenesis PEP is converted to glucose that is subsequently made into sucrose (Taiz and Zeiger, 1991).

A major function of peroxisomes in photosynthetic cells is photorespiration. Photorespiration involves three organelles: chloroplasts, peroxisomes and mitochondria (Figure 1.3). During photorespiration, ribulose bisphosphate carboxylase/oxygenase (RuBisco) oxygenates ribulose 1,5-bisphosphate resulting in a loss of CO_2 that is normally fixed by RuBisco during the carboxylation events of photosynthesis. These two diametrically opposite

reactions of photosynthesis and photorespiration occur concurrently and are a result of the competition between oxygen and CO_2 for the reaction with ribulose 1,5 bisphosphate. It is the oxidation of the transported glycolate to glyoxylate in the peroxisome that leads to the production of H_2O_2. This illustrates the importance of catalase in the peroxisome where approximately 40% of the total peroxisomal protein is catalase.

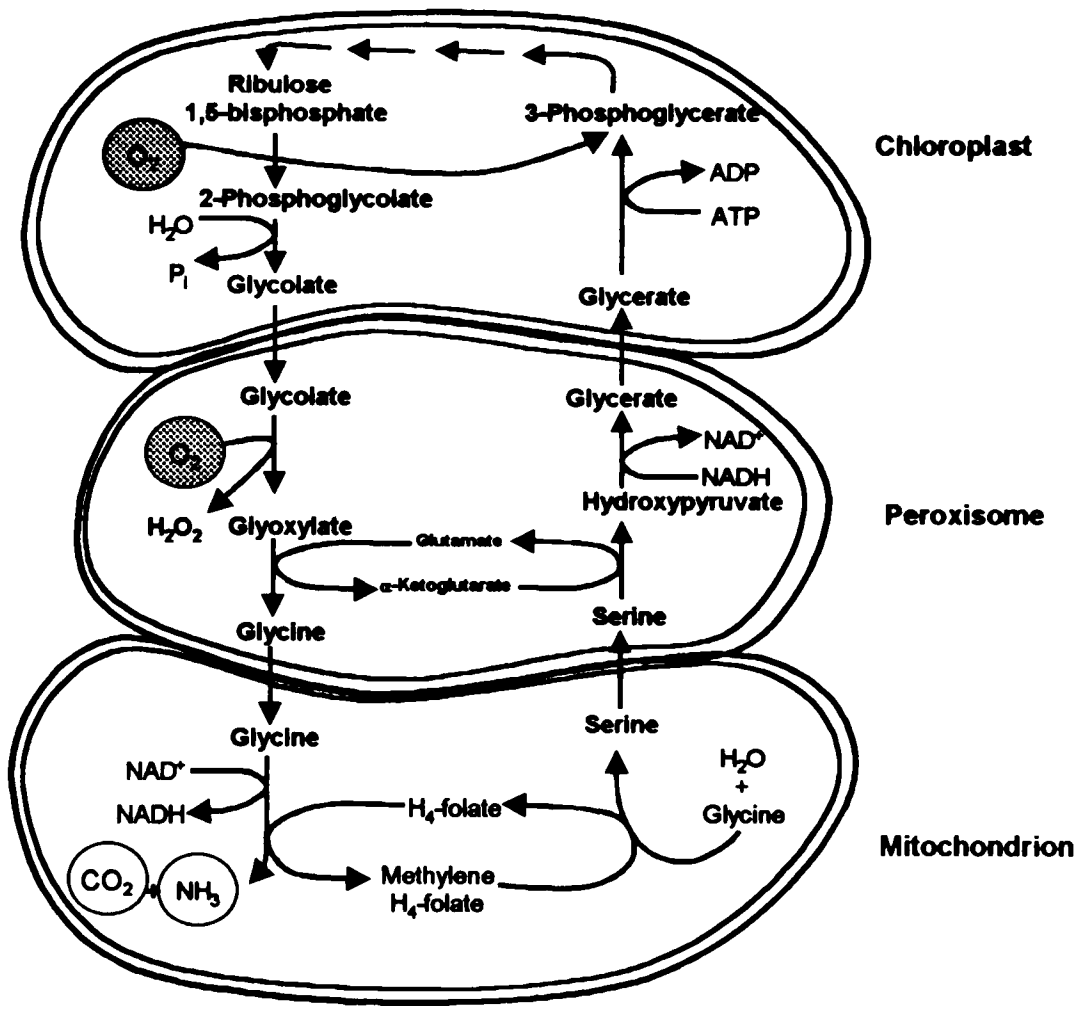


Figure 1.3 – Schematic representation of photorespiration events in three organelles: chloroplasts, peroxisomes, and mitochondria. Note the production of H_2O_2 in the peroxisome during the oxidation of glycolate to glyoxylate by glycolate oxidase (Taiz and Zeiger, 1991).

Several catalase isozymes have been found in maize (Scandalios 1965), *Nicotiana tabacum* (Havir and MacHale, 1987), cotton (Ni et al., 1990), tomato

(Gianninetti et al., 1993) and *Arabidopsis thaliana* (Zhong et al., 1994). It appears that multiple forms of catalase may each serve a protective role in different environments and during different developmental stages.

Maize catalases have been the most extensively studied eukaryotic catalases to date. Three different isoforms have been identified in maize: CAT1, CAT2 and CAT3 (Scandalios, 1965) and are encoded by three unlinked nuclear structural genes *Cat1, Cat2* and *Cat3*, respectively (Roupakias et al., 1980). Expression studies indicate that each *Cat* gene has specific temporal and spatial patterns of expression (Scandalios et al., 1984). Maize CAT1, a peroxisomal isozyme, is expressed exclusively during early kernel development in the scutellum, milky endosperm, aleurone, and in mature pollen (Scandalios 1983; Wadsworth and Scandalios, 1989; Acevedo and Scandalios, 1990). The peroxisomal CAT2 is found localized in the scutellum and in bundle-sheath cells of mature green tissues (Scandalios, 1974). In germinating seedlings CAT3 and CAT1 are the only catalase isozymes detected in both etiolated leaves and in the coleoptiles (Scandalios, 1997). Additionally both Maize CAT3 and CAT1 enzymes are present in mesophyll cells of mature green leaves (Tsaftaris et al., 1983) and in the pericarp of developing ovules (Scandalios et al., 1997). CAT3 is the only CAT isoform detected in mature plant stems (Acevedo and Scandalios, 1991). Specifically, maize CAT3 has been found to co-isolate with mitochondrial cell fractions (Scandalios et al., 1980a). However, it has been suggested that the hypothetical location of CAT3 in the mitochondria could be inaccurate due to the appearance of catalase in all fractions of isolated organelles most likely caused

by the fragile nature of peroxisomes. Previous reports have also localized catalase in chloroplasts as questionable for similar reasons (Havir, 1990). Furthermore, the expression patterns of these three maize isoforms corresponds with the activity patterns of each respective isozyme in developing maize seedlings (Redinbaugh et al., 1990; Acevedo and Scandalios, 1992).

Time course studies indicate a tissue-specific gene expression pattern for maize catalases. In developing maize seedlings, mRNA levels of the three maize catalases are as follows: CAT*1* mRNA increases in the scutellum from day 2 to day 10 and then disappears as compared to CAT2 mRNA transcripts that accumulate from day 2 through day 5 and then drop off (Scandalios et al., 1997). Declining expression patterns of CAT2 mRNA that coincide with the rise of CAT*1* mRNA in the scutellum have been found to be under the control of two regulatory elements, *Car1* (Scandalios et al., 1980b) and *Car2* (Chandlee and Scandalios 1984), that are implicated in controlling the expression of *Cat2* and *Cat1* respectively.

1.3 Catalase Activity and Kinetics

Biochemical studies have found unique properties for the maize catalases. These catalase enzymes exist as tetramers consisting of 4 subunits of approximately 60-kD each that are similar in structure to other catalase subunits of other organisms (Chandlee et al., 1983). Each subunit binds one FeIII heme group. Catalases can function either as a peroxidase or as a catalase depending on the H_2O_2 concentration. With less than 10^{-6} M H_2O_2 catalase catalyzes a

peroxidatic reaction by oxidizing many different proton donors (ethanol, ascorbic acid) as follows (Scandalios et al., 1997) (Figure 1.4):

$$RH_2 + H_2O_2 \rightarrow R + 2\ H_2O$$

Figure 1.4 - The peroxidatic reaction catalyzed by catalase.

Whereas with high concentrations of H_2O_2, catalase has "catalatic" activity capable of detoxifying H_2O_2 at a very high rate, with H_2O_2 acting both as a proton acceptor and donor (Figure 1.5):

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Figure 1.5 - The catalatic reaction catalyzed by catalase.

A two-step mechanism has been suggested for both modes of action by catalase (Deisseroth and Dounce, 1970; Dounce, 1973) where each heme iron interacts with the H_2O_2 forming an oxygen-rich iron peroxide (Figure 1.6):

$$CATFe\text{-}OH + H_2O_2 \rightarrow CATFe\text{-}OOH + H_2O$$

Figure 1.6 - The formation of compound I.

The intermediate, CATFe-OOH, known as compound I determines if the reaction will proceed as a catalase or peroxidase (Chance et al., 1979). In the peroxidatic reaction, under low H_2O_2 levels, compound I is reduced by proton donors such as methanol, ethanol, formaldehyde, formate or nitrite in the following manner (Figure 1.7):

$$CATFe\text{-}OOH + C_2H_5OH \rightarrow CATFe\text{-}OH + H_2O + CH_3CHO$$

Figure 1.7 - Compound I reduction under low H_2O_2 levels during the peroxidatic reaction of catalase.

When high H_2O_2 levels exist, a second H_2O_2 can react with compound I to produce water and molecular oxygen (Figure 1.8):

$$CATFe\text{-}OOH + H_2O_2 \rightarrow CATFe\text{-}OH + H_2O + O_2 \uparrow$$

Figure 1.8 - Compound I oxidation under high H_2O_2 levels during the catalatic reaction of catalase.

The ratio of peroxidatic to catalatic activity of catalase is expressed as Rp/c, where Rp/c = (mU peroxidatic/U catalatic) X 10 (Havir and MacHale, 1989). Interestingly, of the three maize catalases, only the maize CAT3 has a higher peroxidatic activity than catalatic activity with an Rp/c = 17.6 (Havir, 1990). Other

catalase isozymes with enhanced peroxidatic activity have been identified in tobacco (Rp/c = 9.2) with tobacco CAT1 exhibiting 65% catalatic and 15% peroxidatic activities and tobacco CAT3 having 15% catalatic and 85% peroxidatic activities (Havir and McHale, 1987). It has also been shown that CAT3 of *N. sylvestris* has Rp/c values that are 50 times greater than that of *N. tabacum* CAT3 (Havir and McHale, 1987). In barley, two forms of catalase exist with one isoform having an enhanced peroxidatic/catalatic ratio (Rp/c = 11.8) (Havir, 1990). Additionally, the peroxidatic reaction catalyzed by catalase has been placed in a completely different class than that containing horseradish and other plant peroxidases (Sichak and Dounce, 1986). In general, plant catalases with low-peroxidatic catalase activity have been termed LP-CAT, and plant catalases with enhanced-peroxidatic catalase activity are termed EP-CAT (Havir et al., 1996).

1.4 Inhibition of Catalases

Maize catalases display different sensitivities to a variety of inhibitory compounds. For instance, cyanide (CN), azide (N_3), and aminotriazol (AT) all inhibit CAT1 and CAT2 at relatively low levels while CAT3 is the least sensitive (Chandlee et al., 1983). Thus, CAT3 may have a unique function in other cellular reactions, e.g., alternative oxidase pathway, otherwise known as cyanide resistant respiration (Elthon and McIntosh, 1987). In addition, other catalases with enhanced peroxidatic activity in microorganisms that are classified as catalase-peroxidases are also insensitive to AT (Havir, 1990).

1.5 Evolution of Plant Catalases

All plant catalases are thought to have evolved from a common ancestral catalase gene (Guan and Scandalios, 1996). Three major classifications of plant catalases exist. Included in the first group are mainly enzymes from dicots with the exception of maize *Cat1*, barley *Cat1* and rice *CatB*. Maize *Cat2* has more sequence identity with animal catalases and is only loosely associated with this first group. The second group classified as a dicot-specific group includes tomato *Cat* and tobacco *Cat2*. The third group contains monocot-specific catalases including barley *Cat2, rice* catA and maize *Cat3*. Additionally, codon usage data reveals that group 3 (maize *Cat3*) and maize *Cat2* have the highest G+C (≥ 94%) content at the third codon position (Guan and Scandalios, 1996) suggesting a strong codon bias. Higher GC contents at the third position is commonly seen in monocot genes but not in dicot genes. This codon bias may play a role in gene regulation and gene expression since *Cat2* and *Cat3* products are expressed in a tissue specific manner and at certain times during development (Gaun and Scandalios, 1996).

1.6 Literature Review

Plant catalases are universal in their primary role of H_2O_2 removal both in the glyoxylate cycle and during photorespiration. Recent reports have hinted to secondary roles of plant catalases during periods of oxidative stress. Studies involving low temperature or chilling-induced oxidative stress provide speculative roles for antioxidant enzymes, including catalase. In pre-emergent dark-grown

maize seedlings (chilling-sensitive) chilling acclimation-responsive (CAR) genes have been found to be differentially expressed during acclimation at 14°C for 3 days followed by 4°C for 7 days (Prasad et al., 1994a). Maize C*at3*, which co-purifies with mitochondria, has been identified as one of the CAR genes leading to the hypothesis that chilling treatments lead to oxidative stress in maize seedlings. Both exogenous application of H_2O_2 and the *in vivo* production of H_2O_2 during periods of oxidative stress were found to lead to increases in CAT3 and peroxidase activities (Prasad et al., 1994a). Therefore, it was hypothesized that, at low temperatures, H_2O_2 served to stimulate production of antioxidant enzymes during acclimation. Later efforts examined maize seedlings after an acclimation period followed by severe chilling conditions of 4-6°C for 2-10 days (Anderson et al., 1994). The researchers propose an additional role for abscisic acid (ABA) since treatment of non-acclimated maize seedlings with ABA (1mM) leads to increased resistance to cold temperature stress.

In an effort to determine if the oxidative stress generated from a chilling treatment directly affects mitochondria, researchers treated maize seedlings to an acclimation period, and/or treatment with H_2O_2 or ABA (Prasad, 1996). Acclimated seedlings had higher CAT3 and peroxidase levels than non-acclimated seedlings that were hypothesized to provide a protective mechanism that allowed the mitochondria to recover from the chilling-induced oxidative stress (Prasad, 1996). To understand the specific role of catalase in protection against oxidative stress in pre-emergent maize seedlings, treatment with aminotriazole (AT), a catalase inhibitor, was found to increase protein carbonyl

content used as an indicator of oxidative stress (Prasad, 1997). Therefore, elevated catalase activity, specifically of CAT3, is believed to correlate with chilling tolerance in maize seedlings after acclimation (Prasad, 1997). In contrast, when several species of cold tolerant, cold sensitive and very cold sensitive plants were subjected to chilling temperatures in light, a decrease in catalase activity along with a decrease in H_2O_2 was seen (MacRae and Ferguson, 1985). A direct relationship between catalase activity and H_2O_2 levels was not apparent suggesting other H_2O_2 removal systems are important. It should be noted, however, that this study measured catalase non-specifically by monitoring only the evolution of oxygen. Additionally, photoinactivation of catalase has been attributed to lowered catalase activity levels in rye leaves that were subjected to chilling and light (Volk and Feirabend, 1989). A diurnal examination of catalase activity in 'Rutgers' tomato found higher catalase activities in plants chilled after light exposure than plants chilled after a dark period (Kerdnaimongkol et al., 1997).

Light serves as an important signal in establishing autotrophic metabolism in plants and can also signal the expression of antioxidant genes to protect against ROIs. In maize, each catalase responds to light signals in a different manner. *Cat1* gene expression is light independent while *Cat2* and *Cat3* are both light mediated (Polidoros and Scandalios, 1997). Transcriptional regulation of *Cat3* occurs in a circadian pattern and a photoreceptor for blue light/UV-A and UV-B is presumed to mediate this response (Polidoros and Scandalios, 1997). These authors believe that since *Cat3* is expressed late in the light period and

through the dark period, therefore *Cat3*'s may not be involved in photorespiration. The function of *Cat3* during these metabolic periods remains a mystery. It has been suggested that *Cat3* is involved in cyanide sensitive respiration in the mitochondrion since this respiration follows the same pattern as *Cat3* gene expression (Polidoros and Scandalios, 1997). Furthermore, maize seedlings growing under either constant darkness or constant illumination have been found to have a 10-fold increase in transcript levels of *Cat3* as compared to *Cat2* transcripts (Boldt and Scandalios, 1997). Plants, without evidence of a circadian pattern, grown under similar conditions led to induction of a *Cat3* circadian rhythm by exposure to constant UV-light. This suggests UV-light might serve as another environmental signal for *Cat3* (Boldt and Scandalios, 1997).

Salicylic acid's (SA) action in plants is not completely understood. For instance, SA promotes temperature elevation by activating the alternative respiratory pathway as discovered in thermogenic voodoo lily, *Amorphophallus rivieri* (Raskin et al., 1987). Exogenous application of SA leads to increased leaf surface temperatures of *N. tabacum* suggesting a possible role of SA might be to increase the alternative respiration pathway elevating leaf temperatures (Van Der Staeten et al., 1995). Modulation of catalases in tobacco by SA has been theorized to occur by a dual system. At low H_2O_2 (μmolar range), concentrations, it is proposed that SA inhibits tobacco catalase, while at higher, more damaging concentrations of H_2O_2 (mmolar range), SA protects catalase against inactivation allowing removal of H_2O_2 (Durner and Klessig, 1996). This theory proposes that healthy tissues that normally maintain low H_2O_2 levels undergo elevation of

defense-related genes, known as PR (pathogenesis-related) genes, upon infection that is triggered by elevated levels of H_2O_2 brought about by SA's inhibition of catalase. Opposing views hold that H_2O_2 levels do not change during infection (Neuenschwander et al., 1995) and that catalase levels in tobacco remain unchanged after infection (Bi et al., 1995). In addition, SA has been found not to inhibit soybean catalases, but instead, SA has been found necessary in establishing the hypersensitive reaction (HR) (Tenhaken and Rübel, 1997). Other researchers, though, believe that SA may act simply as a phytoalexin and not by specifically binding to catalase (Rüffer et al., 1995). Treatments with SA were used to illustrate the effect of SA on maize catalases. During embryogenesis and germination of maize seeds, treatment with SA leads to increases in both catalase transcripts and total catalase activity (Scandalios et al., 1997). Specifically 1 mM SA applied to developing maize seedlings leads to increased transcripts and activity in the *Cat2*/CAT2 isoform. Though SA has been implicated as a catalase binding protein in tobacco, it appears that no such function exists for SA in maize (Scandalios et al., 1997) even though both *Cat1* and *Cat2* have 76% and 72% sequence homology, respectively, with a SA binding protein isolated from tobacco. On the other hand, *Cat3* transcript levels decline with increased levels of SA and have the lowest sequence homology (69%) to the SA binding protein (Guan and Scandalios, 1995).

LP-CATs are the most sensitive to the inhibitory effects of AT while the EP-CATs are less sensitive to inhibition. This reduced sensitivity of EP-CATs to 3-AT is believed to occur for several reasons. First, the protein structure of EP-

CAT may differ slightly from that of the LP-CAT causing an impairment of the binding of the AT (Havir, 1992). Also it has been suggested that the localization of the EP-CATs may be at a site with limiting levels of H_2O_2 (Havir, 1992). Specifically, maize CAT1 and CAT2 activities are inhibited by 98% with 5 mM AT while CAT3 activity is reduced only by 32% (Prasad et al., 1994). The use of AT during acclimation studies has allowed for the inhibition of the active catalase enzyme, not the synthesis of catalase, leading to the production of H_2O_2 and enhancement of oxidative stress (Prasad, 1997).

Use of gene transfer technology has allowed examination of the specific effects of altered catalase expression. Sense and antisense transgenic tobacco (*N. tabacum*) were developed to express the CAT1 of *N. tabacum* and the subunit 1 of cottonseed (*G. hirsutum*) catalase (Brisson et al., 1998). These transgenic plants that overexpressed CAT1 led to increases in specific catalase activities that were 1.25 to 2-fold higher than the non-transformed wild-type plants. The transgenic tobacco plants expressing the antisense CAT1 construct had reduced specific catalase activities that were 0.2 to 0.75 times less than the levels found in the controls. There is evidence that an increase in the stoichiometry of CO_2 produced per mole of glyoxylate oxidized during photorespiration events that are favored by increases in temperature or O_2 could also be triggered in leaves that are lacking catalase activity. The purpose of this research study was to monitor the photorespiration events in the antisense transgenic plants. Therefore, by measuring the CO_2 compensation point (the CO_2 concentration at which CO_2 fixed by photosynthesis balances CO_2 loss by

photorespiration and net CO_2 exchange is zero) at 38°C in the transgenic plants the effects of catalase activity on photorespiration could be determined. The antisense transgenic plants had increased CO_2 compensation points that corresponded with decreasing levels of catalase activity. Whereas in the transgenic tobacco plants that overexpressed CAT1 a temperature dependent linear decrease in the CO_2 compensation point was reported. Therefore, the researchers suggested that wildtype catalase levels were not high enough to deal with accumulating H_2O_2 during periods of high photorespiration as measured at 38°C. At high temperatures catalase levels are able to modify the photorespiration utilization of CO_2 by regulating the stoichiometry of the photorespiratory pathway (Brisson et at., 1998).

Transgenic tobacco plants were evaluated in order to determine the role of catalase during pathogen attack. It is believed that catalase plays a role, along with salicylic acid, in the induction of a pathogenesis-related (PR) response. Previous reports hypothesized that H_2O_2 acted as an intracellular signal in both infected and uninfected leaves to trigger the PR responses (Chen et al., 1993). In addition SA produced after pathogen attack has been documented as binding to and inactivating catalase *in vitro.* One study developed transgenic tobacco (*N. plumbaginifolia*) plants that expressed antisense *Cat1* and/or *Cat2* (Chamnongpol et al., 1996). Transgenic plants deficient in catalase were found to have about 10% of the normal catalase activity in the antisense *Cat1* plants and about 80% of the normal catalase activity in the antisense *Cat2* plants. These antisense transgenic plants showed no detectable phenotype when grown

under low light conditions. Necrotic lesions became evident when the antisense *Cat1* along with the antisense *Cat1/Cat2* transgenic plants were subjected to light intensities greater than 250 µmol m^{-2} sec^{-1}. Antisense *Cat2* transgenic tobacco plants did not show this phenotype indicating necrosis was associated with *Cat1* specifically. Lack of *Cat1* and *Cat2* activity in these antisense transgenic tobacco plants was not sufficient to induce PR gene expression when exposed to low light intensities. Even with exogenous application of 10 µM SA leaf disks from the antisense *Cat1* and *Cat2* transgenic tobacco plants did not induce PR gene expression. These two findings indicate that it is not the suppression of catalase itself that signals PR gene expression. Additionally, antisense *Cat1* transgenic tobacco plants grown under light intensities of 1000 µmol^{-2} sec^{-1} suffered massive leaf damage in the lower and middle leaves with the formation of white necrotic lesions. These tissues were found to have induced PR-1 gene expression that correlated with levels of necrosis (Chamnongpol et al., 1996). The researchers concluded that since *Cat1* had been implicated in the removal of H_2O_2 produced during photorespiration supporting the fact that the antisense *Cat1* transgenic tobacco plants phenotypic effects were greatest under high light conditions. In addition, the inactivation model of catalase by SA is not supported in this study using antisense transgenic *Cat1* and *Cat2* tobacco plants. Further studies involving the antisense *Cat1* transgenic tobacco plants were conducted to better understand the role of H_2O_2 in the activation of PR proteins (Chamnongpol et al., 1998). With increased exposure to high light intensities excess H_2O_2 led to increases in tissue damage, triggered production of both SA and ethylene, and

led to PR protein expression that mimics the HR in plants in intensity and timing. The SA was produced in two phases. The first phase, where an increase in ethylene was also detected, was during the first few hours of exposure to the light treatment when necrosis had yet to develop. If these antisense *Cat1* transgenic plants were exposed for shorter periods to high light and subsequent H_2O_2, the plants did not exhibit necrosis, but had induction of localized PR proteins and enhanced tolerance to pathogens. It was therefore concluded that sublethal levels of H_2O_2 were enough to induce PR protein expression and provide increased tolerance to pathogens (Chamnongpol et al., 1998).

Transgenic plants expressing various antioxidant enzymes have been developed and analyzed. For instance, various isoforms of SOD, chloroplastic and mitochondrial, have been overexpressed in tobacco. Several groups have developed transgenic plants that express chloroplast-localized (Chl) Cu/Zn SOD. Sen Gupta et al. (1993) found increased levels of pea Chl Cu/Zn SOD led to increased resistance to MV-induced oxidative damage. Additionally, these plants were found to have greater protection against photooxidative damage caused by exposure to low temperatures and high light intensities as compared to the controls. Work from Perl and co-workers (1993) developed transgenic potato plants that overexpressed a tomato Chl Cu/Zn SOD that also had increased resistance from MV-induced membrane damage. Development of transgenic plants expressing different SOD isoforms was carried out by Bowler et al. (1991). Here a Mn SOD isoform from *Nicotiana plumbaginifolia* was targeted for expression in the chloroplast instead of the native mitochondria. These plants

were found to have increased protection from ozone and MV-induced membrane damage. Other attempts to express MnSOD targeted to the chloroplast in tobacco were developed by Allen and co-workers. The Chl Mn SOD transgenic plants were found to have increased tolerance to MV treatments especially at higher concentrations (Schake, 1994). These Chl Mn SOD plants had no increased protection against photooxidative damage induced by treatment of high light intensity and chilling temperatures.

Plants expressing other antioxidant enzymes such as APX have also been developed. Tobacco plants expressing either cytosolic (Cyt) or Chl targeted APX were found to have increased resistance to MV induced membrane damage and increased tolerance to photooxidative stress from high light and chilling temperatures (Allen et al., 1997). Further analysis is currently being conducted on tobacco plants that express *Arabidopis thaliana* stromal and thylakoid APXs. Initial data indicate increased protection against photooxidative stress from high light and chilling temperatures in the stromal transgenic tobacco plants.

Studies with transgenic tobacco plants that express a GST with GPX activity provided increased seedling growth during conditions of chilling (15°C and 4°C) and 100 mM and 150 mM salt stress (Roxas et al., 1997). Additionally, transgenic GST seedlings subjected to chilling or salt stresses were found to have lower levels of lipid peroxidation as compared to control seedlings. Increased amounts of oxidized glutathione were also detected in GST overexpressing seedlings that were subjected to both chilling and salt stress. Leaf disks from transgenic tobacco plants expressing GST were not found to

have increased protection against either MV-induced membrane damage or photooxidative treatments of high light and low temperatures.

1.7 Purpose and Rationale

Plants are exposed to a variety of biotic and abiotic stresses that lead to production of ROIs that all contribute to a dramatic loss of crops yearly. The mechanisms involved in protection against these stresses are currently being studied in many laboratories. Knowledge gained from the study of plant antioxidant mechanisms will enhance our comprehension of how to increase the resistance of plants to different forms of oxidative stress. Specifically, assessment of the role of a high-peroxidatic form of catalase will aid in determining the specific functions of maize CAT3 during oxidative stress. Additionally a greater knowledge of plant defense systems against oxidative stress will shed more light on the role of catalase as an antioxidant. Ultimately, if genetic modification of plants with increased and appropriately expressed enzymes leads to increased oxidative stress resistance, future crop plants could then be modified to increase plant productivity.

The characteristics of endogenous maize CAT3 have been well documented. In seedlings, maize CAT3 has been shown to have increased activity during periods of chilling stress. Therefore, I hypothesized that maize CAT3, with enhanced peroxidatic activity, could provide enhanced protection against oxidative stress in seedlings. To analyze the role of maize CAT3 expressed in *Nicotiana tabacum L.* a multi step approach was taken. Our

primary goal was to have a clearer understanding of maize CAT3's role during seedling germination. These experiments follow similar analyses as in the GST-overexpressing tobacco lines that were found to have increased seedling vigor under chilling and salt stress (Roxas et al., 1997).

The first stage of this study involved the isolation of the maize CAT3 cDNA. Total RNA extractions of 8-day old maize seedlings were used to perform RT-PCR resulting in a 1500 bp fragment. Subcloning steps resulted in the introduction of maize CAT3 cDNA into gene constructs that encoded CAT3.

The second step of the study was the transformation of wild type tobacco plants to encode for the maize CAT3 cDNA. The induction of the maize CAT3 cDNA in the wild type tobacco plants was evaluated by performing both Northern blot analysis using the previously isolated maize CAT3 cDNA and by total enzyme activity studies. We propose that any increase in protection found in CAT3 expressing transgenic tobacco plants will be linked to the enhanced peroxidatic ability of maize CAT3. Tobacco plants expressing maize *Cat3* have been found to have at least a 2-fold increase in catalase activity and a 7-fold increase in peroxidatic activity.

The third tier of the study was to evaluate the stress tolerance of the transgenic plants that express maize CAT3. These plants, at both the seedling and mature plant stages, were tested for increased protection against chilling induced oxidative damage. Here transgenic seedlings expressing maize CAT3 cDNA were found to have increased tolerance to low and high temperatures along with protection against salt stress. Mature plant tissue of transgenic

tobacco plants that express maize CAT3 cDNA had little or no increased protection against photooxidative stress treatments or paraquat compared to control plants.

The fourth part of the study involved evaluation of the transformed tobacco plants expressing a maize CAT3 cDNA treated with different chemicals in order to provide an understanding of the introduced cDNA's effect on the endogenous catalases.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant Materials for Cloning

Maize seeds (*Zea mays ssp. mays* variety Mo17) were germinated in the dark on moist filter paper in petri dishes at room temperature. Approximately 1.36 g of epicotyls were removed from 8-day-old dark-grown maize seedlings and frozen in liquid N_2 for RNA extraction.

2.2 RNA Extraction

Total RNA from the maize seedlings was isolated using the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Briefly, maize seedlings were ground to a fine powder in liquid N_2, transferred to a solution of equilibrated phenol (pH8.0), 24:1 chloroform:isoamyl alcohol, 4M LiCl, 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl and 0.1 M β-mercaptoethanol, well mixed and placed on ice. Homogenization with a polytron grinder followed the ice treatment. After centrifugation at 10,000g the resulting aqueous phase was transferred to a fresh tube. With addition of isopropanol the solutions were placed in the –20°C freezer for 1 hour. A pellet was obtained after centrifugation as before that dissolved in a solution of 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl and 0.1 M β-mercaptoethanol. Again isopropanol was added and place in –20°C freezer for 1 hour. A pellet was obtained after centrifugation as before that dissolved in a solution of DEPC-treated water.

Equal volumes of 4M LiCl were added to each sample and placed on ice and kept at 4°C for 4 hours. The samples were then centrifuged as before and again the resulting pellet was resuspended in DEPC-treated water. Next, 0.1 volume of 3M NaOAc was added and mixed along with 2 volumes of EtOH. The samples were placed at –20°C for 1 hour. After centrifugation as before, the resulting pellet was washed with 400 µL of 70% EtOH. The resulting pellet after centrifugation was then dried and resuspended in an appropriate amount of DEPC-treated water.

Total RNA for transgenic tobacco plant material was collected as follows. Briefly, four #7 cork borer (~1.5 cm in diameter) leaf punches were ground in liquid Nitrogen with the addition of 100 mM Tris (pH 9.0), 1% Sarcosine, 200 mM NaCl, 20 mM EDTA (pH 9.0) and 5 mM DTT with a hand grinder. Phenol and 24:1 chloroform:isoamyl alcohol extractions immediately followed allowing collection of the aqueous phase at each step. Next, with the addition of 6 mM of the samples were placed on ice and kept at 4°C overnight. After centrifugation the pellet was resuspended in 200 µL of 2 % (w/v) KoAC, followed by the additions of 100 µL of 7.5 M NH_4OAC and 200µL of 100% EtOH. The samples were mixed well and stored at –20°C for 1 hour. After centrifugation at the resulting pellet was rinsed with 70% EtOH, centrifuged, and dried. The pellet was resuspended in 40 µL of DEPC-treated water.

2.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Approximately 4-6 µg of RNA suspended in a total volume of 25 µL DEPC-treated water were incubated at 67°C for 10 min in a water bath. Preparation for the first strand cDNA synthesis from this population of total RNA was performed by the addition of Moloney murine leukemia virus (MmLV) 5X buffer, 2.5 µM dNTPS (Promega, Madison, WI), oligo dT primer (Promega, Madison, WI), RNAsin, and enough DEPC-treated water for a total volume of 50 µL. This reaction was kept at room temperature for 10 minutes followed by placement in ice for 5 minutes. Next, 2.5 µL of MmLV reverse transcriptase (Promega, Madison, WI) were added to the reaction and placed at 37°C for one hour in order to allow production of the 1st strand cDNAs. A PCR reaction with a 5 µL aliquot of the first strand cDNA products serving as templates was performed in a final volume of 50 µl using Takara Ex Taq DNA Polymerase (PanVera Corportation, Madison, WI). A slight modification using 2 mM MgCl was made with all other components following established protocols. Published sequence information coding for maize CAT3 cDNA (Redinbaugh et al., 1988) was used to design and synthesize 5' and 3' primers that were utilized in RT-PCR (GIBCO BRL Custom Primers, Life Technologies, Inc. Rockville, MD). The 5' primer (5'- CTA GCT AGG TGA ATG ACA ATG -3') included the initiation codon. The 3' primer was designed based on the following sequence (5'-CCT GGC GAC GAC ATG CAT GCG-3'). The following PCR conditions were used beginning with 35 cycles of 95°C (30 sec), 55°C (1 min), 72 °C (1.5 min) and 1 cycle of 72 °C (1 hr). The PCR products obtained were ligated into PCR™ II

(Invitrogen, San Diego, CA) vector according to the TA cloning protocol. Transformation of the PCR II products using DH5α competent cells were plated on LB plates with 5-bromo –4-chloro-3indolyl-B-D-galactosidase (X-gal) and isopropyl β-D-thiogalactopyranoside (IPTG). White colonies were then selected and cultures were grown in LB media containing 50 mg/ml ampicillin in a 37°C shaking incubator overnight in order to perform STET minipreps. The cultures were centrifuged for 30 seconds in order to pellet the cells. The resulting supernatant was discarded allowing the addition of 300 µL of a STET solution containing, 0.1 M NaCl, 0.1 mM Tris (pH 8.0), 0.5% Triton-X-100, 10 mM EDTA and 2 mg/ml lysozyme to the pellet. The pellet and solution were then vortexed to completely resuspend the pellet. The samples were allowed to sit at room temperature for 2-5 minutes allowing degradation of the bacterial cell wall by lysozyme. The solutions were then placed in a boiling water bath for 1 minute. Following a 15-minute centrifugation the resulting supernatent was collected and 2 volumes of cold EtOH were added, and mixed well. After precipitation at room temperature for 5 minutes the samples were again centrifuged for 15 minutes. The pellet was then rinsed with 700 µL of 70% EtOH followed by centrifugation. The pellets were dried and resuspended in 30 µL of water. The miniprep DNA was then digested with EcoRI at 37°C for at least 4 hours to release the insert. Electrophoretic separation of the digestions was performed on 1% TAE agarose gels allowing separation of digested plasmids and inserts. Plamids with inserts of ~ 1500 bp matching the expected length of the maize *Cat3* cDNA were sequenced. Sequence analysis performed by Dr. Susan San Francisco at the

Biotech Core Facility of Texas Tech University using dideoxy chain termination method (Sanger et al., 1977) confirmed that the purified 1498 bp fragment was maize *Cat3*.

2.4 Construction of *Cat3* Constructs

The *Cat3* gene construct was prepared by digesting the pCRII-CAT3 vector with *Sph*I. The resulting linear DNA was modified by blunt ending the *Sph*I ends in the following manner. Purified DNA (15µL), 2.5 µL of Klenow buffer and 1 µL of Klenow fragment of DNA Polymerase (Promega, Madison, WI), were mixed and incubated at 37°C for 5 minutes. With the addition of 2.5 µL of dNTPs (1:1:1:1) the reaction was allowed to proceed at room temperature for 1 hour followed by first a 5 minute incubation at 37°C and then a 10 minute incubation at 55°C. Next the DNA was digested with EcoRI and purified to allow ligation of the maize Cat3 cDNA into the *Sma*I /*Eco*RI sites of the pRTL 2 expression vector (provided by Dr. James Carrington, Dept. of Biology, Texas A&M University) that contains an enhanced Cauliflower Mosaic Virus (CMV) 35S promoter and a CMV 35S terminator polyadenylation signal (Figure 2.1). The CAT3 gene cassette was removed as a *Hind*III fragment and subsequently ligated into the plant binary transformation vector pCGN 1578. The pCGN-CAT 3 plasmid was transformed into *Agrobacterium tumefaciens* strain EHA 101 (van Haute et al., 1983).

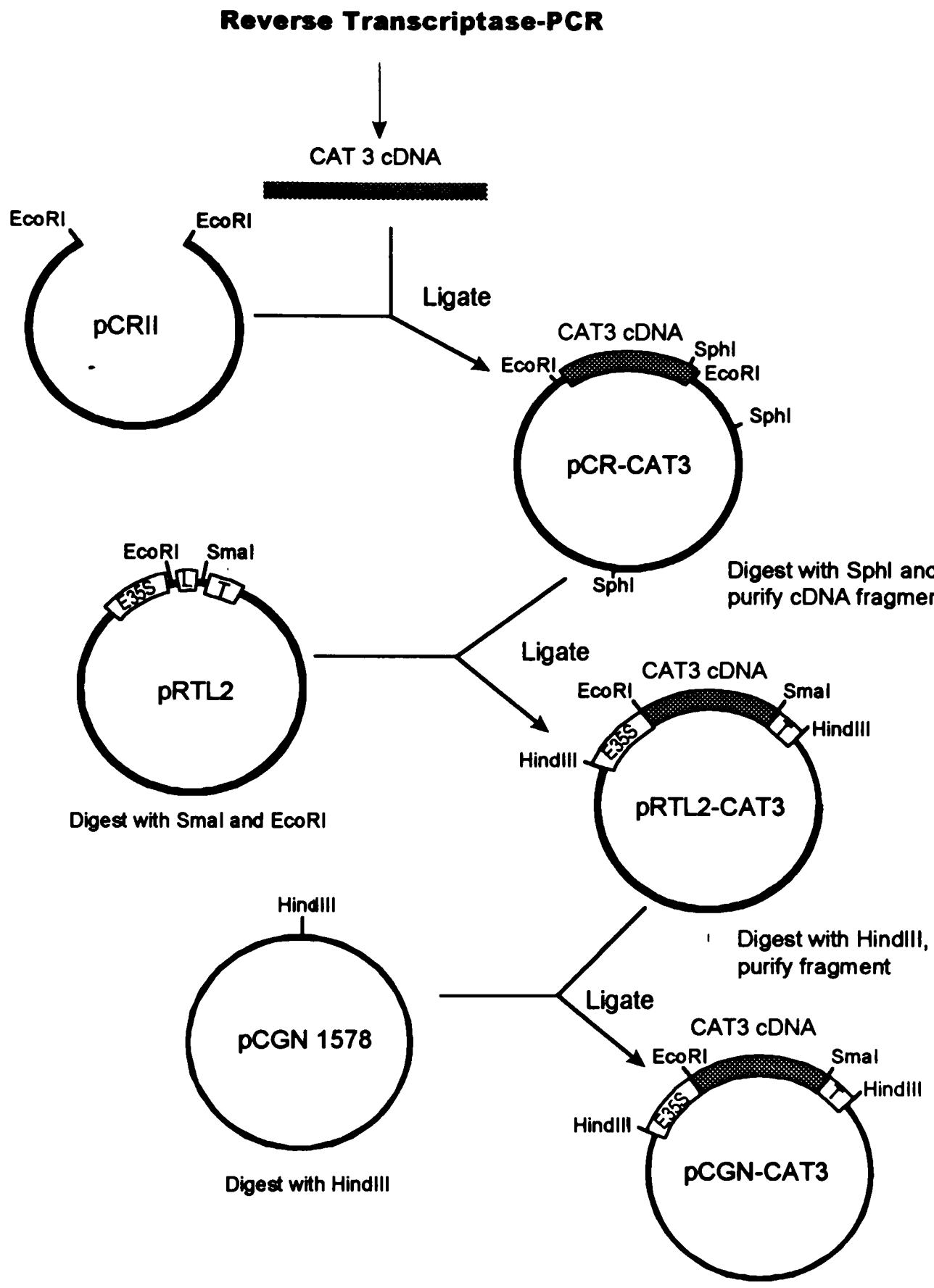


Figure 2.1 Strategy for the development of the Maize *Cat3* construct.

2.5 *Agrobacterium*-mediated Leaf Disk Transformation

Through direct transformation methods, the pCGN-CAT3 construct was transferred to the *Agrobacterium tumefacians* strain EHA 101. A single colony was inoculated into 50 ml of MG/L media (Appendix A) with 50 µg/ml kanamycin. A 1 ml aliquot was removed from a 12-hour culture grown at 30°C in a shaking incubator (250 rpm) and transferred into fresh MG/L media without antibiotics and grown for 6 h. Cultures were centrifuged for 10 min at 11,000g to collect cells that were then resuspended in 2 ml of fresh MG/L media. The purified plasmid containing the specific chimeric gene construct (~ 1 µg) was combined with 200 µl of the resuspended cells. The mixture was frozen in liquid N_2, thawed at 37°C for 5 min, transferred to 1 ml of MG/L and grown for 2 h at 30°C in a shaking incubator. The culture was then plated on MG/L plates with 100 µg/ml gentamycin and allowed to grow at 30°C. After growth, plasmids were used for tobacco transformation and analyzed using restriction digests and Southern blotting to confirm the presence of pCGN-CAT3 plasmids. Approximately 2 ml cultures were used for transformation of tobacco plants with the *Agrobacterium*-mediated leaf disk method.

Inoculation of tobacco (*Nicotiana tabacum* cv. Xanthi NN) leaf disks was carried out as described by Horsch et al. (1985). Briefly, leaves from ~3 month old tobacco plants (~ 50 cm tall) were collected and immersed in an anti-fungal agent Benlate (Benomyl, DuPont, Wilmington, DE) solution (1.2 g/l) for 10 minutes. The leaves were then immediately disinfected with a 14% commercial bleach solution for 5 minutes and rinsed 4 times in sterile water. The leaves

were blotted dry on sterile 3M paper. Leaf disk punches from a #7 cork borer (~1.5 cm in diameter) were immersed for 5 minutes in the *Agrobacterium* suspension (1:17 dilution) containing the chimeric gene construct CAT3 (pCGN 1578-Cat3), blotted on sterile filter paper and placed upside down on MSA nutrient plates (Appendix A) for 2 days in order to allow infection to occur. Each disk was cut into four pieces and transferred to MSB plates (Appendix A) containing hormones and antibiotics to promote callus and shoot growth. Shoots that were grown from the callus and resistant to antibiotics were transferred to MSC plates (Appendix A) to promote root growth. The regenerating plantlets were transferred to 4-inch pots, covered with a plastic bag and allowed to acclimate slowly. Once the initial transgenic plants (T_0 plants) were hardened, they were transferred to 2-gallon containers in the greenhouse grown to maturity and allowed to seed. Seeds (T_1) were plated on Kanamycin germination plates in order to select for positive transformants (Appendix A). These T_1 plants were used for all whole plant assays.

2.6 Northern Blot Analysis

Total RNA was separated by gel electrophoresis on 1% agarose gels containing 20% formaldehyde in a 1X MOPS buffer (0.2 M MOPS, 8 mM NaOAc, 1 mM EDTA pH 7.0). The gels were soaked in 75 mM NaOH for 30 minutes, washed in DEPC water for 20 minutes, soaked in 20X SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA pH 8.0) for 45 minutes and transferred to nitrocellulose membranes using 10X SSPE as a transfer buffer. The nitrocellulose filters were

dried at 82°C for 1 hour in a vacuum oven. The filters were prehybridized for 2 hours at 42°C in a solution of 50% (v/v) formamide, 2X Denhardt's (1X Denhardt's is 0.02% PVP, 0.02% Ficoll, 0.02% BSA), 0.1% SDS, and 5X SSPE (1X SSPE is 0.15M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA (pH7.4)). Northern blot hybridization (Sambrook et al., 1989) using an α-^{32}P-labelled probe derived from the *Cat3* cDNA clones was carried out in identical buffer solution at 42°C overnight. The filters were washed with 2X and 1X SET for 30 minutes each at 42°C and 50°C respectively. The filters were dried and immediately exposed to X-ray film (Amersham, Arlington Heights, IL).

2.7 Determination of Enzyme Activities

Leaf disks from transgenic plants and transgenic seedlings undergoing different treatments were assayed for catalase activity as described by Havir and McHale (1987) by monitoring the decrease in absorbance at 240 nm of 12.5 mM H_2O_2 in 50 mM K-phosphate (pH 7.0). One unit is defined as the amount of enzyme necessary to catalyze the decomposition of 1 µmol H_2O_2 per minute. The reaction rate was calculated using the molar coefficient of 0.036 cm^2 μmol^{-1} (Luck, 1963). Additionally, the peroxidatic activity of catalase was determined by following the method of Zamocky et al. (1995) by measuring the oxidation of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma, St Louis, MO) by the peroxidatic activity of catalase which was followed for 10 minutes at 660 nm. Slight modifications were made according to Barr and Aust (1993) with a 1 ml reaction mixture of 0.1 M Tris buffer (pH 8.0), 12.5 mM H_2O_2 and 1 mM ABTS.

This reaction is specific to the peroxidatic activity of catalase (Barr and Aust, 1993). The reaction rate was calculated using the molar coefficient of 12,000 cm^2 μmol^{-1}.

2.8 Seedling Stress Assays

Four independently transformed lines of tobacco seedlings expressing a maize *Cat3* cDNA were subjected to a variety of abiotic stresses including high and low temperatures, exposure to salt, and prooxidant chemicals. The growth of these seedlings was monitored at high temperatures (37°C and 30°C), and at low temperatures (15°C and 4°C) and compared to growth of seedlings at 25°C. Seeds from both transgenic expressors and non-expressors (Xanthi and H1.2) were sown on moist seed germination paper (Anchor Paper Company, St. Paul, MN) in Petri dishes. These seedlings were assayed for seedling length along with catalatic and peroxidatic activities of catalase. Chemical treatments were administered to seedlings grown at 15°C and 25°C as follows: 0.5 mM 3-AT, 10 mM 3-AT, 100 µM SA, and both 100 mM and 150 mM NaCl. Twenty seedlings per line were measured for length (mm) 5-7 days post-imbibition (DPI) unless otherwise noted. Immediately after measurement, the seedlings were weighed, frozen in liquid N_2, and stored at –80°C for further analysis.

2.9 Lipid Peroxidation Assay

Malondialdehyde (MDA) levels were determined with modifications of the procedure outlined by Draper and Hadley (1990). Approximately 100 mg of frozen seedling tissue was ground in 10% TCA (Trichloroacetic acid, Aldrich,

Milwaukee, WI) and 0.5 g/L butylated hydroxytoleune (BHT) (Aldrich, Milwaukee, WI) solution in ethanol. Samples were then immersed in a boiling water bath for 30 min and allowed to cool to room temperature. The precipitate was pelleted by centrifugation allowing transfer of the supernatant to a tube containing 0.67 % (w/v) thiobarbituric acid (TBA) (Sigma, St Louis, MO). The solutions were placed in a boiling water bath for 30 min and allowed to cool to room temperature. The concentration of MDA was spectrophotometrically determined by measuring at 532 and 600 nms. A 155,000 ε value was used in the calculations of MDA.

2.10 Photooxidative Stress

T_1 plants were germinated on kanamycin selection plates (Appendix A) to allow selection of positive transformants. Four plants per line were transferred to 4 inch pots until plants were approximately 4-7 cm in length. They were then transplanted to 5 gallon pots and allowed to acclimate under 12 hour light/12 hour dark schedule. Once the plants were about 30-40 cm tall they were transferred to the greenhouse. When the plants reached a bolting stage of approximately 80–120 cm, leaf disk assays were performed. Response to photooxidation was determined following the protocols of Sen Gupta et al. (1993). Briefly, oxygen evolution was monitored using a gas-phase, O_2 electrode system (Hansatech Instruments, Pentney, Kings Lynn, U.K.) under saturating CO_2 conditions. Leaf disks (3.6 cm in diameter) from transgenic and non-expressors (non-transformed and wild-type Xanthi) were collected from the first fully expanded leaf of each plant and transferred to moist filter paper under a

500W quartz halogen lamp at a photon flux density (PFD) of 1000 µmoles $m^{-2}s^{-1}$ for an acclimation period of one hour at 25°C. An initial photosynthetic rate was determined at 1000 µmoles $m^{-2}s^{-1}$. The leaf disks were then subjected to a 4 hour stress treatment at 4°C on a moist block of ice illuminated at 1500 µmoles $m^{-2}s^{-1}$. A final rate of photosynthesis was taken in order to determine an overall percent recovery based on the initial and final photosynthetic rates.

2.11 Membrane Permeability Assay

Methyl viologen (MV or paraquat dichloride, Sigma, St Louis, MO) induces chemical membrane damage and therefore can serve as an indicator of oxidative stress protection. MV assays were carried out according to Sen Gupta et al. (1993a). Three leaf disks (1.5 cm in diameter) were incubated in concentrations of 1.2 and 2.4 µM MV along with a water control. The samples were vacuum infiltrated for 5 minutes, incubated at 27°C in the dark for 16 h, illuminated at 500 µmoles $m^{-2}s^{-1}$ for 2 h at 27°C and then incubated in the dark at 30°C for 16 h. Initial cell leakage was measured using an Orion model 120 conductivity meter. The MV solutions and leaf disks were autoclaved for 25 min, cooled, and measured for a final conductivity reading. Percent electrolyte leakage was calculated based on initial and final readings.

2.12 Statistical Analysis

Physiological experiments were carried out in a randomized design using at least 3 replicates per treatment. Four maize Cat3-expressing lines were evaluated independently to determine an average mean for each line (Appendix B). Additionally, t-test analyses were used to determine if differences existed between the CAT3-expressing lines (Appendix B). Although some significant differences were detected between these transgenic lines, the data from these lines was pooled since similar trends were evident. The pooled data of these four expressing lines were calculated as means and evaluated for statistical differences. These data were used to compare to the means of 2 non-expressing lines that were also calculated as means. Standard deviations were calculated and used to represent error bars in all graphical representations of the data. Significant differences were determined using a defined t-test for comparing samples of different sizes (Glantz, 1997).

CHAPTER III

RESULTS

3.1 Cloning of Maize *Cat3*

Fragments approximately 1500 bp long were produced using RT-PCR amplification of total RNA from maize epicotyls (Figure 3.1) and were then cloned into a pCRII vector system. DNA sequencing confirmed that the cloned PCR fragments matched published sequences of maize *Cat3*. This cDNA was used to develop the *Cat3* gene construct that was introduced into tobacco via *Agrobacterium*-mediated gene transfer as described in Chapter II.

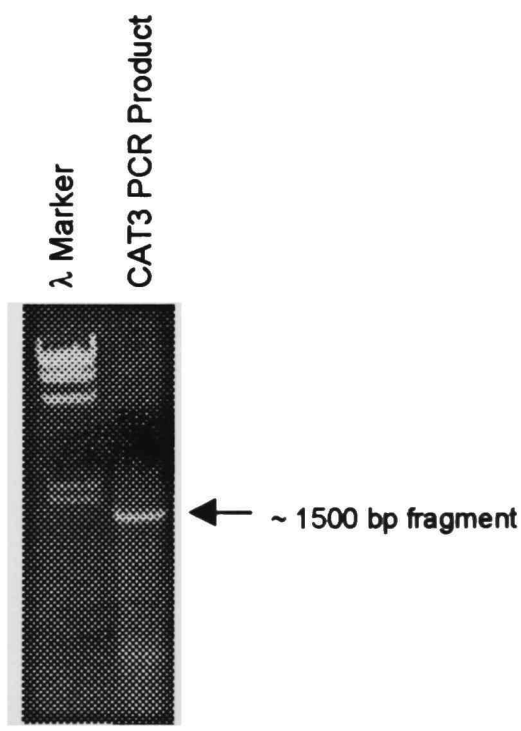


Figure 3.1 Agarose gel electrophoresis of the RT-PCR products of *Cat3* using total RNA extracted from 8-day epicotyls of maize.

3.2 Expression of Maize *Cat3* in T_0 Tobacco Plants

Transgenic tobacco plants that contain the *Cat3* gene construct were regenerated and transferred to the greenhouse. A total of 20 independently transformed lines was obtained. T_0 plants that expressed maize CAT3 were initially identified by Northern blot hybridization using a α-^{32}P-labelled partial CAT3 cDNA probe. Analysis of autogradiograms, as shown in Figure 3.2, indicated high levels of maize CAT3 mRNA in several of the transgenic plants while little radioactive signal from native CAT3 mRNA was seen in wild-type control plants. A total of four independently transformed lines expressing maize CAT3 (A1.9, M1.15, Z1.12 and T2.17) were identified by a several-fold increase in transcript levels than found in non-expressing (H1.2) or non-transformed control plants (Xanthi NN).

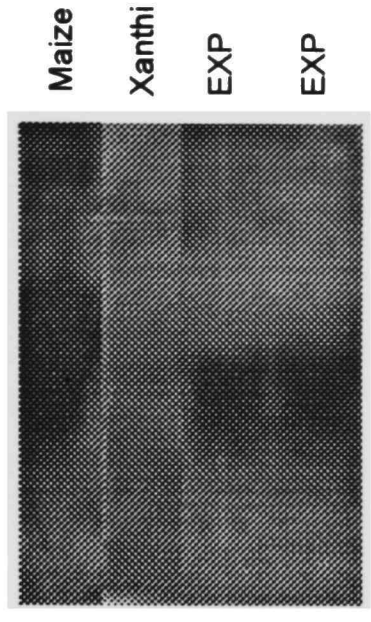


Figure 3.2 Northern blot analysis of maize *CAT3* in T_0 tobacco plants. Total RNA (20 µg) was isolated from leaves of tobacco plants expressing maize CAT3 (EXP) and control plants (maize and Xanthi NN), separated, blotted onto nitrocellulose, and hybridized with a ^{32}P-labeled *Cat3* cDNA.

T_0 plants were self-pollinated to produce T_1 seeds that were used for further analysis. T_1 seeds were sown on kanamycin germination media to allow selection of transformants (Appendix A). Control seedlings (Xanthi and H1.2) were sown on germination media minus the antibiotic. These transgenic and control seedlings were transplanted to soil and subsequently analyzed by Northern blot analysis, enzyme activities and various physiological experiments. High levels of mRNA accumulated in T_1 transgenic plants as compared to the little or no signal seen in the non-expressor and wild-type Xanthi as indicated in Figure 3.3.

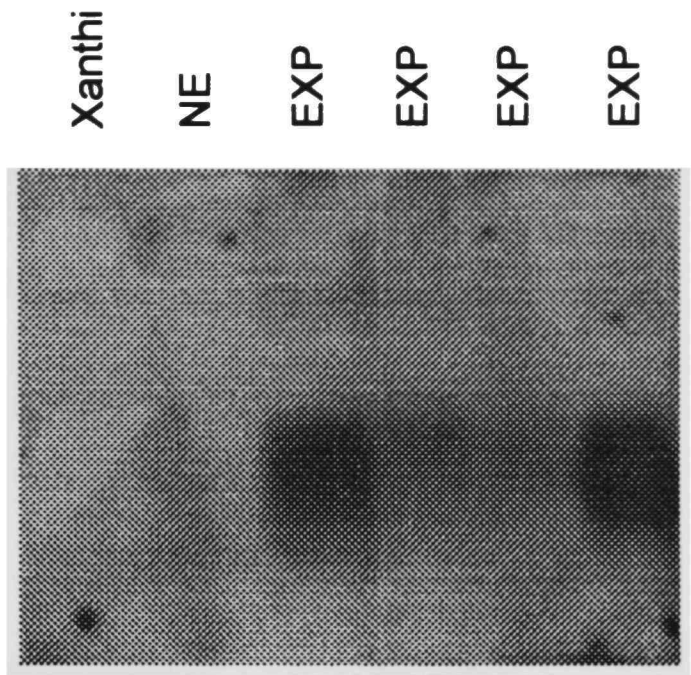


Figure 3.3 Northern blot analysis of *CAT3* in T_1 tobacco plants. Total RNA (20 µg) was isolated from leaves of T_1 tobacco plants expressing maize CAT3 and control plants (Xanthi and NE), separated, blotted onto nitrocellulose, and hybridized with a ^{32}P-labeled *Cat3* cDNA.

In order to ascertain the expression levels of maize CAT3 mRNA in tobacco, total RNA was extracted from various plant organs. Total root, stem, leaf, and flower RNA was used for Northern blot analysis. Hybridization was carried out using a α–^{32}P-labeled maize C*at*3 cDNA. Analysis of the autoradiogram shown in Figure 3.4 indicates expression of CAT3 transcripts in seed, flower, root, and stem tissues.

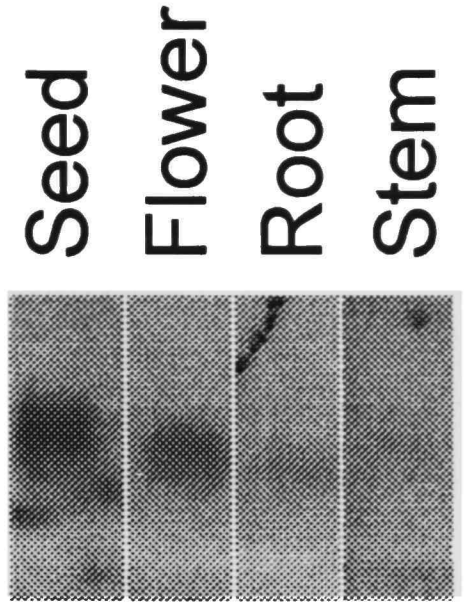


Figure 3.4 Accumulation of maize CAT3 transcripts in a variety of different tobacco tissues. Total RNA was extracted from roots, stems, seeds and flowers; separated on 1% agarose-formaldehyde gels; transferred to a nitrocellulose membrane; and hybridized with a α–^{32}P-labeled maize *Cat3* cDNA.

Catalase activity levels were determined by monitoring the disappearance of H_2O_2 in reactions containing extracts of transgenic (T_1) and control tobacco plants. The results from three independently transformed lines are shown in Figure 3.5. Only a minimal increase (approximately 1.2-fold) in catalase activity was detected in leaves of T_1 tobacco plants that express maize CAT3 as compared to the control plants, and this difference was not statistically significant (P=0.2461).

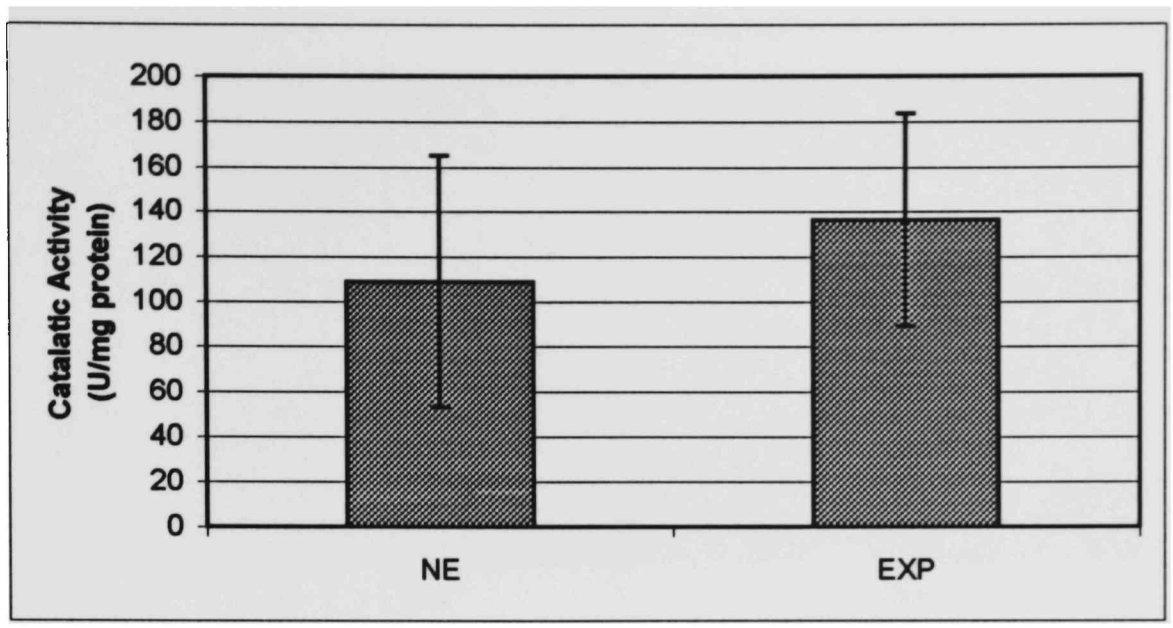


Figure 3.5 Spectrophotometric catalase activity assay of leaf extracts from non-expressing and T_1 transgenic tobacco plants. Standard deviations (SDs) are indicated by error bars, n=15 CAT3 expressors (EXP) and n=7 control and non-expressing (NE) *CAT3* lines. No significant differences were detected between EXP and NE.

Since maize CAT3 has enhanced peroxidatic activity, the peroxidatic activity of extracts from transgenic (T_1) and non-expressing plants was measured by monitoring the oxidation of ABTS. Tobacco plants that express maize CAT3 exhibited a 12-fold higher peroxidatic activity than the control plants (Figure 3.6). These results demonstrate that the maize *Cat3* transgene encodes a functional catalase with enhanced peroxidatic activity in transgenic tobacco plants.

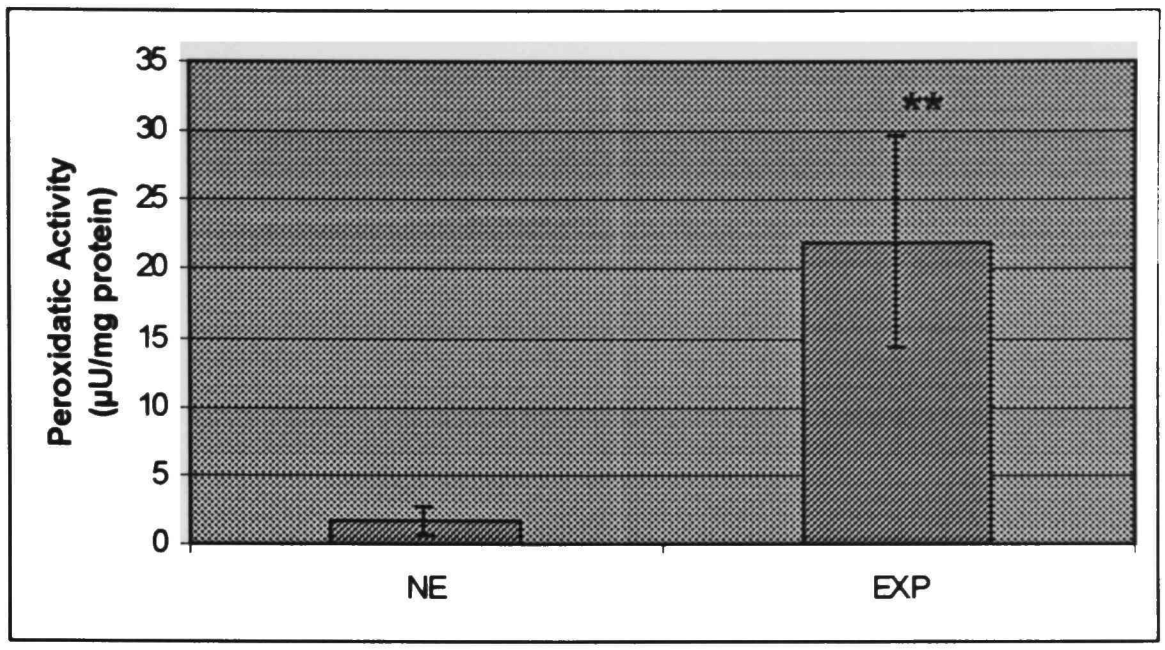


Figure 3.6 Spectrophotometric peroxidatic activity assay of leaf extracts from non-expressing and transgenic tobacco plants. SDs are indicated by error bars, n=13 CAT3-expressors (EXP) and n=7 control and non-expressing (NE) lines. ** denotes significant differences at $P < 0.001$.

Comparison of the peroxidatic and catalatic activities of catalase enzymes can be represented by the expression Rp/c, where p=mU of peroxidatic activity and c=U of catalatic activity. Total catalatic activity of transgenic tobacco plants that express maize CAT3, which has enhanced peroxidatic activity, would be expected to have higher Rp/c values compared to non-expressing control plants. As illustrated in Table 3.1, transgenic (T_1) tobacco plants that express maize CAT3 had Rp/c values that were 14-fold higher than control plants.

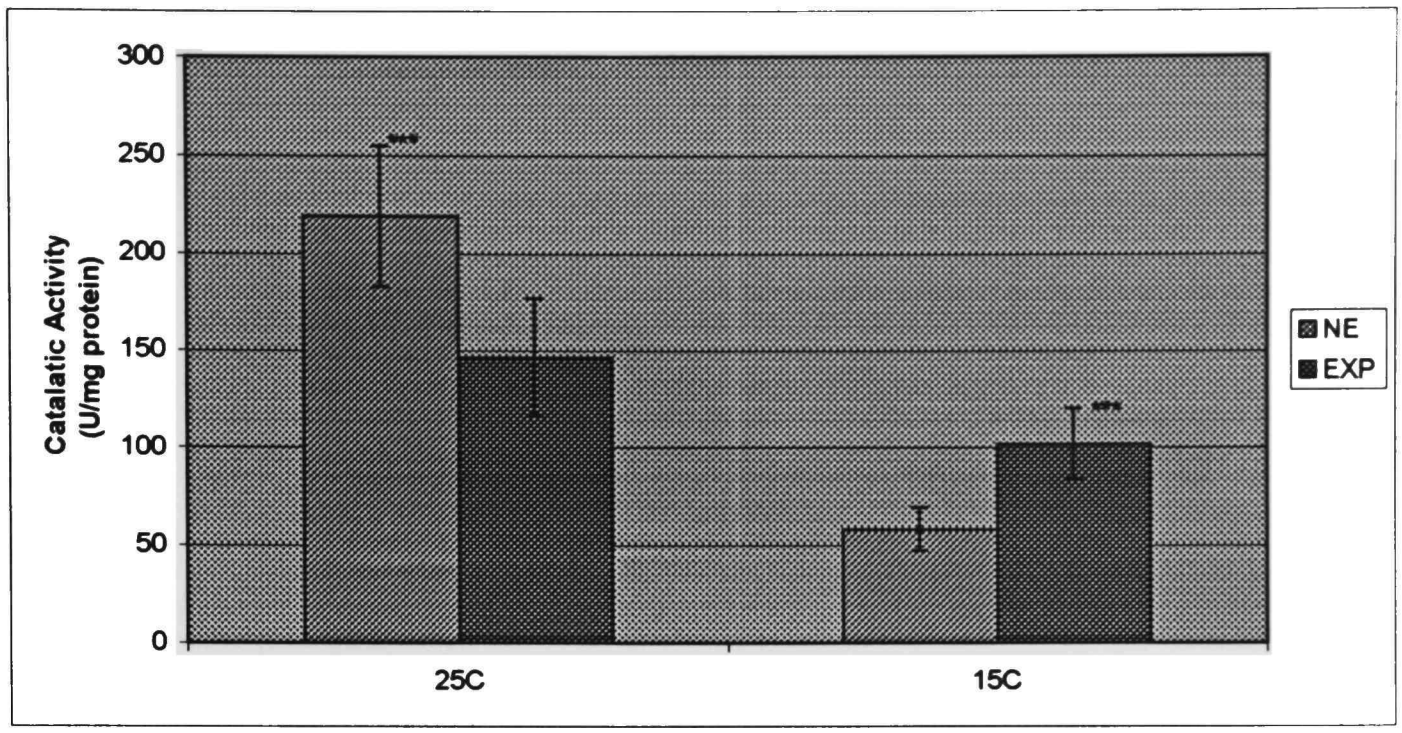
Table 3.1 Catalase activity expressed as Rp/c in mature leaf tissue. Catalatic and peroxidatic activities of transgenic and non-expressing plants were determined in order to calculate Rp/c values. Rp/c is defined as mU/U X10.

Plant	Rp/c (mU/U X 10)
NE	0.0001594
EXP	0.0022376

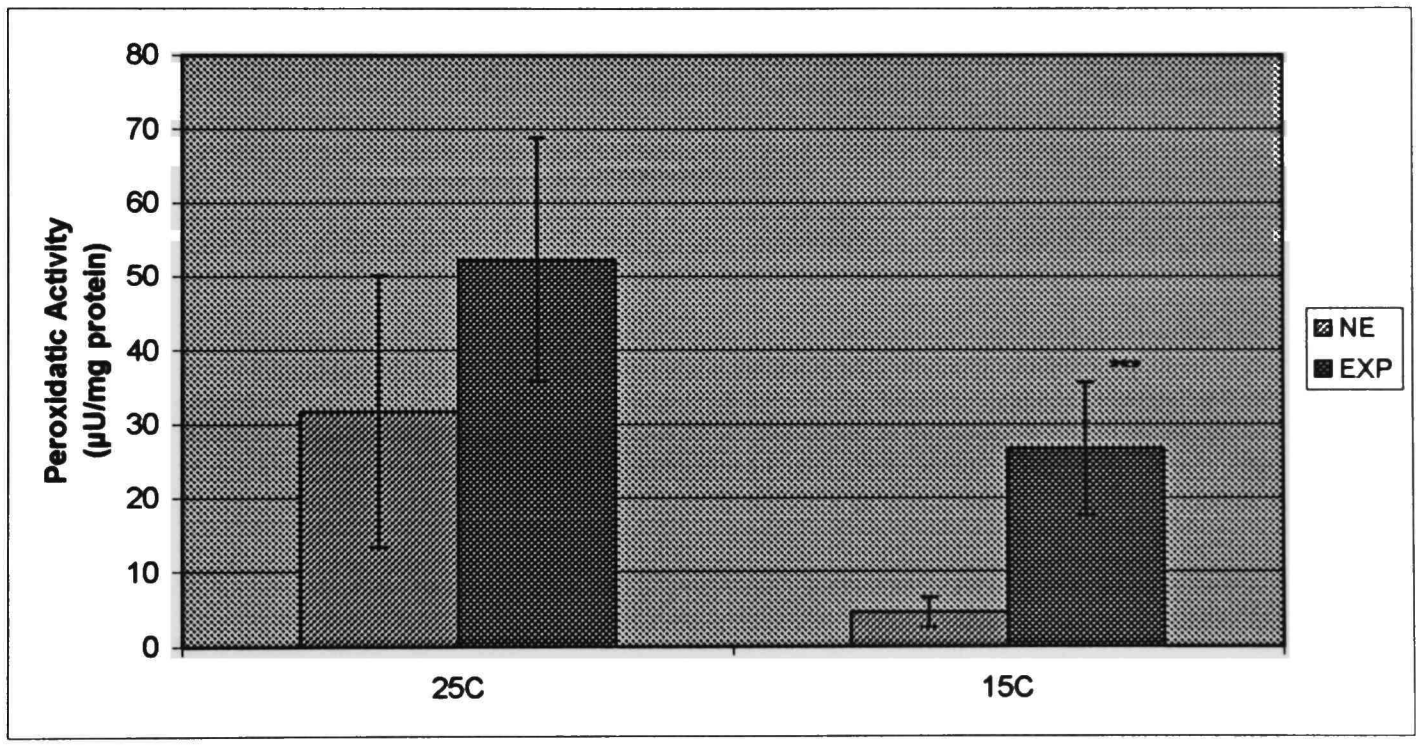
To determine the effects of chilling on catalase expression in tobacco seedlings, total catalatic and peroxidatic activities were measured for seedlings grown in the dark at 25°C or 15°C for 7 days (Figure 3.7). Total catalatic activity in control seedlings grown at 25°C was slightly higher than the CAT3-expressing seedlings grown under the same conditions. Catalatic activity of control seedlings grown at 15°C was less than 25% of that activity at 25°C. While catalatic activity of CAT3-expressing seedlings grown at 15°C was also lower, they retained more than 66% of the activity seen at 25°C. Therefore, CAT3-expressing seedlings had approximately two-fold higher catalatic activity than control seedlings when grown at 15°C. In addition, seedlings were assayed for peroxidatic activity. Transgenic tobacco seedlings grown at 25°C had almost 2-fold higher peroxidatic activity compared to control seedlings treated identically. Although a trend toward higher peroxidatic activity is exhibited in the transgenic plants, this difference was not found to be statistically significant between the transgenic and control seedlings grown at 25°C due to high variability in the samples. Peroxidatic activity levels in seedlings grown at 15°C were reduced in both

CAT3-expressing and control tobacco seedlings, but transgenic seedlings retained approximately 50% of their activity at 25°C while peroxidatic activity of control seedlings was only about 15% of that activity at 25°C. Therefore, while the levels of catalatic and peroxidatic activity in transgenic CAT3-expressing seedlings grown at 25°C is similar to that of control plants, levels of both catalatic and peroxidatic activity is significantly higher in transgenic seedlings grown at chilling temperatures.

Calculation of Rp/c values for catalase enzymes from seedlings grown under normal and chilling conditions is shown in Table 3.2. The Rp/c values for control seedlings grown at 25°C were nearly 10-fold higher than in leaf tissue of plants grown in the greenhouse (Table 3.2). This reflects the lower peroxidatic activity in leaf tissue. Rp/c values for transgenic seedlings grown at 25°C were only slightly higher than extracts from leaf samples of greenhouse grown plants. At 15°C, Rp/c values for control seedlings are about 20% of that at 25°C, while in transgenic seedlings Rp/c values remain relatively high.



A



B

Figure 3.7 – Total catalatic (A) and peroxidatic (B) activities measured spectrophotometrically of transgenic and control (Xanthi and NE) tobacco seedlings grown at 25°C and 15°C. SDs are indicated by error bars, n=4 *Cat3* independently transformed lines and n=2 non-expressing lines. *** denotes significant at $P<0.001$.

Table 3.2 – Ratio of peroxidatic activity to catalatic activity (Rp/c) of transgenic tobacco seedlings (EXP) expressing CAT3 and control seedlings (Xanthi and NE) grown at 25°C and 15°C for 7 days. Rp/c is calculated as p(mU)/c(U)X10. n=4 *Cat3* independently transformed lines and n=2 non-expressing lines.

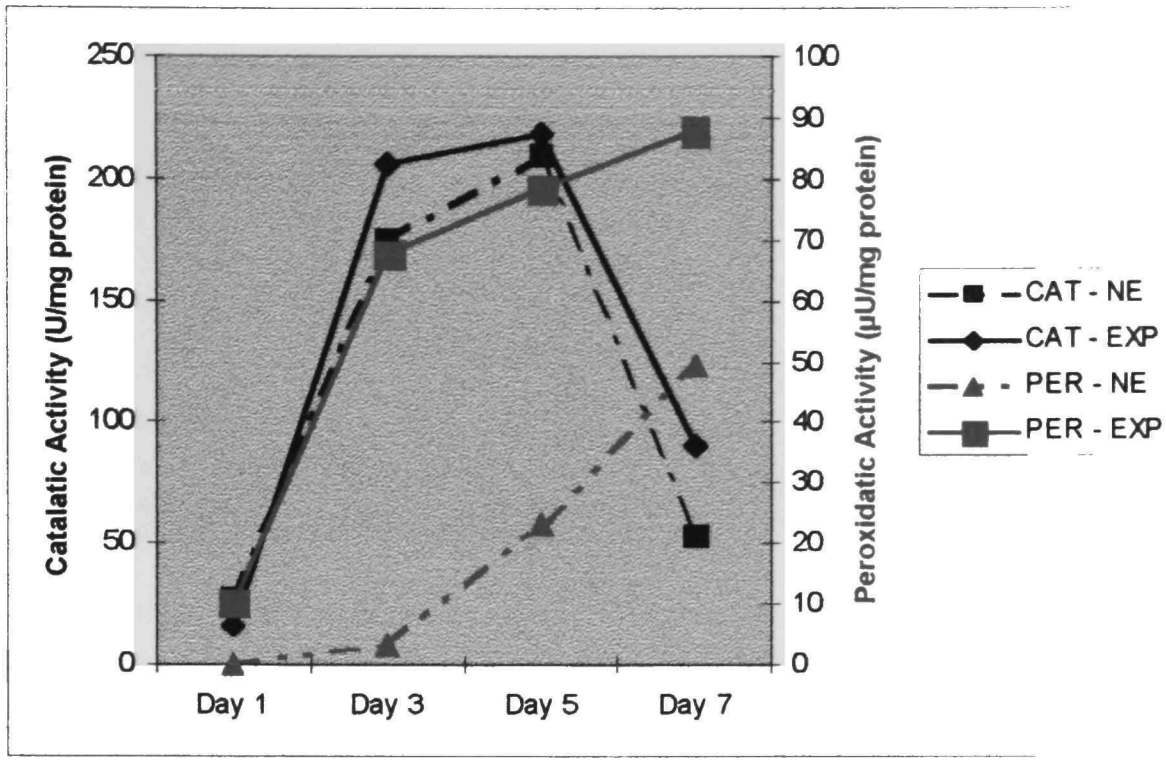
	Temperature	
	25°C	15°C
NE	0.00145	0.000782
EXP	0.00356	0.00261

Since the activity of native catalases in germinated seeds and growing seedlings is developmentally regulated, a time course analysis of both catalatic and peroxidatic activities was performed on transgenic and control tobacco seedlings grown at a normal temperature (25°C) and under chilling conditions (15°C). At 25°C, levels of catalatic activity were similar for both transgenic CAT3-expressing seedlings and control seedlings at all time points tested (Figure 3.8A). Catalatic activities were uniformly low at day 1 post-imbibition and increased by seven to eight fold in both control and transgenic seedlings by day 3 and remained high at day 5 post-imbibition. By day 7 post-imbibition, levels of catalatic activity dropped sharply in both genotypes.

Levels of peroxidatic activity in CAT3-expressing seedlings grown at 25°C also increased sharply between day 1 and day 3 post-imbibition and continued to increase through day 7. However, peroxidatic activity in control seedlings showed a slow increase in activity through day 7 post-imbibition. Therefore,

while there were no substantial differences in catalatic activity between control and transgenic seedlings at 25°C, differences in peroxidatic activity were detected throughout seedling development. At 15°C, the increase in catalatic and peroxidatic activities was delayed in both control and CAT3-expressing seedlings (Figure 3.8B). But, by day 7 post-imbibition, catalatic and peroxidatic activities were nearly two-fold higher in CAT3-expressing seedlings than in control seedlings. The peak and subsequent decline in catalatic activity in control seedlings at 25°C reflects changes in expression of endogenous catalase genes. While the more stable increase in peroxidatic, activity in CAT3-expressing seedlings is the result of transgene expression under control of the constitutive CaMV 35S promoter.

Transgenic and control tobacco seedlings grown at elevated temperatures (30°C and 37°C) were assayed for catalatic (A) and peroxidatic (B) activity (Figure 3.9). Though catalatic activity was lower in both CAT3-expressing and control seedlings grown at 30°C and 37°C (Figure 3.9A), no differences were seen in catalatic activity between CAT3-expressing seedlings and control seedlings (Xanthi and NE). Peroxidatic activity was higher in transgenic seedlings than in control seedlings at all temperatures (Figure 3.9B). Peroxidatic activity declined in control seedlings at both 30°C and 37°C relative to seedlings grown at 25°C but it increased slightly in CAT3-expressing seedlings at 30°C. While peroxidatic activity was reduced in CAT3-expressing seedlings at 37°C, activity levels were nearly 10-fold higher than in control seedlings at this temperature.



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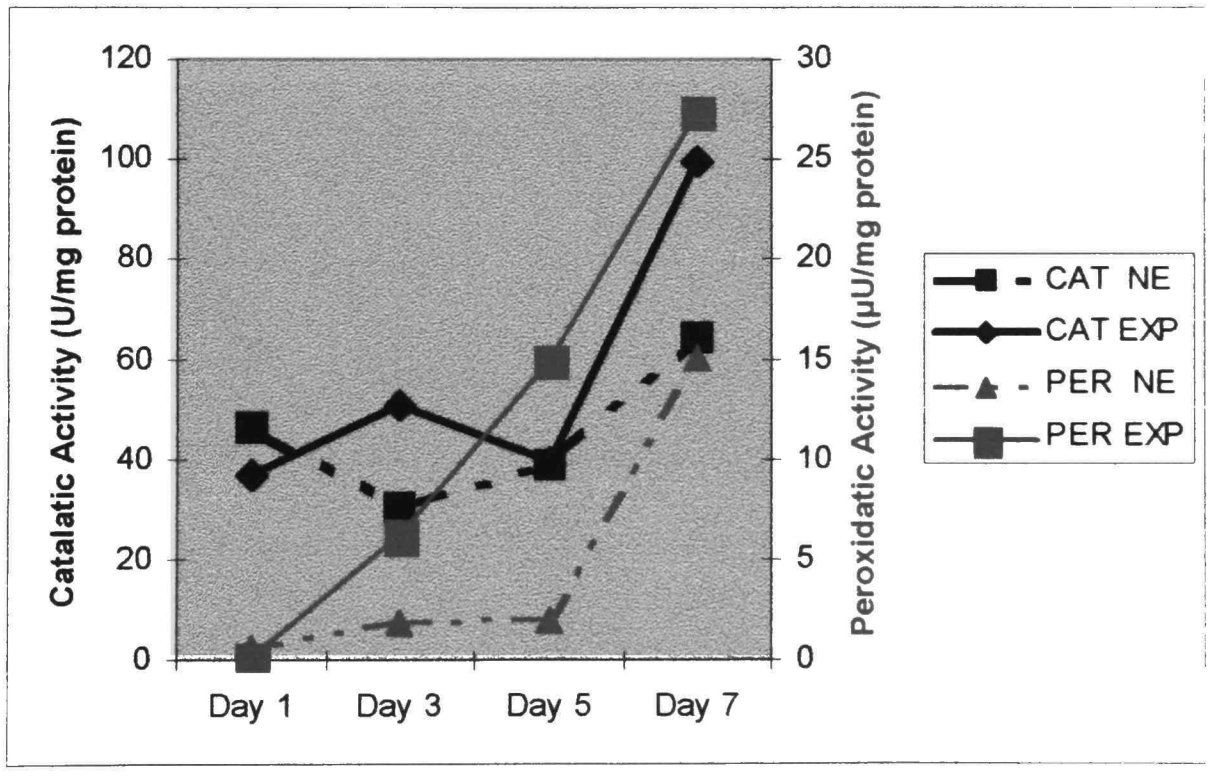
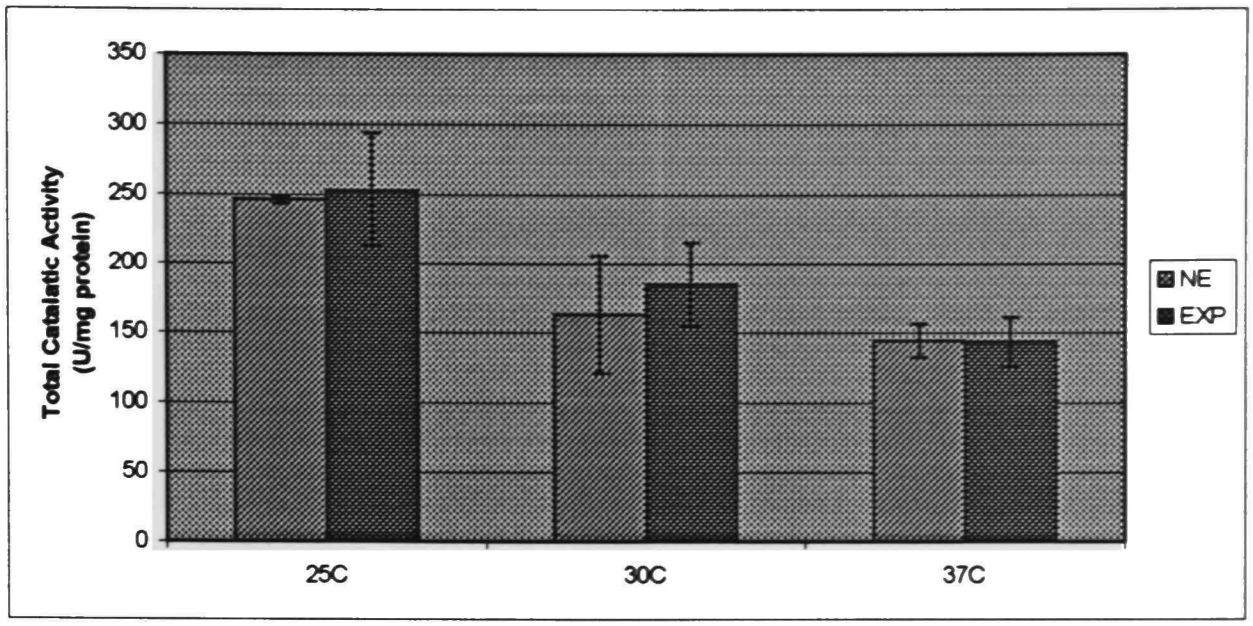


B

Figure 3.8 – Time course analysis of total catalatic and peroxidatic activities of transgenic seedlings (EXP) and control seedlings (NE) germinated at 25°C (A) and 15°C (B) for a period of 1, 3, 5, and 7 days. Data shown are means of two transgenic lines and one control line (Xanthi).



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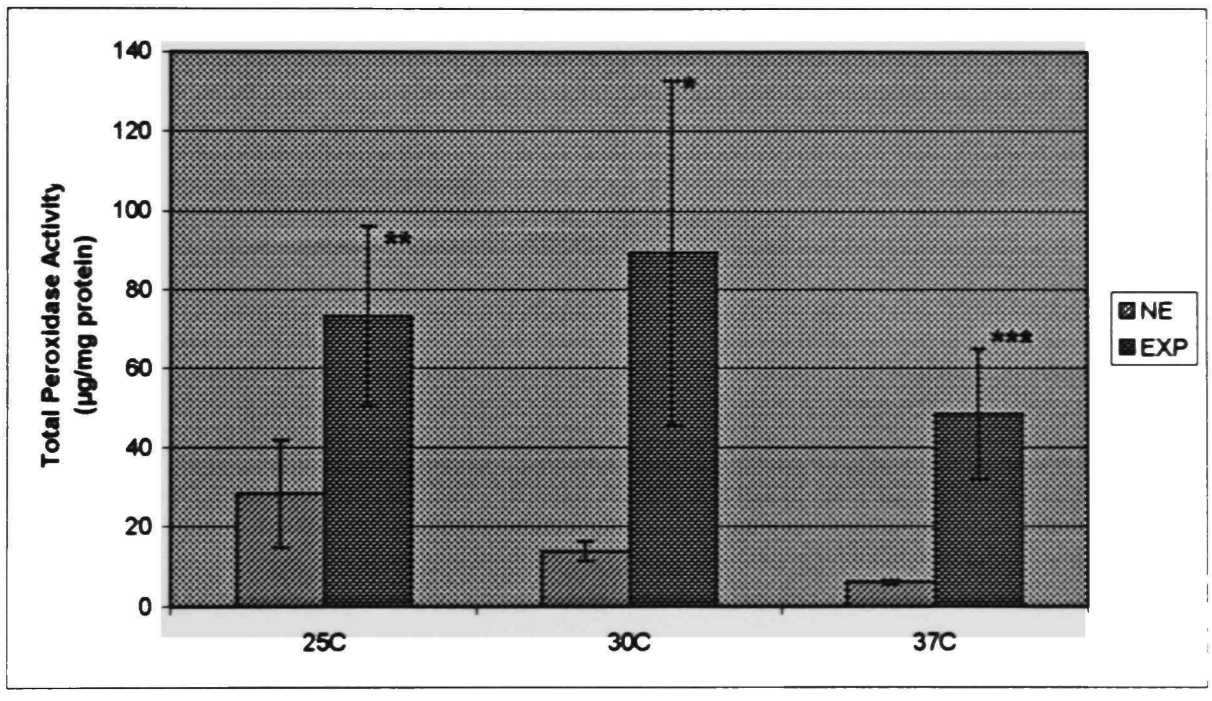


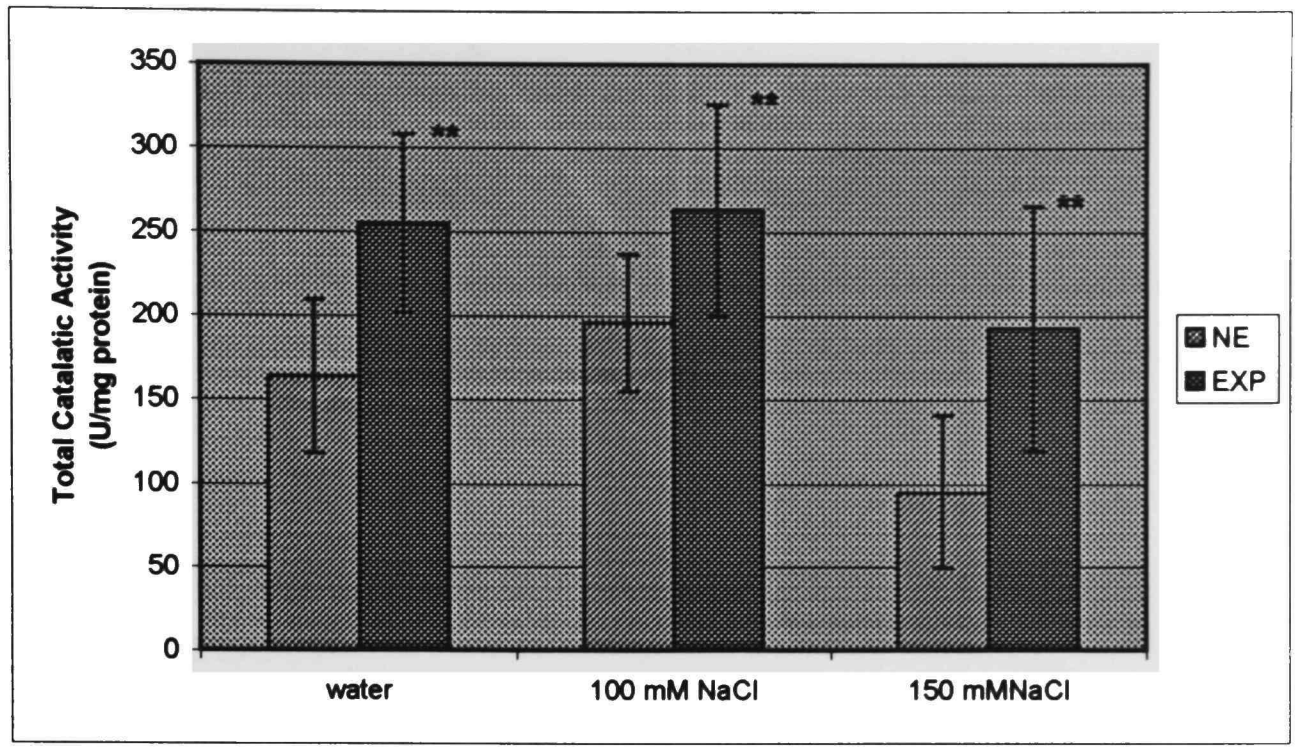
B

Figure 3.9 – Total catalatic (A) and peroxidatic (B) activities of seedlings grown at 25°C, 30°C and 37°C for 5 days. Four independently transformed lines were compared to two control lines (Xanthi and NE) for all treatments. No significant differences were evident in the catalatic activity levels between the CAT3-expressing transgenic and control seedlings. SDs are indicated by error bars. * denotes significant difference at $P<0.05$, ** denotes significant difference at $P<0.01$ and *** denotes significant difference at $P<0.001$.

Levels of catalatic and peroxidatic activity were also monitored in CAT3-expressing and control seedlings exposed to salt stress by growth in 100 mM and 150 mM NaCl (Figure 3.10). Catalatic activities in transgenic and control lines at 100 mM NaCl were unchanged compared to those grown without salt (Figure 3.10A). Catalatic activity was decreased in seedlings grown at 150 mM NaCl and the loss of activity in control seedlings was more substantial than in CAT3-expressing seedlings. Similar results were noted for peroxidatic activity in salt-stressed seedlings. However, the peroxidatic activity of CAT3-expressing seedlings was approximately 4-fold higher than control seedlings in all cases (Figure 3.10B).

3.3 Analysis of Photooxidative Stress

Photooxidative stress occurs when leaves undergo periods of high light intensity and low temperatures (Allen, 1995) leading to inhibition of carbon fixation. When an increase in the photoreduction of O_2 occurs leading to photooxidative stress, it can result in increased damage of photosynthetic components with a reduction in overall photosynthetic capacity. One way to simulate this process is by subjecting leaf tissue to a stress treatment of high light and cold temperature and monitoring the loss of photosynthetic capacity. Leaf disks from tobacco plants (T_1) that expressed maize CAT3 exhibited no increased photooxidative stress protection as compared to control tobacco plants after a four-hour period of photooxidative stress under high light and low temperature (Figure 3.11).



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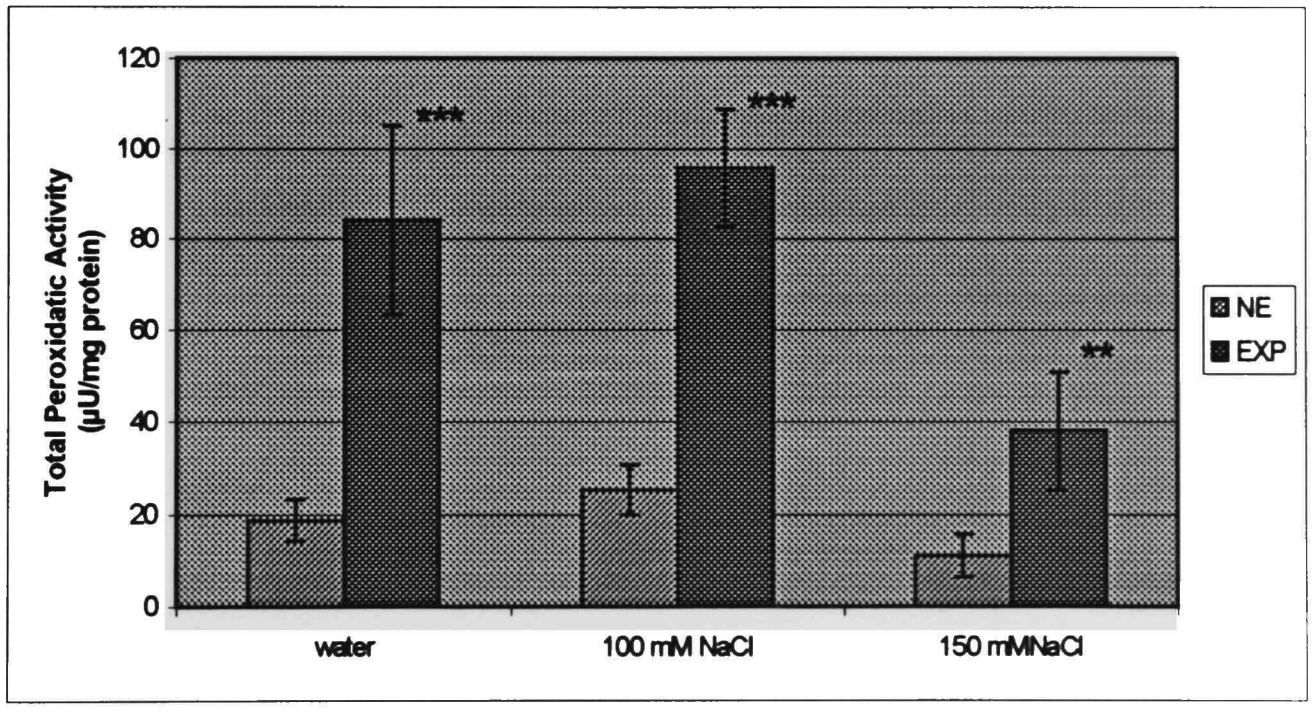

B

Figure 3.10 – Total catalatic (A) and peroxidatic (B) activities in seedlings grown under salt stress. Four independently transformed lines expressing CAT3 and 2 control lines (Xanthi and NE) were grown in 100 mM and 150 mM NaCl for a period of 5 days. SDs are indicated by error bars. ** denotes significant difference at P<0.01 and *** denotes significant difference at P<0.001.

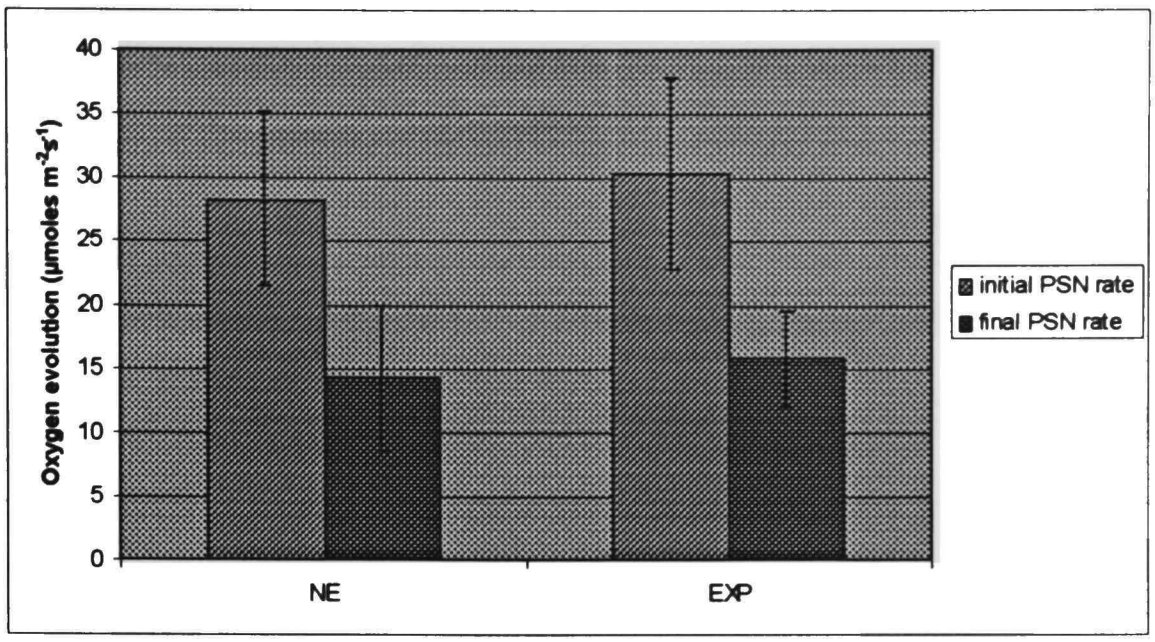

Figure 3.11 Photosynthetic rates of control plants and transgenic tobacco plants expressing maize CAT3. O_2 evolution was monitored from leaf disks under saturating CO_2 conditions after a 1-hour acclimation period at 25°C and a PFD of 1500 µmole $m^{-2}s^{-1}$. An initial photosynthetic rate was taken immediately following acclimation and a final photosynthetic rate was determined following the 4 h stress treatment on ice (4°C) and a PFD of 1500 µmole $m^{-2}s^{-1}$. SDs are indicated by error bars. n=17 for transgenic plants and n=7 for control plants. No significant differences were found between expressors and non-expressors.

3.4 Methyl Viologen Assay

In order to evaluate the protective effects of expression of a maize *Cat3* gene product in transgenic tobacco against oxidative stress, leaf tissue from plants grown in the greenhouse was treated with the herbicide methyl viologen (MV), also known as paraquat. Electrons that are normally transported from reduced ferredoxin (Fd) to $NADPH^+$ in photosystem I of the electron transport chain during photosynthesis are instead seized by MV to produce a MV radical. These MV radicals are then able to react with molecular oxygen to produce superoxide radicals leading to the production of H_2O_2 and $^{\cdot}OH$ radicals that can

cause physiological damage. Induction of oxidative stress *in vitro* was carried out by incubating leaf disks of CAT3-expressing and control tobacco plants at increasing concentrations of MV. Electrolyte leakage assays were performed on the MV solutions in order to determine the amount of membrane damage to the tissues. An overall percent of electrolyte leakage was measured allowing comparison between expressors and non-expressors. No increased protection from MV-induced oxidative stress in the 1.2 µM MV treatment was seen in CAT3-expressing tobacco plants compared to the control plants (P=0.36031) (Figure 3.12). A slight protection from MV-induced oxidative stress in the 2.4 µM MV treatment was found to be statistically significant in the CAT3-expressing plants compared to the control plants (P=0.02011).

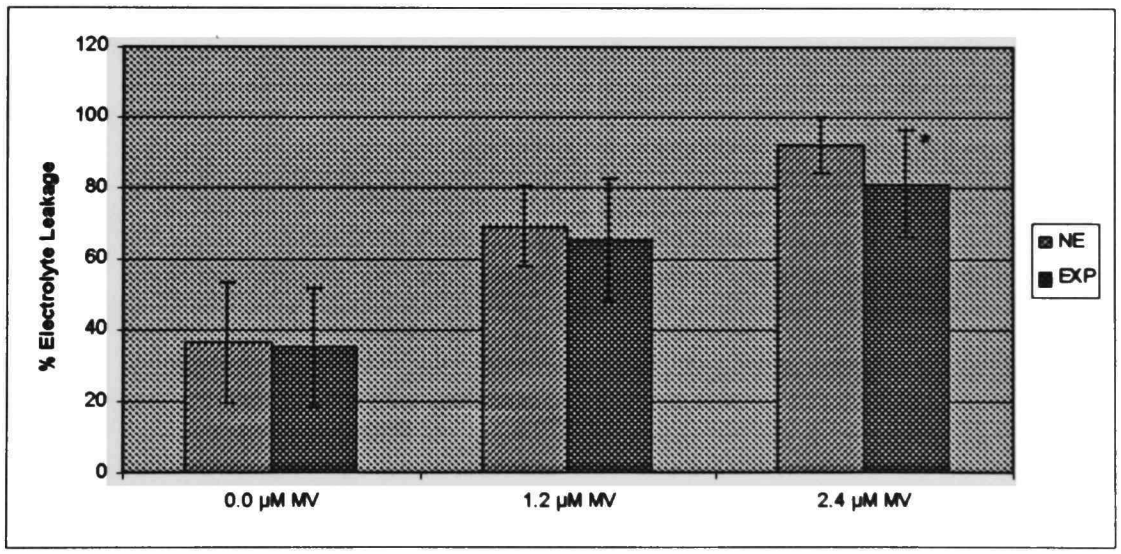

Figure 3.12 Methyl viologen induced oxidative stress damage in CAT3-expressing and control tobacco plants. SDs are indicated by error bars. Data shown are means of 19 transgenic plants and 8 control plants. * denotes significant difference at P<0.05.

3.5 Effects of Stress on T_1 Tobacco Seedling Growth

In order to determine the protective effects of maize *Cat3* expression in transgenic tobacco seedlings, seeds of CAT3-expressing and control plants were germinated and evaluated under a variety of different stress conditions such as low temperatures, high temperatures, salt and chemical treatments.

Analysis of chilling tolerance was determined by incubating the imbibed seeds of CAT3-expressing tobacco plants along with control seeds at 4°C, 8°C, and 15°C in the dark. Transgenic seedlings grown in the dark at 4°C for 70 days were observed to have increased ability to germinate and increased seedling length compared to the control seedlings that exhibited little germination (Figures 3.13 and 3.14). Twenty seedlings were randomly picked for seedling length measurements. These results suggest the protective capabilities against chilling induced oxidative stress in tobacco plants expressing maize CAT3.

Seedlings, both transgenic and control, were grown at 8°C for 28 days in the dark. CAT3-expressing seedlings had increased seedling growth that was approximately double that of control (Xanthi and non-expressor) seedling growth at 8°C (Figure 3.15).

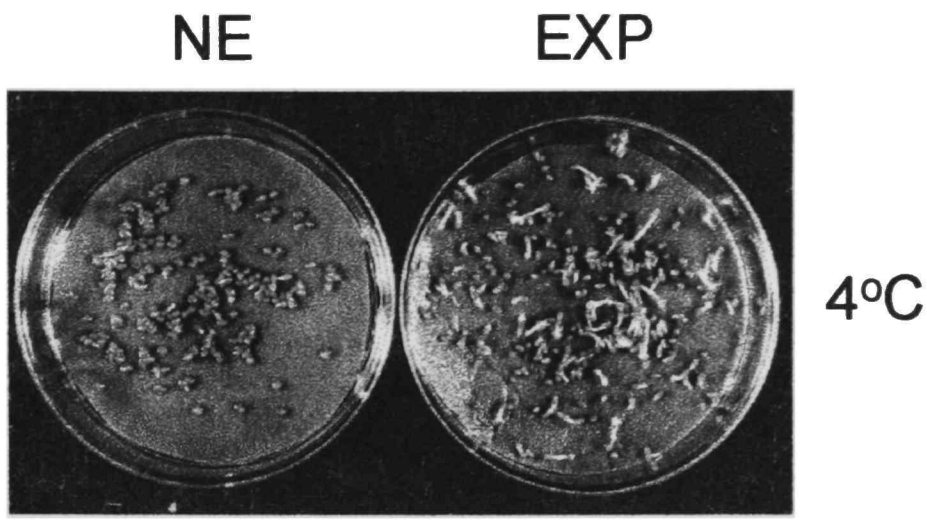

Figure 3.13 – Seedling growth at 4°C in the dark for 70 days of CAT3-expressing tobacco seedlings compared to control seedlings (Xanthi and non-expressors). Seeds were germinated on moist filter paper in Petri dishes sealed with parafilm.

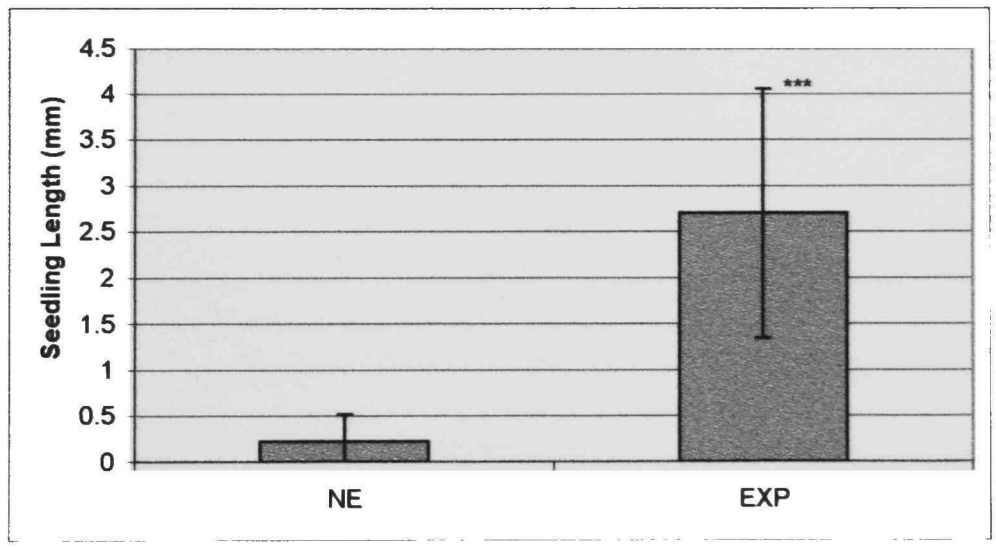

Figure 3.14 – Seedling length of CAT3-expressing and control tobacco plants. Seeds were sown on moistened germination paper in Petri dishes and incubated at 4°C for 70 days. n=4 independently transformed lines expressing maize CAT3, and n=2 control (Xanthi and non-expressing line). Error bars denote SDs. *** indicate significant differences at $P<0.001$.

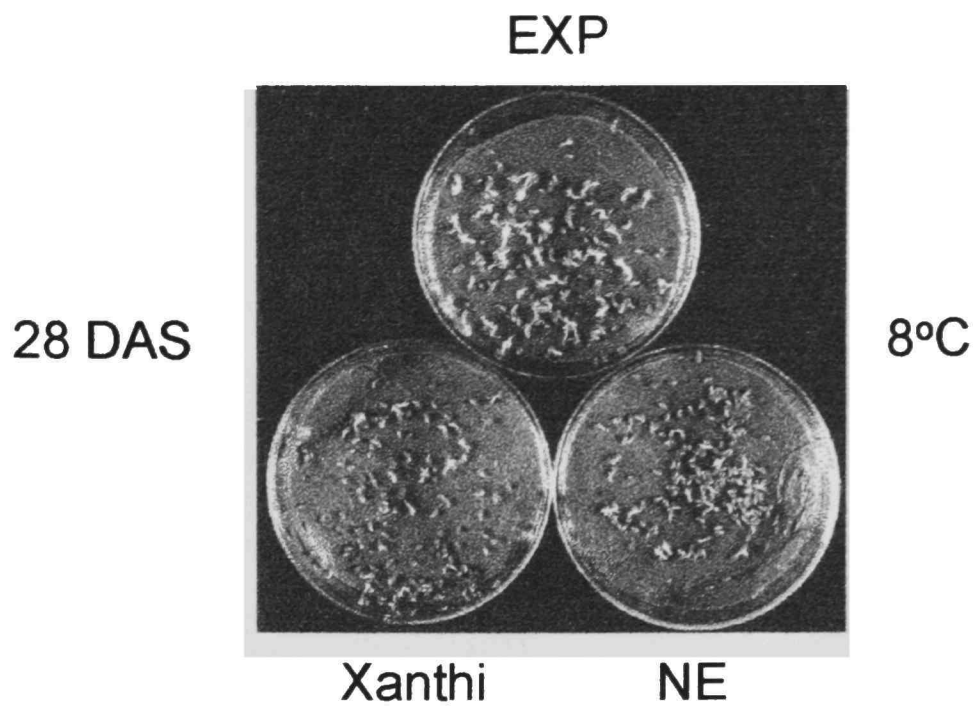

Figure 3.15 – Seedling growth of CAT3-expressing and control (NE and Xanthi) seedlings grown in the dark at 8°C for 28 DPI. All seedlings were germinated in Petri dishes lined with germination papers moistened with 800 µl of sterilized water.

Tobacco seedlings expressing maize CAT3 at 15°C were also found to have increased seedling growth compared to control tobacco plants (Figures 3.16 and 3.17). Approximately twenty seedlings of four independently transformed lines of CAT3-expressing maize were randomly selected for seedling length measurement and compared to controls (non-expressors and Xanthi). Although a substantial loss of seedling vigor was seen in CAT3-expressing and control seedlings grown at 15°C compared to 25°C, the CAT3-expressing seedlings germinated faster and were longer than control seedlings. Differences seen between the CAT3-expressing and control seedlings grown at 15°C were significantly different. Transgenic seedlings with increased protection

against cold temperature stress appeared to be segregating in a 3:1 Mendelian fashion.

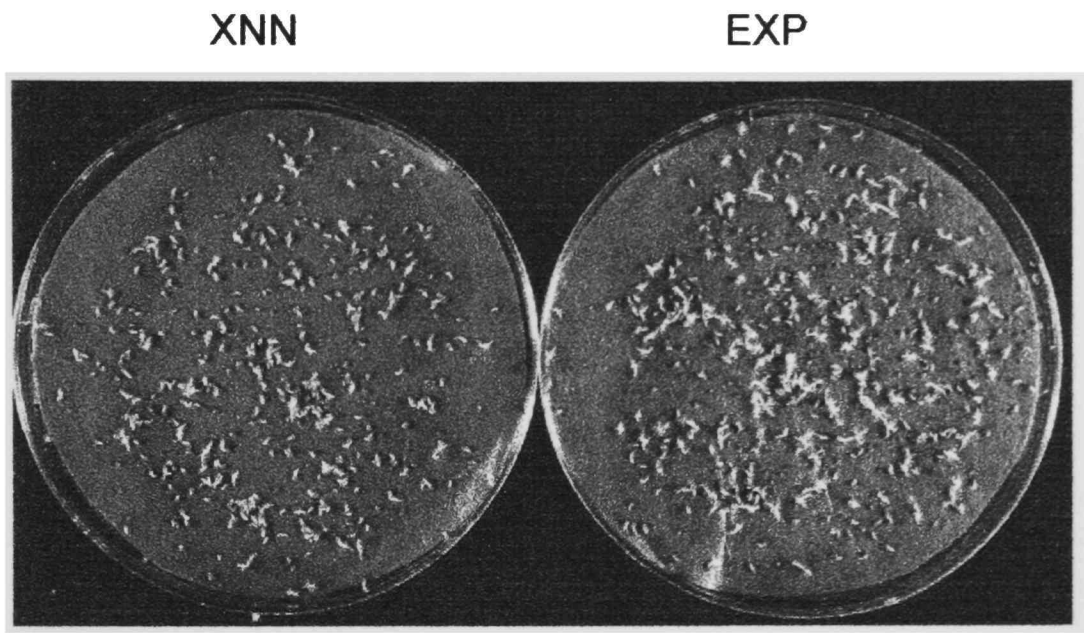

Figure 3.16 – Comparison of seedling growth between control and CAT3-expressing tobacco seedlings. Seedlings were germinated in the dark on moist filter paper and grown in Petri dishes at 15°C for 7 days. Four independently transformed expressing lines were used for germination studies.

In order to better understand the growth patterns of the two populations of seedlings at 25°C and 15°C, a time course study was performed using two of the four transgenic lines that express maize CAT3 and control (Xanthi) seedlings. Seedling measurements were recorded and photographed at three, five, and seven days post-imbibition (Figure 3.18). Interestingly, at day 5 a detectable difference in seedling length was evident between control and transgenic seedlings grown at 25°C. This difference between the CAT3-expressing and control seedlings disappeared by day 7. No differences were noted at 3 days

post-imbibition between the transgenic and control seedlings grown at 15°C. Significant differences were seen beginning at 5 days through 7 days post-imbibition, where CAT3-expressing seedlings were longer than control seedlings grown at 15°C .

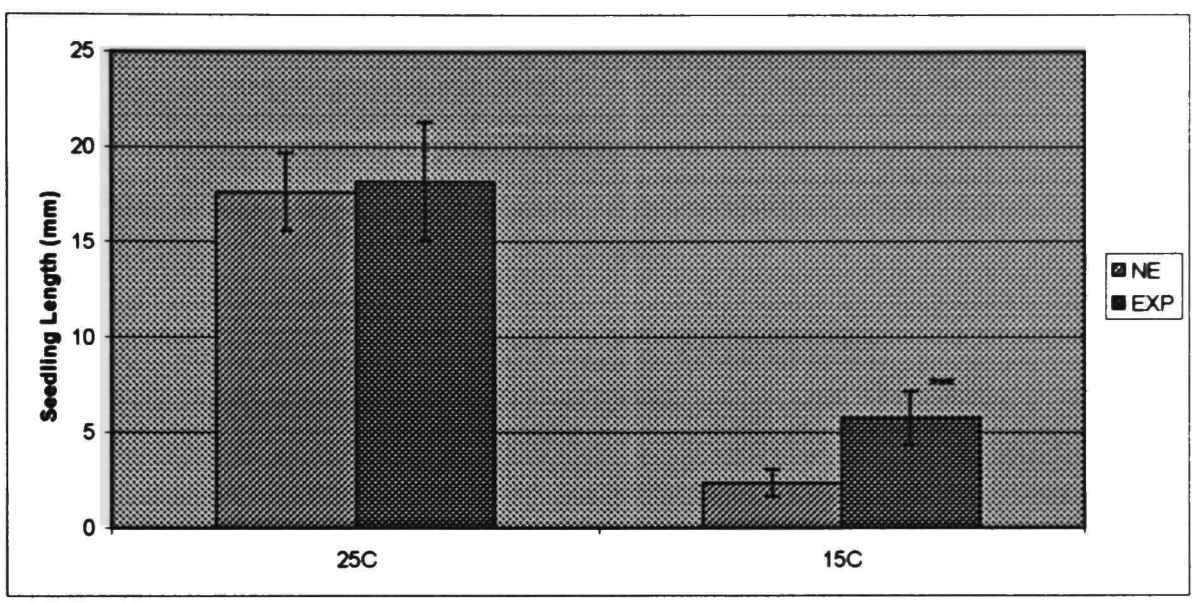

Figure 3.17 – Seedling length of transgenic CAT3-expressing and control seedlings grown at 25°C and 15°C for 7 days in the dark. Increased protection against cold temperatures was evident in the transgenic seedlings. SDs are indicated as error bars for the four replicates. *** significant at P<0.001.

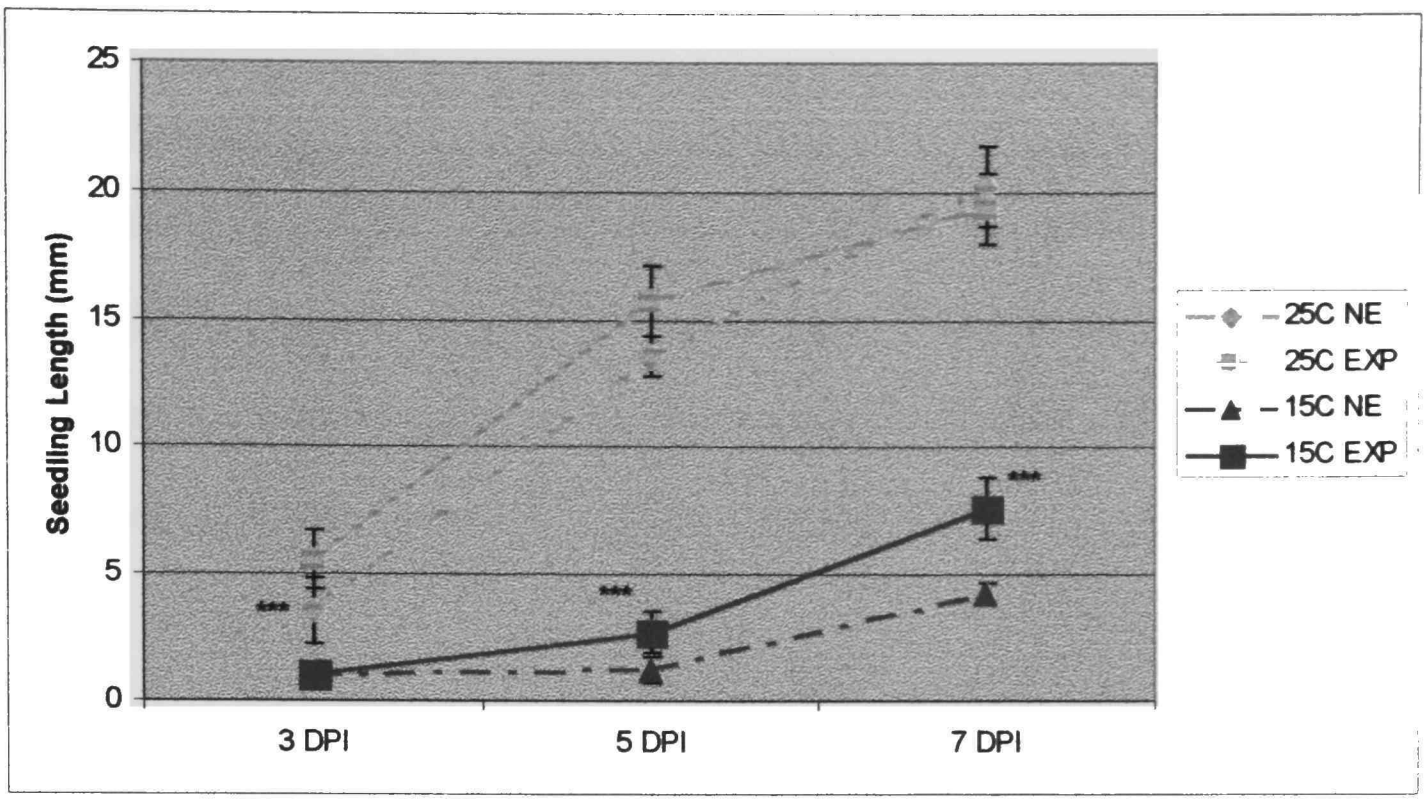

Figure 3.18 – Growth at 25°C and 15°C of maize CAT3-expressing seedlings and control seedlings at 3, 5, and 7 days post-imbibition. n=2 transgenic lines, and n=1 for control lines (Xanthi). Error bars indicate SDs. *** denote significant differences at P<0.001.

Tobacco seeds expressing maize CAT3 were subjected to high temperature germination trials in order to determine the protective capabilities of maize CAT3 at 30°C and 37°C (Figures 3.19 and 3.20). Seedlings were placed on moist germination papers in Petri dishes, sealed with parafilm, and grown in the dark at 25°C, 30°C and 37°C for 5 days when detectable differences were seen between transgenic and control seedlings (Figure 3.20). At 30°C, both the CAT3-expressing tobacco seedlings and control seedlings were longer than the seedlings grown at 25°C. Tobacco seedlings expressing maize CAT3 were found to have increased growth at both 30 and 37°C compared to control

samples. The differences in seedling length between the CAT3-expressing and control seedlings grown at 30°C and 37°C were significant. An inhibition of growth was seen in both CAT3-expressing seedlings and control seedlings grown at 37°C compared to the seedling growth of these same seedlings at 25°C. These results indicate that the introduction of a maize *Cat3* transgene confers seedling tolerance to high temperature stresses.

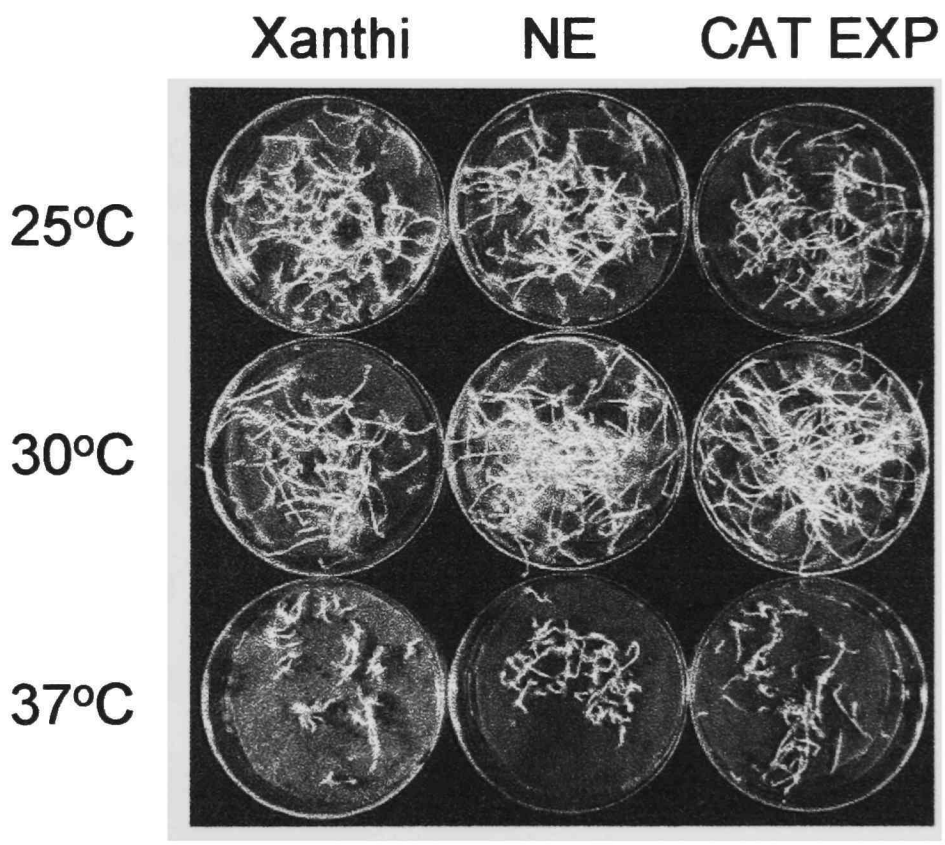

Figure 3.19 – Tobacco seedlings grown at high temperatures (30°C and 37°C) for 5 days. Transgenic tobacco and control seedlings were grown on moist filter paper in Petri dishes, sealed with parafilm, and grown at indicated temperatures.

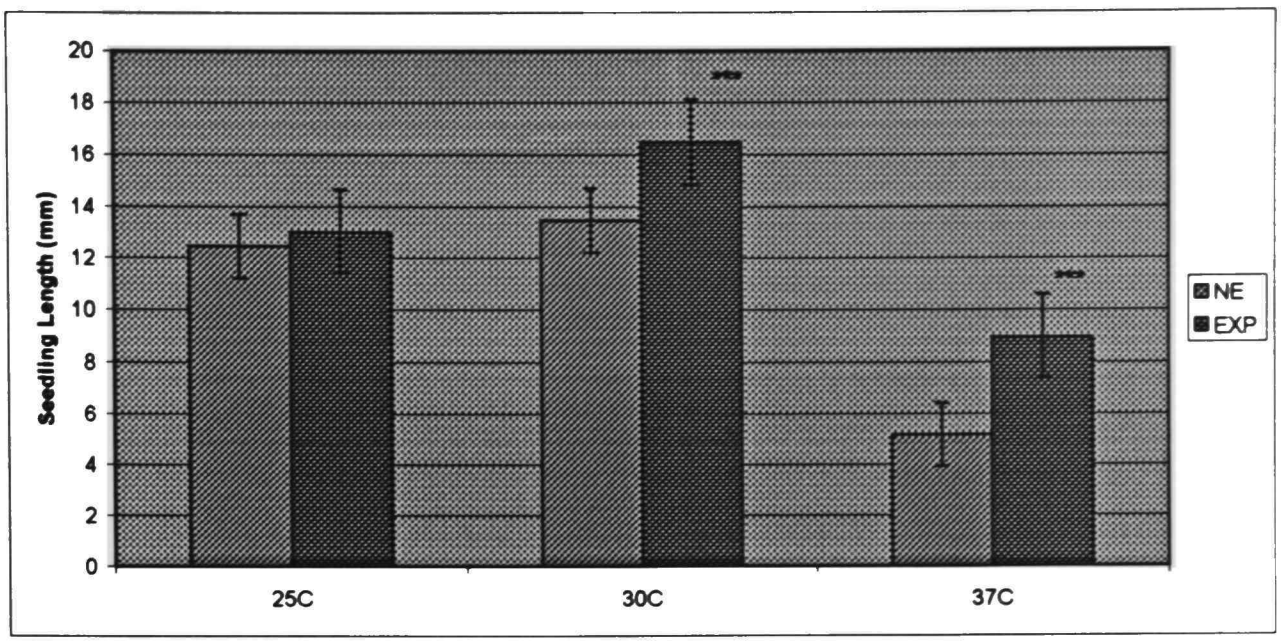

Figure 3.20 – Length of transgenic and control tobacco seedlings grown at 25°C, 30°C and 37°C for 5 days. n=4 maize *Cat3* independently transformed expressing tobacco lines, and n=2 control (Xanthi or non-expressors). Error bars indicate SDs. *** denotes significant differences at P<0.001.

A time course study was conducted for seedlings grown at high temperatures in order to surmise the protective effects of the introduced maize *Cat3* transgene into tobacco plants. Again, seedlings were germinated in Petri dishes on moist filter papers, parafilmed and grown at 25°C, 30°C and 37°C and monitored at days 2, 3, 4, 5,and 6 post-imbibition (Figure 3.21). CAT3-expressing seedlings were longer compared to control seedlings at each of the time points.

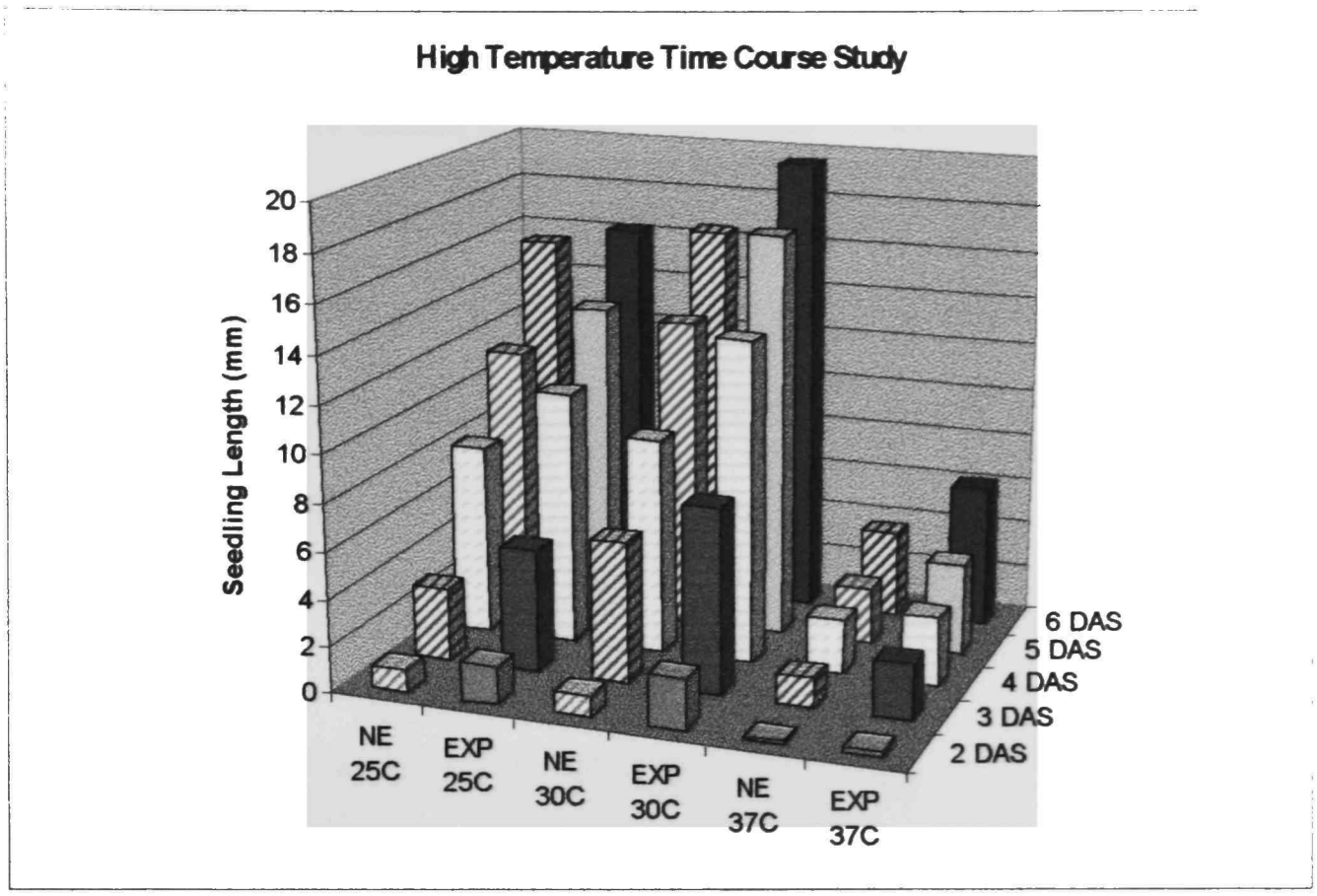

Figure 3.21 – Time course growth analysis of tobacco seedlings, transgenic (EXP) and control (NE), at 25°C, 30°C and 37°C over a 6 day period. Tobacco CAT3-expressing and control (Xanthi) seedlings were germinated on moist filter papers and grown at the mentioned temperatures. n=1 independently transformed line for EXP, n=1 control line.

Prior experiments showed that the growth of control (Xanthi) seedlings was substantially reduced when grown in NaCl solutions of 100 mM and 150 mM (Roxas et al., 1997). Therefore, CAT3-expressing seedlings were analyzed for growth under salinity stress. Transgenic and control seedlings were grown either in water or 100 mM and 150 mM NaCl for a period of 5 days. CAT3-expressing seedlings and control seedlings had similar results when grown in water (control) (Figure 3.22). CAT3-expressing seedlings grown under 100mM salt concentrations grew 40% longer than control seedlings (Xanthi and NE) (Figure 3.23). These differences were significantly different between the transgenic and

control seedlings grown in 100 mM NaCl treatments. Overall, though, a substantial decrease in seedling growth was seen in the CAT3-expressing and control seedlings grown in the 150 mM NaCl treatment. Nevertheless, the CAT3-expressing seedlings were significantly longer than the control seedlings. These results indicate that the introduction of a maize *Cat3* transgene confers seedling tolerance to salt stresses.

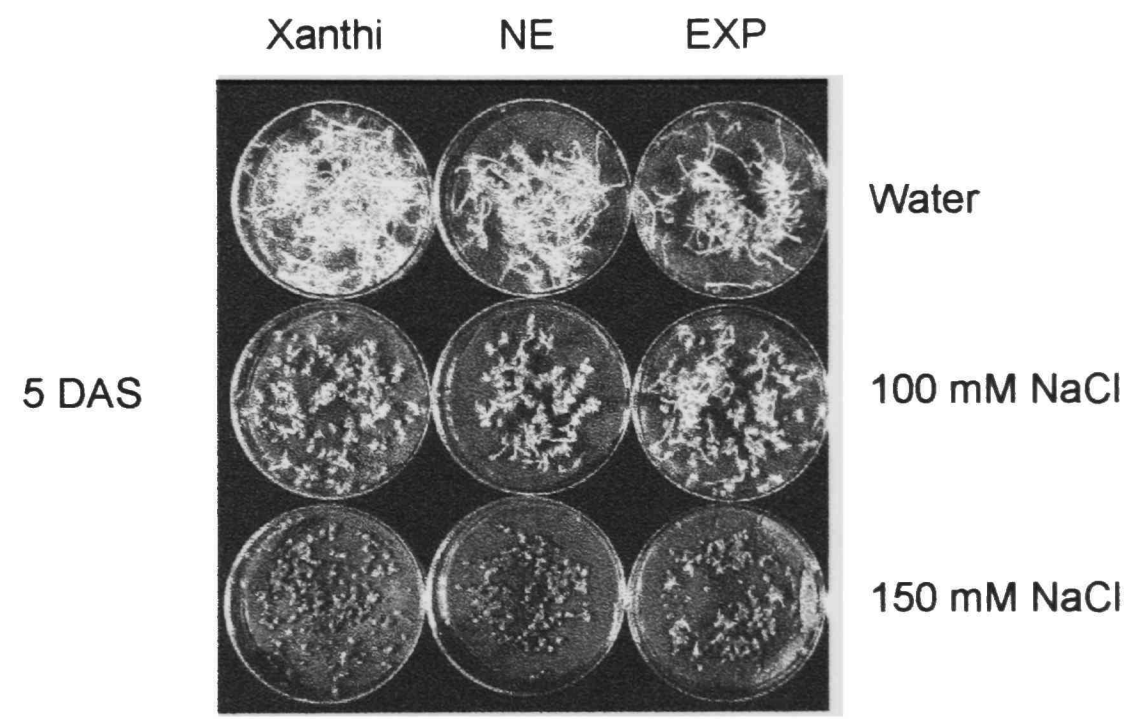

Figure 3.22 – Seedling growth of CAT3-expressing and control (Xanthi and NE) seedlings grown in water, 100 mM and 150 mM NaCl for 5 days. Transgenic and control seedlings were grown on Petri dishes lined with germination papers that were pre-wetted with different NaCl solutions and incubated at 25°C.

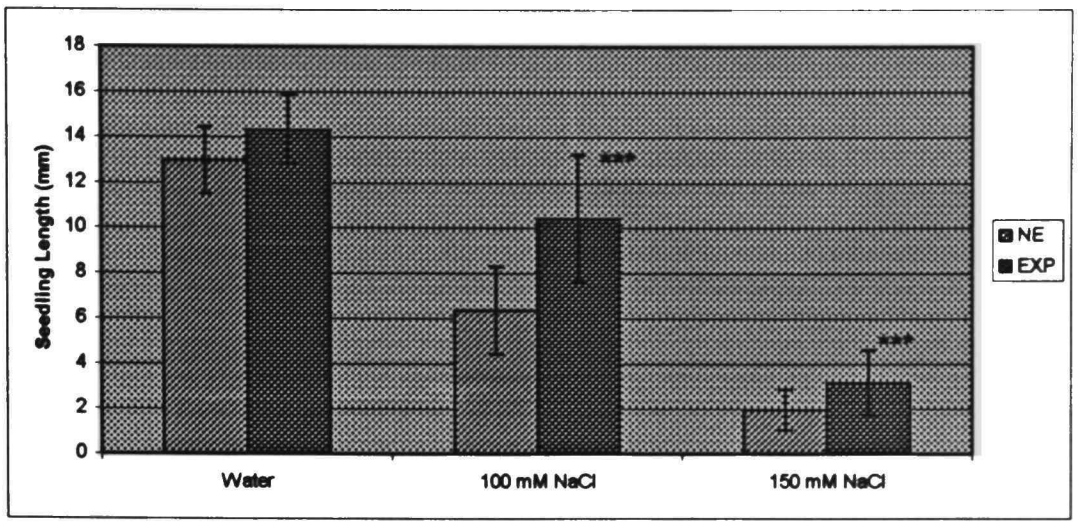

Figure 3.23 – Seedling length of CAT3 expressors and control (Xanthi and NE) plants grown in two different concentrations of NaCl for 5 days. Seeds were germinated on Petri dishes lined with germination papers that were pre-wetted with NaCl solutions and incubated at 25°C. SDs are indicated as error bars. *** significant at $P<0.001$.

3.6 Effects of Seedling Stress on MDA Levels

Malondialdehyde (MDA) levels were measured in order to estimate lipid peroxidation levels in the CAT3-expressing and control seedlings subjected to 25°C and 15°C. Surprisingly, transgenic seedlings grown in the dark for 7 days at 25°C and 15°C had higher MDA levels than in control seedlings (Figure 3.24). These data indicate that greater levels of lipid peroxidation have occurred in the CAT3-expressing seedlings especially at 15°C.

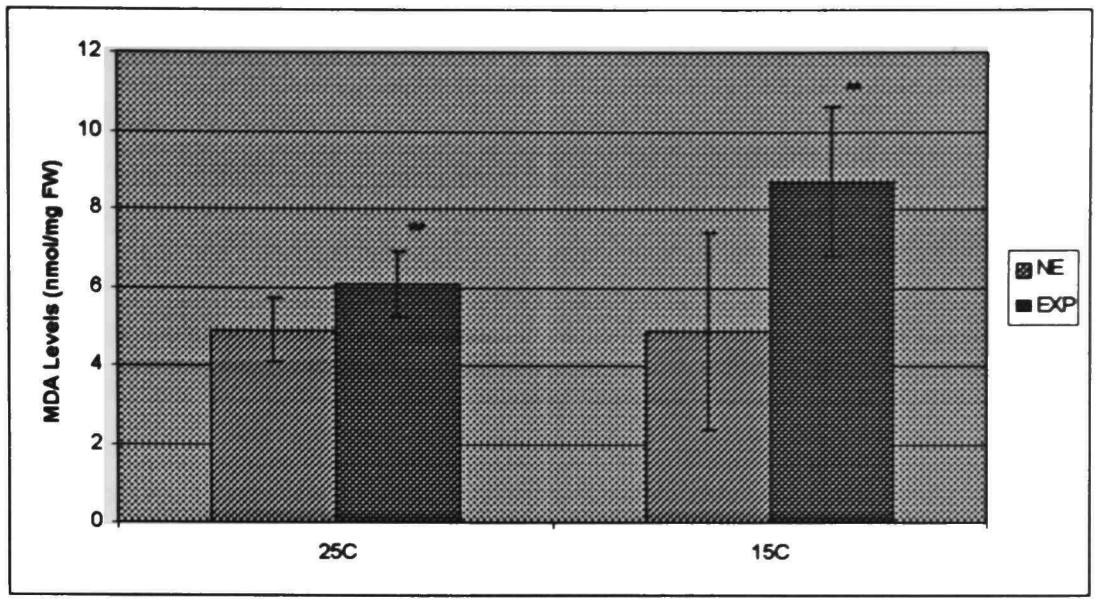

Figure 3.24 – MDA levels in CAT3-expressing and control seedlings grown at 25°C and 15°C for 7 days. Four transgenic (EXP) lines were analyzed and compared to two control (NE) lines. SDs are indicated by error bars. ** denotes significant at P<0.01.

Additionally, MDA levels were assayed in the CAT3-expressing and control seedlings subjected to high temperatures to determine the amounts of lipid peroxidation (Figure 3.25). While levels of MDA declined slightly in the 30°C grown seedlings for both the CAT3-expressing and control seedlings, MDA levels increased in the 37°C treatment for both the CAT3-expressing and control seedlings compared to MDA levels at lower temperature treatments. No significant differences were seen between the MDA levels of CAT3-expressing and control seedlings under any of the treatments.

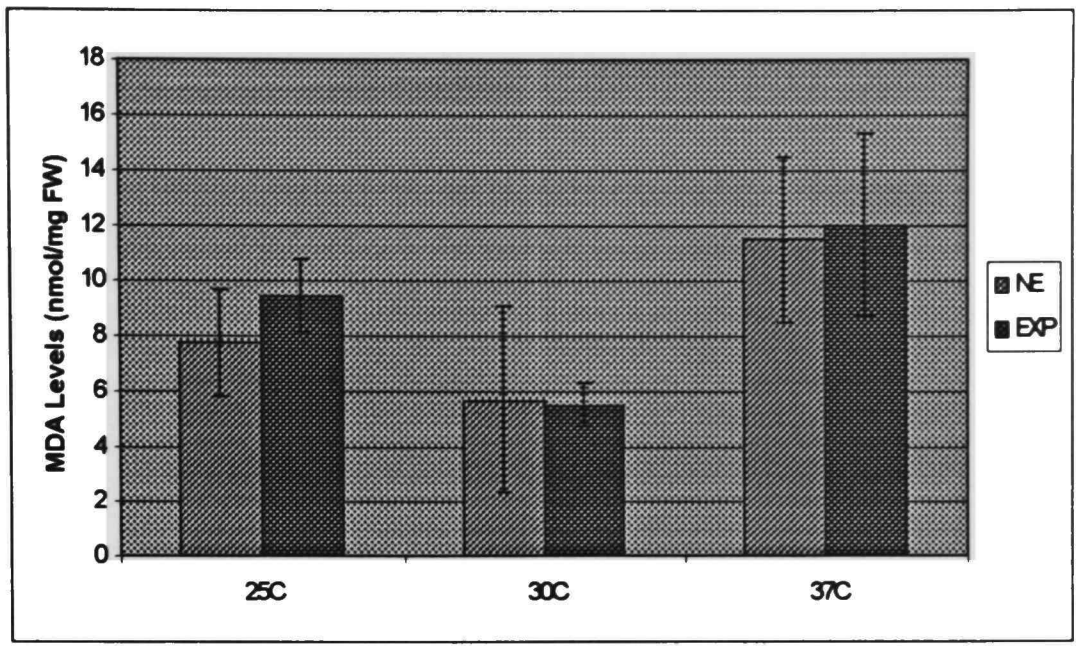

Figure 3.25 – MDA levels of transgenic (EXP) and control (NE) seedlings grown at high temperatures, 30°C and 37°C for 5 days. n= four independently transformed lines and n= 2 controls (Xanthi and NE). SDs are indicated by error bars. No differences were found between seedling groups.

Malondialdehyde (MDA) levels were also assayed to monitor levels of lipid peroxidation in the CAT3-expressing and control seedlings subjected to salt treatments of 100 mM and 150 mM NaCl (Figure 3.26). A slight reduction in MDA levels was noted in both the CAT3-expressing and control seedlings grown in 100 mM NaCl. Although levels of lipid peroxidation do appear to be higher in CAT3-expressing seedlings grown in 150 mM NaCl compared to control seedlings, these differences were not found to be significant due to a large variability in sampling. Differences between CAT3-expressing and control seedlings were not significant for either treatment

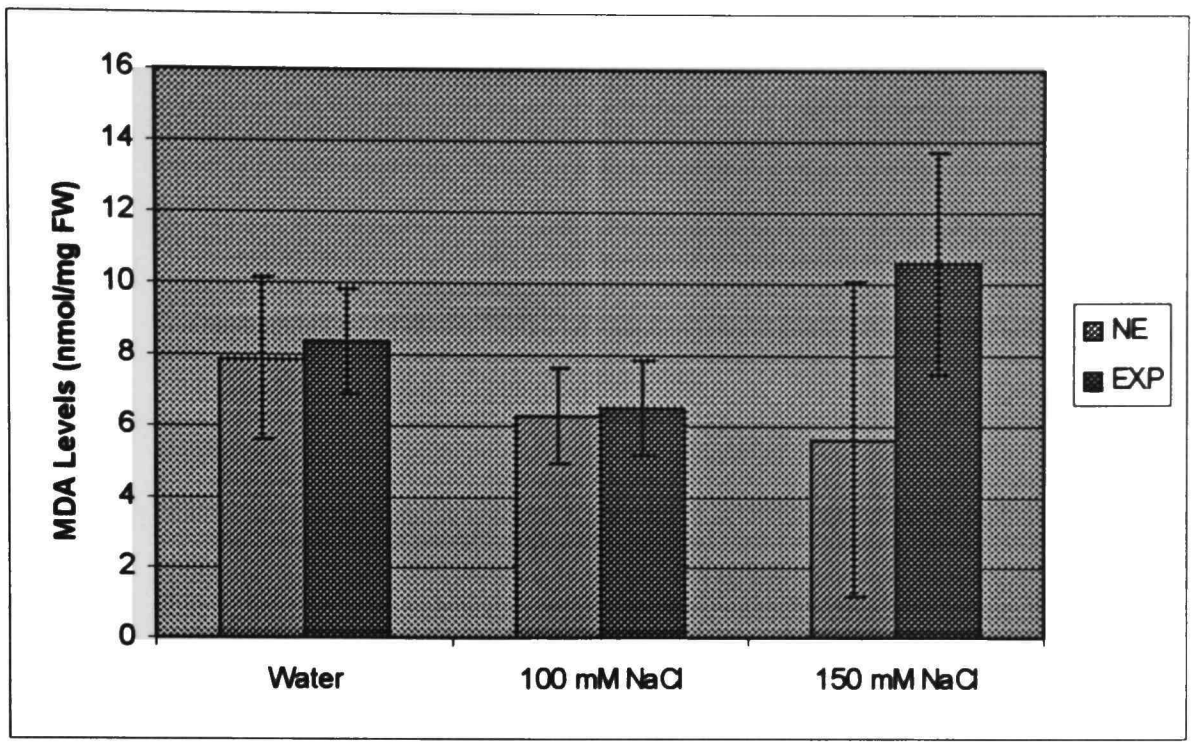

Figure 3.26 – MDA levels in seedlings exposed to salt stresses. Transgenic seedlings expressing maize CAT*3* and control seedlings were monitored for lipid peroxidation after germination in water, 100 mM NaCl and 150 mM NaCl. No differences between control seedlings (NE) and transgenic seedlings (EXP) were found to be significant. SDs are indicated by error bars.

3.7 Effects of Inhibitory Compounds on Seedling Growth

To examine the effects of catalase inhibitors on seedlings expressing maize CAT*3* during seedling germination, seeds were germinated in solutions containing either 0.5 mM AT, 10 mM AT, or 100 µM SA. These data will help to establish a better understanding of the protective capabilities attributed to the expression of maize *Cat3* during various growth conditions. Additionally, previous findings have determined the differential effects of AT on the three isoforms of catalase. A concentration of 10 mM AT was reported to inhibit purified CAT1 and CAT2 by 93% and 98%, respectively, while purified CAT3 was

inhibited by only 32% (Chandlee et al., 1983). Therefore, CAT3-expressing and control seedlings were germinated in 10 mM AT at 15°C and 25°C for 7DAS in order to evaluate the role of maize *Cat3* in these transgenic seedlings (Figure 3.27). In addition, a concentration of 0.5 mM AT was determined to inhibit seedling growth by ~50% in control plants (Xanthi) grown at 25°C (data not shown). Length of CAT3-expressing and control seedlings grown in solutions of 0.5 mM AT at 15°C and 25°C for 7 days is shown in Figure 3.27. At 25°C growth of both the CAT3-expressing and control seedlings was strongly inhibited in both 0.5 mM and 10 mM AT treated seedlings, but transgenic seedlings were found to be significantly longer than control seedlings grown in both concentrations of AT ($P<0.001$). Growth of CAT3-expressing and control seedlings at 15°C was further inhibited by 0.5 mM or 10 mM AT. Again, CAT3-expressing seedlings showed more growth than control seedlings.

Transgenic CAT3-expressing and control seedlings were germinated in water or 100 µM SA solutions at 25°C and 15°C (Figure 3.28). Again seedlings were placed in Petri dishes with moistened filter papers, parafilmed and grown for 7 days. Growth of both transgenic and control seedlings grown at 25°C decreased when treated with 100 µM SA compared to the water treated seedlings. But, CAT3-expresssing seedlings grown in 100 µM SA at 25°C grew significantly more than controls. CAT3-expressing and control seedlings grown in 100 µM SA at 15°C had slight increases in seedling length as compared to the water treatment. Additionally, the transgenic seedlings grown in 100 µM SA at 15°C were significantly longer than control seedlings treated similarly.

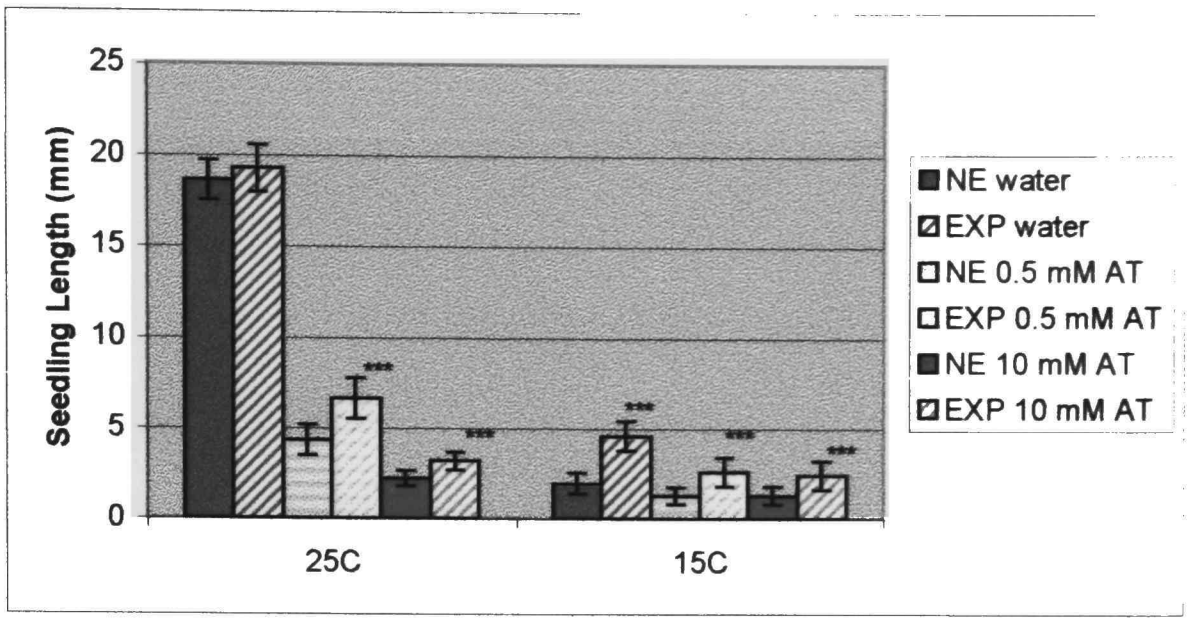

Figure 3.27 – Seedling lengths of CAT3-expressing and control (Xanthi and NE) seedlings grown in solutions of 0.5 mM AT and 10 mM AT at 25°C and 15°C for 7 days. Twenty seedlings each of two transgenic *Cat3* lines and two control lines were used for seedling measurement. SDs are indicated by error bars. *** significant at P<0.001.

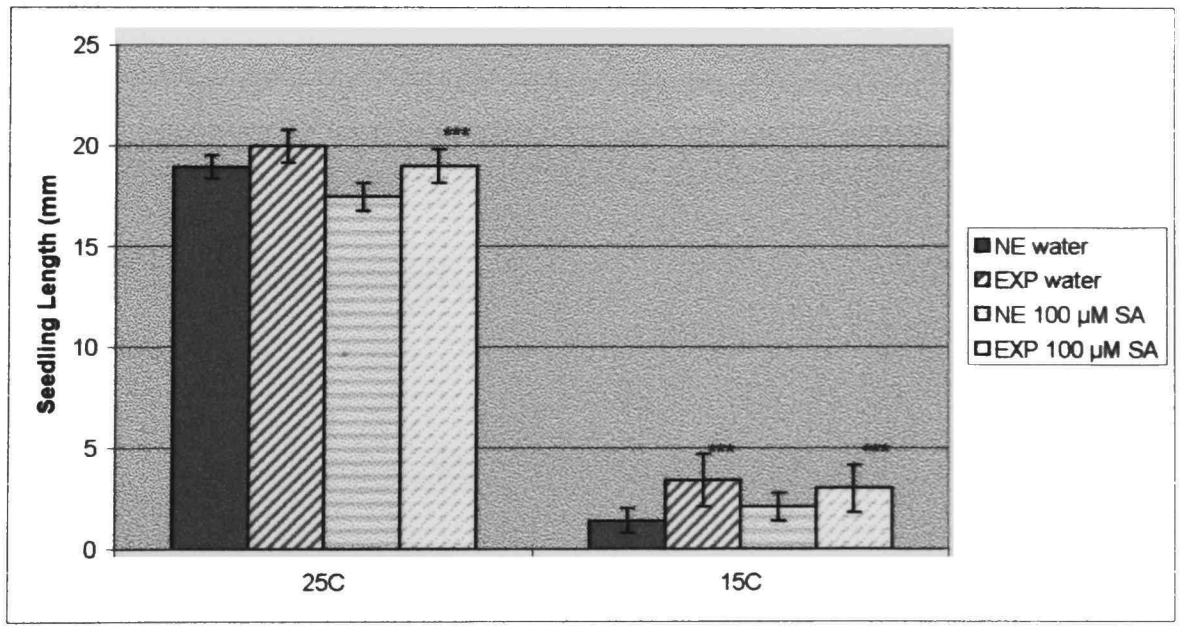

Figure 3.28 – Seedlings expressing maize CAT*3* and controls (Xanthi and NE) were germinated at 25°C and 15°C in a solution of 100 µM SA. Twenty seedlings of three transgenic lines and 2 control lines were used for seedling measurement at 7 DAS. SDs are indicated by error bars. *** significant at P<0.001.

CHAPTER IV

DISCUSSION

4.1 Cloning of Maize *Cat3*

This study describes the cloning of maize *Cat3* cDNA isolated using RT-PCR amplification of total RNA from maize epicotyls. Prasad and coworkers (1994) isolated three chilling acclimation-responsive (*CAR*) cDNAs that were differentially expressed in maize seedlings during acclimation. One of the *CAR* cDNAs was identified as *Cat3* that encodes a mitochondrial catalase. It was also discovered that seedlings acclimated to chilling temperatures had higher *Cat3* transcript levels and increased catalase activity. However, other analyses showed that maize plants exposed to 14°C had increased levels of CAT1 and CAT2 transcripts along with increases in corresponding enzyme activities but both CAT3 transcript and activity levels declined (Auh and Scandalios, 1997). These discrepancies indicate an incomplete understanding for the role of maize *Cat3* in seedling stress tolerance.

4.2 *Cat3* Transgenic Tobacco

Results from the present study show that the maize *Cat3* cDNA encodes a functional catalase in tobacco that has enhanced peroxidatic activity. Higher steady state levels of maize *Cat3* mRNA were detected in transgenic plants (T_0) that express the maize *Cat3* cDNA that were comparable to the native *Cat3* mRNA levels in maize. In addition, transgenic plants grown in a greenhouse

were identical to control plants in development, growth patterns, flowering, maturation, and seed production. Since we relied on four independently transformed lines that all performed similarly for this study, the protective effects provided by maize *Cat3* can therefore be attributed to the expression of the transgene and not a somaclonal or epigenic event.

T_1 transgenic plants that express maize *Cat3* were also found to have higher mRNA transcript levels when compared to control plants. Transcript levels in the T_1 transgenic plants did not always correlate with total enzyme activities, which could be attributed to post-transcriptional regulation events. Previous work with transgenic tobacco plants the overexpress CAT-1 also indicated that catalase specific activity does not necessarily correlate with steady-state levels of catalase transcripts (Brisson et al., 1998). Additionally, Auh and Scandalios (1997) found changes in CAT1 activity in low temperature treated seedlings while levels of *Cat1* transcripts did not change.

Since maize *Cat3* has been characterized as an EP-CAT, enhanced peroxidatic catalase, both catalase and peroxidase activities were measured spectrophotometrically (Chandlee et al., 1983; Zamocky et al., 1995). In the present project, leaf extracts from transgenic tobacco plants (T_1) that express maize *Cat3* were found to have somewhat higher, although not statistically significant, levels of total catalase activities than the control plants. However, peroxidatic activity of the transgenic tobacco plants that express maize CAT3 (T_1) was approximately twelve-fold higher than in control plants. These findings further support the EP-CAT activity assigned to maize CAT3.

The development of transgenic tobacco plants that express a maize CAT3 cDNA that encodes a catalase with enhanced peroxidatic activity has allowed us to examine the potential protective effects of CAT3 overexpression during abiotic oxidative stress. Overexpression of other ROI-scavenging enzymes including SOD and APX has been shown to provide increased protection from oxidative damage in mature leaves (Bowler et al., 1991; Sen Gupta et al., 1993; Allen et al., 1997). However, in the present study there appears to be little or no increased protection in leaves of transgenic plants against oxidative damage caused by MV or photooxidation treatment. Likewise, leaves of tobacco plants that overexpress a GST isozyme that also has GPX activity had no increased protection from MV induced oxidative stress or photooxidative damage (Roxas et al., 1997a). However, leaf disks of transgenic tobacco plants that expressed an *E. coli* catalase in chloroplasts were found, by visual inspection, to have little or no chlorosis after treatment with 0.1 – 1.0 μM MV under illumination of 300 μmol m^{-2} s^{-1} (Shikanai et al., 1998). Since these observations are not quantitative and the concentrations were lower than those used here, direct comparisons are difficult.

Leaf tissue of CAT3-expressing transgenic tobacco plants exposed to high light and chilling temperatures had no increased protection from photooxidative stress. Since the CAT3 isoform is not localized in the chloroplasts this result is not unexpected. This introduced catalase would not be in close proximity to the photosynthetic apparatus and the build up of H_2O_2 occurring in the chloroplast would be scavenged by the native chloroplastic antioxidant enzymes. Therefore,

once the system became overwhelmed, photosystem damage and inactivation of CO_2-fixing enzymes would lead to the loss of photosynthetic capacity known as photoinhibition (Foyer and Mullineaux, 1994). It is generally believed that catalase serves a more important role during photorespiration than during photosynthesis. In support of this view, mutant tobacco plants with enhanced catalase activities were shown to have increases in net photosynthesis under conditions, such as increased O_2 levels or temperatures, that favor photorespiration (Zelitch et al., 1991). The elevation in catalase activity minimized the negative effects of photorespiration and allowed fixation of CO_2 for photosynthesis. Additionally, Brisson et al. (1998), using transgenic tobacco plants with reduced catalase activities, detected a temperature dependent increase in the CO_2 compensation point. And, tobacco plants with increased catalase activities were found to have a reduction in the CO_2 compensation point. Both of these findings illustrate the importance of catalase during photorespiration.

4.3 Analysis of CAT3-expressing Seedlings

Tobacco seedlings that express maize *Cat3* have increased tolerance to abiotic stresses such as low and high temperatures, and salinity. Transgenic seedling vigor, as indicated by growth rate, increased for all transgenic seedlings compared to the control seedlings germinated under the aforementioned conditions.

Total catalase activities in wildtype plants were approximately twice as high in seedlings as in leaves indicating strong developmental regulation of native catalases. Since the *Cat3* transgene is under the control of a constitutive CaMV 35S promoter, approximately equal enzyme activities would be expected between the transgenic seedlings and the greenhouse grown transgenic plants. This is evident in the comparison of catalase activities of transgenic leaf tissue samples (138 U/mg protein) to dark germinated transgenic seedlings at 25°C (146 U/mg protein). The variation in total catalase activity found in the control samples from leaves (110 U/mg protein) compared to the dark germinated control seedlings (218 U/mg protein) could therefore be attributed to developmental differences in gene regulation. Similar expression patterns were seen in earlier work in our laboratory examining the regulation of a ß-glucuronidase reporter gene under control of the soybean catalase promoter. Increasing levels of GUS activity in dark germinated seedlings were observed that peaked at day 3 and were maintained through day 6 and then diminished (Isin, 1992). Therefore, increases in both catalatic and peroxidatic activity of catalase that occur from day 1 to day 5 in seedlings germinated at 25°C in this study are attributed to the typical gene expression patterns of catalase in germinating seedlings. Also recall that post-transcriptional events have been implicated in regulating catalase activities in tobacco and maize (Brisson et al., 1998; Auh and Scandalios, 1997).

It is generally believed that seedlings exposed to chilling temperatures have increased levels of H_2O_2. Reports confirm that the production and

consumption of H_2O_2 occur early during normal germination in soybean embryonic axes, and catalase was implicated as the primary H_2O_2 scavenger (Puntarulo et al., 1988). Therefore, as the H_2O_2 levels begin to rise in chilling treated seedlings, the protective role of catalase becomes extremely important. In this project, maize CAT3-expressing seedlings that were exposed to 15°C were found to have increased growth, compared to the control seedlings, which corresponded with increased levels of both catalatic and peroxidatic activities. Therefore, the increased vigor of these seedlings is most likely the result of their greater capacity to scavenge H_2O_2 that provides increased protection against oxidative stress. Although H_2O_2 levels were not measured in this study, earlier findings provide supportive evidence for such protection. Maize seedlings exposed to low temperatures were reported as having elevated levels of H_2O_2 (~7.5 µmol/g FW) that was believed to be primarily scavenged by *CAT3* (Prasad et al., 1994). Later work in maize suggested that the buildup of H_2O_2 lead to increased activity levels in CAT1 and CAT2 but not CAT3 (Auh and Scandalios, 1997). However, only catalatic activity was assayed and, since maize CAT3 has enhanced peroxidatic activity, it is possible that the importance of CAT3 in maize seedlings exposed to low temperature stress was underestimated. Additionally, chilling stress during all stages of plant development was found to increase H_2O_2 production in winter wheat, maize, and cucumber while lower levels of H_2O_2 were detected in pea and mung bean (Scandalios et al., 1997). Interestingly the total catalase activities increased in maize, but declined in cucumber, pea and mung bean.

Growth of transgenic CAT3-expressing tobacco seedlings under chilling temperatures (4°C, 8°C and 15°C) was significantly greater than control seedlings under the same conditions. These results are similar to those with GST/GPX expressing transgenic tobacco seedlings reported by Roxas and coworkers (1997a). Additionally, CAT3-expressing seedlings exposed to chilling stresses had increased catalatic and peroxidatic activities at 15°C when compared to control seedlings treated identically. Examination of seedlings grown at 15°C over a seven-day period revealed that the most substantial differences occurred in the transgenic seedlings between 5 DPI and 7 DPI. This pattern corresponds with the highest levels of peroxidatic activity in these seedlings. The transgenic CAT3 expressing seedlings exhibited a continuous rise in catalatic and peroxidatic activity that is attributable to the transcriptional control of the promoter (CaMV-E35S). Both catalatic and peroxidatic activities were more severely reduced in control seedlings germinated at 15°C than in CAT3-expressing seedlings.

The higher levels of both catalatic and peroxidatic activity in CAT3-expressing seedlings compared to control seedlings at 15°C could be factors in providing increased protection from chilling stress under these conditions. However, it seems most likely that it is the increased peroxidatic activity of maize CAT3 that is the most critical. Results from the treatment of seedlings with the catalase inhibitor, 3-aminotriazole (AT), support this hypothesis. Since AT has differential effects on different catalase isoforms, it is possible to draw inferences from this finding. For example, 10 mM AT was reported to inhibit purified CAT1,

CAT2 and CAT3 by 97%, 98% and 32% respectively. While seedling length declined for both transgenic and control seedlings treated with 10 mM AT, the transgenic seedlings retained the ability for significantly more growth than control seedlings. The reduced seedling growth evident in both CAT3-expressing transgenic and control seedlings treated with AT support the importance of both CAT1 and CAT2 during seedling development. However, the ability of CAT3-expressing seedlings to grow more rapidly than control seedlings during AT treatment could be due to its relative insensitivity to inactivation by AT.

Comparison of growth of CAT3-expressing seedlings and control seedlings at elevated temperatures also indicated that CAT3 expression provided increased seedling vigor during stress. As in GST/GPX-expressing seedlings (Roxas et al., 1997a), growth of both transgenic and control seedlings was increased relative to that at 25°C. However, significant protection of seedling growth at 37°C was detected in CAT3-expressing seedlings that was not apparent in GST/GPX-expressing seedlings.

Catalatic activity in both transgenic CAT3-expressing seedlings and control seedlings showed a steady decline with elevated temperatures. Ghadermarzi and Moosavi-Movahedi (1997) reported that the rate of irreversible inactivation of Compound I to Compound II of catalase is temperature sensitive. Dat et al. (1998) showed decreased catalase activity in mustard seedlings exposed to a 1.5 h heat shock treatment at 55°C that also led to increases in H_2O_2. Additionally, *N. plumbaginifolia* exposed to 37°C for five hours showed decreased transcript levels for both CAT1 and CAT2 that were found to increase

when the plants were returned to 22°C. Interestingly, the transcript levels of tobacco CAT3 remained constant at both temperatures (Willekens et al., 1994). While catalatic activity levels were identical for both transgenic and control seedlings at all temperatures, peroxidatic activity of CAT3-expressing seedlings increased somewhat at 30°C compared to 25°C and was more than five-fold higher than in control seedlings. At 37°C, a decline in peroxidatic activity was seen in CAT3-expressing seedlings but the activity levels remained much higher than in control seedlings. These results could indicate that the peroxidatic activity of maize CAT3 is more heat tolerant than native tobacco catalases. Furthermore, the correlation between the markedly higher peroxidatic activity levels in CAT3-expressing seedlings than in control seedlings at both 30°C and 37°C provides strong support for the hypothesis that the increased peroxidatic activity provided by CAT3 expression is responsible for enhanced seedling vigor during stress.

The apparent relative stability of the peroxidatic activity of maize CAT3 in transgenic tobacco plants at elevated temperatures could indicate that EP-CAT enzymes are intrinsically more heat stable than LP-CAT isoforms. However, purified EP-CAT enzymes were reported to be more heat-sensitive than "typical" catalases (Havir, 1990). Therefore, it is possible that some protective mechanism could exist in vivo. Binding of NADPH to the active site of bovine liver catalase prevents deactivation and can even reverse the build-up of the inactive form of Compound II (Hillar and Nicholls, 1992). Since NADPH could be

used as an electron donor by peroxidatic catalase in vivo it is possible that the association of NADPH with this enzyme could provide increased thermal stability.

With increased salinity, overall growth of both control and transgenic CAT3-expressing seedlings decreased as compared to seedlings grown in water. However, as with other stress treatments, growth of CAT3-expressing seedlings was significantly greater than control seedlings at both 100 mM and 150 mM NaCl. Overexpression of a GST/GPX in tobacco also led to increased tolerance of seedlings to mild and moderate salt treatments (Roxas et al., 1997a). Changes in the levels of catalatic and peroxidatic activities were also seen in CAT3-expressing seedlings exposed to salt stresses. Levels of both catalatic and peroxidatic activity increased slightly in both genotypes at 100 mM NaCl then deceased at 150 mM NaCl. Levels of both catalatic and peroxidatic activity were significantly higher in CAT3-expressing seedlings than in control seedlings at all salt concentrations. It was noted that while catalatic activity in CAT3-expressing seedlings was reduced by about 20% at 150 mM NaCl compared to water but peroxidatic activity was about 50% lower. Since the peroxidatic and catalatic activities of maize CAT3 are dependent on H_2O_2 concentrations, it is possible that the 150 mM salt treatment leads to levels of H_2O_2 that favor the catalatic activity of catalase.

Earlier studies confirmed that many environmental stresses, such as drought and salt, led to altered amounts and activities of antioxidant enzymes (Gueta-Dahan et al., 1997). For instance, in citrus plants subjected to 46 mM NaCl an increase in GPX activity was noted. Analysis of salt-tolerant species,

Vigna and *Oryza*, indicated that they had decreases in both SOD and catalase after salt treatment (Singha and Choudhuri, 1990). Also, increased mitochondrial Mn SOD activity was detected in a salt-tolerant pea cultivar after salt stress (Hernandez et al., 1993a). Further analysis showed increases in both catalase and Cu/Zn SOD activities in *Vigna unguiculata* treated with 100 mM NaCl for 30 days (Hernandez et al., 1993b).

The extent of lipid peroxidation in seedlings grown under a variety of stress treatments was estimated by measuring MDA levels. Only seedlings exposed to chilling temperatures showed significant differences in MDA levels. Surprisingly, transgenic CAT3-expressing seedlings germinated 25°C for 7 days had slightly higher levels of lipid peroxidation than control seedlings at 25°C and this difference was greater at 15°C. However, transgenic seedlings germinated and grown at elevated temperatures for 5 days or in 150 mM NaCl did not have significantly higher levels of MDA than the control seedlings. Earlier work in our laboratory showed that levels of lipid peroxidation in seedlings that expressed GST/GPX were significantly lower than control seedlings germinated in salt treatments (9 DPI) and under mild chilling stress (Roxas et al., 1997b). Since the stress tolerance phenotypes of GST/GPX-expressing seedlings and CAT3-expressing seedlings are very similar, the differences in levels of lipid peroxidation seem incongruous. One possible explanation for this difference could be derived from the fact that during lipid peroxidation, free radical chain reactions continue to propagate unless they are terminated by competing quenching reactions (Girotti, 1985). Therefore, radical quenching compounds

such as ascorbate, glutathione and phenolic antioxidants are able to terminate lipid peroxidation chain reactions. Since the GST overexpressing seedlings had increased levels of glutathione (Roxas et al., 1997a), it is possible that this could increase quenching and terminate free radical chain reactions resulting in reduced lipid peroxidation. In addition, the formation of the $^{\cdot}OH$, which contribute to autooxidation reactions in lipid peroxidation, from H_2O_2 and $O_2^{\cdot-}$ via the Haber-Weiss reaction, requires Fe (III). One possible source of Fe (III) could be from the inactivation of catalase by the cleavage of the prosthetic group and liberation of the heme iron. Such a release of heme iron has been reported in bovine catalase (Lardinois and Rouxhet, 1996). Since the CAT3-expressing transgenic seedlings have higher levels of catalase, it is likely that more total catalase is inactivated at chilling temperatures than in control plants. This could increase the levels of cellular Fe (III) resulting in increased $^{\cdot}OH$ production and increased lipid peroxidation.

The data presented in this study show that expression of maize CAT3 in tobacco can provide enhanced protection from a variety of stresses including chilling, high temperatures, and salt, as indicated by increased seedling vigor. These plants show higher levels of peroxidatic activity under normal conditions and during each of the stress treatments. This increased activity correlates with increases in seedling growth. Therefore, it seems likely that the protective capability of maize CAT3 is dependent on this increase peroxidatic activity of catalase. H_2O_2 levels of $<10^{-6}$ favor the peroxidatic activity of catalase while higher concentrations of H_2O_2 shift the activity of a peroxidatic catalase, such as

maize CAT3, to the catalatic mode. Physiological levels of H_2O_2 have been reported to be approximately 10^{-9} M (Scandalios et al., 1997). Although H_2O_2 levels would be expected to increase during periods of stress, it is possible that the H_2O_2 levels do not reach higher than 10^{-6} M that would begin to favor the catalatic mode of catalase. Thus, we see higher levels of peroxidatic activity than catalatic activity in the transgenic seedlings that express maize CAT3. Additionally, it has been proposed that the peroxidatic catalases may not function solely as H_2O_2 scavengers; but, since this peroxidatic group of catalases are induced by several stresses, they may play a pivotal role in protection against adverse conditions (Willekens et al., 1995). These results complement those from GST/GPX-expressing tobacco seedlings (Roxas et al., 1997a). In addition to GST activity, many plant GSTs have peroxide scavenging activity. Together, these reports strongly indicate that increased levels of peroxidative scavenging of cellular peroxides in seedlings can provide protective effects.

Plant peroxidases serve many important functions in physiological processes such as leaf and flower abscission, aging and senescence, apical dominance, cold tolerance, fruit development and ripening, and germination and early development (Melhourn et al., 1996). In addition, it has been proposed that peroxidation events can be viewed either with the peroxidase using reduced substrates with the goal of H_2O_2 removal, or that peroxidases act primarily to oxidize specific substrates that result in the dismutation of H_2O_2 (Willekens et al., 1995).

One might then ask, why would an enzyme as efficient as catalase take on an additional role as a peroxidase and what factors would promote such a change? (or perhaps peroxidases evolved to include catalatic activity). One possibility may be that different protein structures exist between catalatic and peroxidatic catalases. For instance, Zamocky et al. (1995) mutagenized the major substrate channel in yeast CAT-A to develop a catalase with increased peroxidatic activity and decreased catalase activity. The heme groups in catalases are deeply buried in non-polar pockets and are connected to the surface of the tetramer by a narrow channel while the heme groups in peroxidases are located in polar environments that are easily accessible to substrate. It is believed that these narrow channels in catalase may be one reason why catalases oxidize peroxidase substrates very slowly. Therefore, it is possible that catalases with peroxidatic activity are able to undergo conformational changes that make the active site more accessible to the peroxidatic substrates. Perhaps dual function enzymes like EP-CAT catalases allow the enzyme to adapt to differing H_2O_2 concentrations at a cellular level. Since H_2O_2 is required in many reactions such as initiation of lignification, alternative oxidase thermogenesis, membrane transport, etc. (Rao et al., 1998), the cellular levels of H_2O_2 must be monitored to meet the requirements of the cell. Since catalase is very effective in the efficient scavenging of H_2O_2, a additional system to insure the maintenance of minimal levels H_2O_2 must also be present. Perhaps this is the role of peroxidatic catalases found in many plant species.

4.4 Conclusions

The results of this study demonstrate the expression of maize *Cat3* in tobacco plants that express a functional catalase with enhanced peroxidatic activity. Little or no protection was seen against MV-induced oxidative stress or photooxidative stress in these transgenic plants as compared to control plants. Additionally, CAT3-expressing transgenic tobacco plants had increased seedling vigor during abiotic stresses such as low and high temperatures and salinity that corresponded with increased peroxidatic activities.

Information derived from this study and others will help to provide a broader knowledge base to enhance our understanding of plant oxidative stress. Therefore, many agronomically important crop species that are susceptible to these abiotic stresses could be modified to develop oxidative stress protective mechanisms. Since ROIs scavenging involves multiple antioxidant enzymes and multiple isoforms, a transgenic system that employs a variety of combinatorial antioxidant enzymes may provide even greater protection against periods of oxidative stress. Additionally, targeting of these combinations of enzymes to appropriate organelles could also enhance the native protective mechanisms.

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APPENDIX A

TRANSFORMATION AND TISSUE CULTURE MEDIUM

MG/L Medium

To 800 ml of deionized water, add:

5.00 g Mannitol
1.00 g L-glutamic acid or 1.15 g Sodium glutamate
5.00 g Tryptone
2.50 g Yeast Extract
0.25 g KH_2PO_4
0.10 g NaCl
0.10 g $MgSO_4 \cdot 7H_2O$
Mix well, pH to 5.7 with 1.0 M NaOH. Bring volume to 1 L. Add 2.00 g Phytagel if preparing plates. Autoclave.

MSA Plates (for inoculation)
To 800 ml of deionized water, add:
4.33 g MS Salts (Murashige and Skoog Basal Salts)
30 g Sucrose
1 ml 1000X vitamin B-5
50 µl 10 mg/ml NAA
100 µl 5 mg/ml BA
Mix well, pH to 5.7 Adjust volume to 1 L. Add 2.00 g Phytagel. Autoclave. Pour into 10X200 tissue culture plates.

MSB Plates (for shoot and callus formation)

Identical to MSA plates except for the addition of antibiotics as described here:
50 µg/ml Kanamycin
250 µg/ml Cefotaxime
100 µg/ml Carbenicillin

MSC Plates (for shoot and root formation)

Identical to MSB plates with the omission of hormones, NAA or BA. The antibiotics remain at the same concentrations.

Kanamycin Germination Selection Plates
To 800 ml of deionized water, add:

4.33 g MS Salts (Murashige and Skoog Basal Salts)
1 ml 1000X vitamin B-5
Mix well, pH to 5.7 Adjust volume to 1 L. Add 2.00 g Phytagel. Autoclave. Add 400 µg/ml Kanamycin. Pour into 10X200 tissue culture plates.

APPENDIX B

STATISTICAL ANALYSES

Table B.1 Catalase Assay of Leaf Extracts

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	120.37	40.01		0.81	0.04	0.68
Line M	ND	ND		ND	ND	ND
Line Z	126.34	46.53			0.08	0.56
Line T	188.58	37.75				0.05
NE line	108.98	56.03				

Table B.2 Peroxidase Assay at 25°C for Cold Treatment Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	19.87	7.19		0.39	0.41	2.5×10^{-5}
Line M	ND	ND			ND	ND
Line Z	24.28	9.09			0.93	8.1×10^{-5}
Line T	25.01	8.57				5.5×10^{-5}
NE line	1.74	0.98				

Table B.3 Catalase Assay at 25°C for Cold Treatment Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	145.3	29.0	0.59	0.24	0.14	0.02
Line M	159.8	31.6		0.5	0.07	0.05
Line Z	177.0	8.0			0.0008	0.17
Line T	114.5	2.5				0.002
NE line	218.9	36.3				

Table B.4 Catalase Assay at 15°C for Cold Treatment Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	96.7	1.9	0.15	0.027	0.77	0.0001
Line M	128.6	18.2		0.02	0.06	0.0001
Line Z	90.2	3.6			0.67	0.002
Line T	94.3	15.7				0.004
NE line	58.1	11.0				

Table B.5 Peroxidase Assay at 25°C for Cold Treatment Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	47.0	12.2	0.74	0.15	0.8	0.27
Line M	43.8	0.6		0.15	0.97	0.43
Line Z	68.8	17.7			0.21	0.04
Line T	43.4	18.6				0.05
NE line	31.7	18.4				

Table B.6 Peroxidase Assay at 15°C for Cold Treatment Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	19.8	5.1	0.93	0.02	0.006	0.0001
Line M	19.5	4.5		0.03	0.007	0.0001
Line Z	34.5	6.9			0.93	$1.6x10^{-5}$
Line T	34.9	2.8				$2.9x10^{-7}$
NE line	4.5	2.0				

Table B.7 Catalase Assay at 25°C control for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	218.4		ND	ND	ND	ND
Line M	280.2	33.1		0.79	0.19	0.15
Line Z	264.2	69.0			0.59	0.65
Line T	232.0	14.5				0.17
NE line	246.1	2.8				

Table B.8 Catalase Assay at 30°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	173.9	34.5	0.91	0.21	0.89	0.78
Line M	177.6	22.6		0.13	0.79	0.69
Line Z	220.0	9.6			0.18	0.15
Line T	168.3	35.9				0.89
NE line	163.5	42.3				

Table B.9 Catalase Assay at 37°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	143.7	21.3	ND	ND	0.78	0.82
Line M	128.8			ND	ND	ND
Line Z	130.3				ND	ND
Line T	154.4	20.5				ND
NE line	144.5	12.4				

Table B.10 Peroxidase Assay at 25°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	63.5	15.1	0.34	0.25	0.95	0.04
Line M	77.4	0.1		0.41	0.79	0.0007
Line Z	85.7	11.8			0.65	0.01
Line T	102.6					ND
NE line	5.1	2.7				

Table B.11 Peroxidase Assay at 30°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	124.0	25.2	0.75	ND	ND	0.004
Line M	107.7	60.4		ND	ND	0.05
Line Z	263.5				ND	ND
Line T	64.7					ND
NE line	11.6	4.2				

Table B.12 Peroxidase Assay at 37°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	48.1	8.7	0.81	ND	ND	0.0005
Line M	54.5	33.2		ND	ND	0.02
Line Z	38.7				ND	ND
Line T	46.5					ND
NE line	5.7	2.5				

Table B.13 Catalase Assay for Water Treatment of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	265.3	50.9	0.11	0.10	ND	0.005
Line M	234.2	32.7		0.97	ND	0.14
Line Z	227.4	46.3			ND	0.08
Line T	239.2	99.9				0.36
NE line	163.8	45.5				

Table B.14 Catalase Assay at 100 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	219.2	56.6	0.11	0.94	0.21	0.22
Line M	353.5	20.1		0.005	0.42	0.002
Line Z	241.3	15.8			0.06	0.11
Line T	318.3	49.5				0.005
NE line	196.0	40.9				

Table B.15 Catalase Assay at 150 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	237.7	117.6	0.76	0.86	0.64	0.05
Line M	203.7	80.1		0.84	0.85	0.06
Line Z	181.7	76.0			0.61	0.02
Line T	190.3	38.4				0.05
NE line	95.2	45.8				

Table B.16 Peroxidase Assay for Water Treatment of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	77.8	12.4	0.99	0.08	ND	0.0001
Line M	94.3	49.1		0.13	ND	0.003
Line Z	89.5	41.1			ND	$2.2x10^{-5}$
Line T	64.0	32.9				0.01
NE line	18.6	4.4				

Table B.17 Peroxidase Assay at 100 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	90.8	10.3	ND	ND	ND	ND
Line M	138.7	24.4		ND	ND	ND
Line Z	102.3	ND			ND	ND
Line T	64.6	23.5				ND
NE line	25.2	5.3				

Table B.18 Peroxidase Assay at 150 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	46.4	13.7	0.52	ND	ND	0.007
Line M	57.9	24.4		ND	ND	0.04
Line Z	40.8	ND			ND	ND
Line T	65.9	76.8				ND
NE line	10.9	4.6				

Table B.19 Methyl Viologen Assay (2.4 μM MV)

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	72.2	12.5	0.01	0.01	ND	0.08
Line M	87.3	14.2		0.74	ND	0.43
Line Z	89.6	8.01			ND	0.26
Line T	ND	ND				ND
NE line	92.1	7.9				

Table B.20 Germination Assay at 25°C for Chilling Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	19.2	2.0	0.05	$2.7x10^{-5}$	0.0006	$9.1x10^{-9}$
Line M	18.7	2.1		0.001	0.06	$1.5x10^{-4}$
Line Z	16.9	4.5			0.05	0.09
Line T	18	2.5				0.19
NE line	17.6	2.0				

Table B.21 Germination Assay at 15°C for Chilling Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	5.6	1.3	0.03	0.13	0.006	$2.7x10^{-80}$
Line M	5.3	1.0		$3.8x10^{-4}$	$5.3x10^{-7}$	$9.1x10^{-72}$
Line Z	6.0	1.6			0.28	$2.1x10^{-80}$
Line T	6.3	1.4				$3.6x10^{-81}$
NE line	2.4	0.7				

Table B.22 Germination Assay at 4°C

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	1.6	0.8	0.04	3.0x10-4	$1.7x10^{-13}$	$2.2x10^{-13}$
Line M	2.3	1.3		0.44	$2.5x10^{-7}$	$3.8x10^{-14}$
Line Z	2.5	0.7			$1.3x10^{-9}$	$8.1x10^{-26}$
Line T	4.35	0.7				$1.7x10^{-37}$
NE line	0.225	0.3				

Table B.23 Germination Assay at 25°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	13.1	2.2	0.06	0.86	0.57	0.0004
Line M	12.3	1.3		0.02	0.001	0.09
Line Z	13.2	1.8			0.68	$8.0x10^{-5}$
Line T	13.5	1.6				$2.9x10^{-7}$
NE line	12.5	1.3				

Table B.24 Germination Assay at 30°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	16	1.7	0.07	0.89	0.65	$5.7x10^{-13}$
Line M	16.6	1.1		0.29	0.16	$6.5x10^{-16}$
Line Z	16.1	2.9			0.85	$8.2x10^{-11}$
Line T	16.2	1.7				$3.0x10^{-16}$
NE line	13.5	1.6				

Table B.25 Germination Assay at 37°C for High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	8.6	1.8	0.29	$3.4x10^{-5}$	0.27	$2x10^{-21}$
Line M	8.0	2.6		$1.5x10^{-5}$	0.04	$3.2x10^{-14}$
Line Z	10.4	1.4			0.008	$1.0x10^{-37}$
Line T	9.2	2.3				$1.9x10^{-25}$
NE line	5.2	1.3				

Table B.26 Germination Assay at Water Treatment of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	14.4	1.3	0.01	0.001	0.003	4.5×10^{-14}
Line M	13.9	1.3		1.2×10^{-7}	0.63	1.4×10^{-7}
Line Z	15.1	1.8			4.1×10^{-8}	7.3×10^{-23}
Line T	13.8	1.4				3.3×10^{-8}
NE line	12.9	1.5				

Table B.27 Germination Assay at 100 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test				
			Line A	Line M	Line Z	Line T	NE Line
Line A	10.6	3.0		0.0009	6.8×10^{-5}	0.01	5.7×10^{-34}
Line M	9.3	2.3			6.6×10^{-16}	0.4	1.8×10^{-24}
Line Z	12.2	2.3				4.1×10^{-12}	1.71×10^{-63}
Line T	9.6	2.6					7.9×10^{-26}
NE line	6.4	2.0					

Table B.28 Germination Assay at 150 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	3.3	1.6	0.17	0.5	0.14	$1.4x10^{-10}$
Line M	3.0	1.2		0.04	0.8	$6.9x10^{-9}$
Line Z	3.5	1.5			0.04	$4.2x10^{-6}$
Line T	2.9	1.3				$6.5x10^{-8}$
NE line	1.9	0.8				

Table B.29 Lipid Peroxidation (MDA) Assay for 25°C of Chilling Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	6.2	0.7	0.31	0.64	0.67	0.08
Line M	5.5	0.2		0.79	0.24	0.31
Line Z	5.7	1.1			0.35	0.16
Line T	6.6	0.9				0.02
NE line	4.9	0.8				

Table B.30 Lipid Peroxidation (MDA) Assay for 15°C of Chilling Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	9.1	2.7	0.73	0.73	0.41	0.08
Line M	8.2	3.0		0.47	0.77	0.22
Line Z	9.5	1.4			0.11	0.01
Line T	7.6	1.2				0.15
NE line	4.9	2.5				

Table B.31 Lipid Peroxidation (MDA) Assay for 25°C of High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	10.3	1.8	0.27	0.33	ND	0.09
Line M	8.9	0.6		0.99	ND	0.37
Line Z	8.9	1.3			ND	0.40
Line T	10.2	ND				ND
NE line	7.7	1.9				

Table B.32 Lipid Peroxidation (MDA) Assay for 30°C of High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	5.4	1.0	0.43	0.6	0.51	0.9
Line M	5.9	0.5		0.74	0.1	0.89
Line Z	5.8	0.7			0.2	0.95
Line T	4.9	0.7				0.71
NE line	5.7	3.4				

Table B.33 Lipid Peroxidation (MDA) Assay for 37°C of High Temperature Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	11.5	4.2	0.41	0.98	0.90	0.10
Line M	14.7	2.7		0.38	0.31	0.23
Line Z	11.4	3.9			0.91	0.98
Line T	11.0	2.7				0.85
NE line	11.5	3.0				

Table B.34 Lipid Peroxidation (MDA) Assay for 25°C of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	8.6	1.7	0.96	0.26	0.71	0.57
Line M	8.6	2.3		0.40	0.74	0.67
Line Z	7.5	0.9			0.8	0.73
Line T	9.1	1.0				0.41
NE line	7.9	2.2				

Table B.35 Lipid Peroxidation (MDA) Assay for 100 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	6.1	2.5	0.61	0.65	0.56	0.86
Line M	5.3	0.1		0.57	0.03	0.17
Line Z	6.7	0.9			0.67	0.25
Line T	7.0	0.9				0.41
NE line	6.3	1.3				

Table B.36 Lipid Peroxidation (MDA) Assay for 150 mM NaCl of Salinity Study

Plant line	Mean	SD	t-test			
			Line M	Line Z	Line T	NE Line
Line A	11.8	ND	ND	ND	ND	ND
Line M	6.8	ND		ND	ND	ND
Line Z	8.0	ND			ND	ND
Line T	10.8	2.1				0.21
NE line	5.6	4.5				