CHARACTERIZATION OF MANNITOL ACCUMULATING T4 GENERATION TRANSGENIC WHEAT EXPOSED TO DROUGHT STRESS

By

SHRADDHA SHRIRAM VADVALKAR

Bachelor of Science in Microbiology

Pune University

Pune, India

2001

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2008

CHARACTERIZATION OF MANNITOL

ACCUMULATING T4 GENERATION TRANSGENIC

WHEAT EXPOSED TO DROUGHT STRESS

Thesis Approved:

Dr. Bjorn Martin

Thesis Adviser

Dr. Michael Anderson

Dr. Ramamurthy Mahalingam

Dr. Niels Maness

Dr. A. Gordon Emslie

Dean of the Graduate College

ACKNOWLEDGMENTS

I would like to express my deep and sincere gratitude to my advisor, Dr. Bjorn Martin for his guidance, encouragement and understanding. Without his valuable advice and friendly help during my difficult moments it would have been impossible for me to finish this thesis. I also wish to thank my other committee members, Dr. Michael Anderson, Dr. Ramamurthy Mahalingam and Dr. Neils Maness for their constructive comments and advice during the preparation of this thesis.

I thank Dr. Sathya Elavarthi for his help and guidance throughout this project. It truly was a great pleasure working with him. I would like to extend my gratitude to my lab mates, Emily Cook, Krystal Steel, and Baige Zhao for their untiring help in completing my research work.

I owe my loving thanks to my parents; they have been a constant source of support throughout my academic pursuits. I express a special gratitude to my most optimistic husband Vijay, for his motivation, emotional support and faith in me.

Finally, I thank USDA-CSREES-NRI; grant no. 2005-35100-16068, for a research assistantship and the Department of Plant and Soil Sciences for being very helpful and supportive during my journey as a graduate student.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Plants experience several abiotic and biotic stresses like drought, salinity, insects, and several potential pathogens which can drastically reduce crop productivity. Boyer's research work demonstrated that the loss in crop productivity due to insects and diseases were only 2.6% and 4.1%, respectively, while 71.1% was due to unfavorable physicochemical environments (Boyer, 1982). Boyer's classification also demonstrated that more than 25% of U.S soil area was affected by the drought. A study on effects of temperature and precipitation trends on U.S. drought indicated that that there has been an increase in precipitation since about 1980, without which the drought level in the U.S would have increased by 50% more in recent drought period (Easterling et al., 2007). Drought usually results in reduction in growth rate, stomatal aperture, leaf expansion, stem elongation, plant growth and productivity (Alexieva et al., 2001).

Wheat is one of the most important cereal crops. It is cultivated worldwide and is the principal cereal grain grown in the United States. One of the major factors affecting wheat production is drought.

Plants activate different physiological and biochemical defense systems upon exposure to stress. One of the strategies for surviving under water deficit stress is to accumulate osmolytes (Serraj and Sinclair, 2002). Osmolytes which have been detected

so far include amino acids, sugars and sugar alcohols, quaternary ammonium compounds, and tertiary sulfonium compounds (Goddijn and van Dun, 1999; McCue and Hanson, 1990; Rhodes and Hanson, 1993; Shen et al., 1997). Many crops have limited ability to produce osmoprotectants needed for stress tolerance. Thus engineering plants for production and accumulation of these osmolytes by introducing novel genes from other organisms has become a common strategy of making stress tolerant plants.

Plants genetically engineered for the production of mannitol, trehalose, glycinebetaine, and fructans might increase resistance to drought (Abebe et al., 2003; Bohnert and Jensen, 1996; Pilon-Smits et al., 1995; Rathinasabapathi et al., 1994; Romero et al., 1997; Rontein et al., 2002). Several mechanisms for osmolyte protection have been proposed but the most popular mechanism is through osmotic adjustment.

Plants genetically engineered to increase osmolyte concentration may not accumulate the necessary amounts required for osmotic adjustment, but they still show stress tolerance (Serraj and Sinclair, 2002). Other mechanisms like hydroxyl radical scavenging and protection of proteins have also been reported (Rontein et al., 2002; Smirnoff, 1989). An accumulation of osmolytes in roots help in the root development and allows plants to reach water in deeper wet soils (Serraj and Sinclair, 2002).

Spring wheat (*cv*. Bobwhite) was transformed with a bacterial *mtlD* gene encoding for mannitol-1-phosphate dehydrogenase causing accumulation of mannitol (Abebe et al., 2003). Two different gene constructs were used to target the mannitol accumulation in cytoplasm or chloroplast (Abebe et al., 2003). A negative control was generated containing only the selectable-marker *bar* gene. Improved tolerance to drought

and salinity was observed in calli and T_2 generation transgenic plants (Abebe et al., 2003).

The study was continued with T_4 generation transgenic plants. Transgenic wheat lines grown under well-watered and water-deficit stress conditions were characterized by conducting physiological and biochemical experiments. Physiological characterization on T_4 generation plants included gas exchange measurements conducted with a LI-6400 (LI-COR, Inc., Lincoln, NE) portable photosynthesis system. The LI-6400 system has a chamber which clamps on to the leaf. The machine allows the CO_2 concentration, light intensity air flow rate, relative humidity (RH), and temperature in the chamber to be controlled. Carbon dioxide and water vapor exchange rates of the leaf are measured with the aid of infrared gas analysis (IRGA) technology.

Biochemical characterization included determination of activities of the antioxidant enzymes catalase, glutathione reductase, superoxide dismutase and ascorbate peroxidase and estimation of lipid peroxidation in transgenic and control lines under well-watered and water-deficit-stress conditions.

Levels of sugars and sugar alcohols were determined with the help of high performance liquid chromatography. In this study, performance of the four transgenic lines and one empty vector line was evaluated under well watered and water-deficit stress conditions and compared with non transformed Bobwhite.

A major problem faced by many scientists working on genetically modified plants is silencing of the transgene. Usually in monocots, the biolistic approach is used for transformation with foreign genes. Plants transformed with this particular approach have exhibited transgene silencing (Anand et al., 2003).

CHAPTER II

REVIEW OF LITERATURE

As mentioned above, various biotic and abiotic factors affect plant growth and productivity. Among the abiotic factors, water deficit stress is the major reason for loss of crop productivity worldwide.

Effects of water stress on photosynthesis: Water stress severely reduces net photosynthesis in flag leaf, top internode and ear of wheat (Wardlaw, 1971). Under water-deficit conditions, stomatal closure and inhibition of chloroplast activity reduce photosynthesis (Matthews and Boyer, 1984), but the decrease in chloroplast activity contributes more to the loss in photosynthesis than the closure of stomata (Matthews and Boyer, 1984). Closed stomata and inhibition of chloroplast activity at low leaf water potential decrease the leaf capacity to fix available CO_2 and the non-stomatal component can not be overcome by increase in concentration of CO_2 (Matthews and Boyer, 1984).

At room temperature, chlorophyll fluorescence is emitted exclusively by photosystem II. Fluorescence has been used to study injury to photosynthesis from drought (Araus et al., 1998). As an indicator of stress, fluorescence measurements are

appropriate because PSII is one of the most susceptible processes to stress. Also, chlorophyll fluorescence can be taken as an indicator of oxidative stress as free radicals are known to inhibit repair of photo damage to PSII (Yoshitaka et al., 2001). Fv/Fm is a measure of the maximum quantum yield of photosystem II. The quantum yield has been shown to be very sensitive to photoinhibition caused to photosystem II mainly by reactive oxygen species. Such damage can be revealed by calculating the Fv/Fm ratios in chlorophyll fluorescence measurements (Souza et al., 2004).

Approaches to protect plants from drought stress: Some plant modification efforts focus on manipulating plant genes which normally protect the plants from drought stress (Valliyodan and Nguyen, 2006). Another approach is to introduce foreign biosynthetic enzymes or genes for synthesizing osmoprotecting compounds inside the cell (Rontein et al., 2002). Different compounds like amino acids, polyols, and quaternary ammonium and tertiary sulfonium compounds are known to be good osmoprotectants (Goddijn and van Dun, 1999; Rhodes and Hanson, 1993; Rontein et al., 2002). A relatively new approach of creating transgenic plants by introducing novel genes that do not occur naturally in these plants has emerged as an effective method (Valliyodan and Nguyen, 2006).

Role of osmoprotectants under stress: The osmoprotectants are small molecules that are not toxic to cells even at high concentration.

Usually under water deficit conditions, osmolyte accumulation occurs inside the cell which decreases a cell's osmotic potential and helps maintain cell turgor (Pathan et al., 2004). Maintenance of leaf turgor due to osmotic adjustment helps in reducing water

loss which in turn increases plant survival under stress (Serraj and Sinclair, 2002). This mechanism is popularly known as osmotic adjustment (OA).

Osmoprotectants like sugars and sugar alcohols are known to protect plants from water deficit stress by stabilizing proteins and cell membranes (Valliyodan and Nguyen, 2006). In an early attempt to create stress resistant transgenic tobacco plants, the bacterial enzyme choline oxidase, responsible for synthesizing the amino acid glycinebetaine, was used (Sakamoto and Murata, 2001). Another study conducted on tobacco plants showed that plants transformed with a trehalose synthase gene responsible for increased trehalose accumulation were tolerant to drought and salinity (Zhang et al., 2005). Another study done on transgenic tobacco plants showed that over expression of the inositol methyl transferase (IMT1) cDNA, increased the accumulation of D-ononitol inside the cell, which in turn conferred salt and drought tolerance to these plants (Sheveleva et al., 1997).

Role of mannitol under stress: The sugar alcohol mannitol is found in many plants and is particularly abundant in algae (Loescher et al., 1992). It is naturally found in various higher plants as well, for example in celery. Several potential roles of mannitol under stress have been proposed like as an osmoprotectant, a ROS scavenger, and for the storage and recycling of reducing power (Loescher et al., 1992; Valliyodan and Nguyen, 2006).

A study conducted to understand the role of mannitol in stress protection demonstrated that the presence of mannitol in the chloroplasts of tobacco plants conferred protection from oxidative damage (Shen et al., 1997). Tobacco plants transformed with an *E. coli* gene, *mtlD*, responsible for producing an enzyme, mannitol-1-phosphate dehydrogenase, involved in mannitol biosynthesis, showed increased tolerance to high

salinity (Tarczynski et al., 1993). Another study on transgenic tobacco suggested the possible role of mannitol in stress tolerance other than as an osmolyte (Karakas et al., 1997).

The pathway for mannitol production in transgenic plants is still somewhat unknown. The key metabolites in the proposed pathway include fructose-6-phosphate and mannitol-1-phosphate where mannitol synthesis is catalyzed by *mtlD* and non specific phosphatases (Thomas et al., 1995).

Different mechanisms of mannitol protection have been proposed; in tobacco plants mannitol protected certain molecules such as glutathione and enzymes like thioredoxin, ferredoxin and phosphoribulokinase from the harmful effects of hydroxyl radicals (•OH) (Shen et al., 1997). In some higher plants and algae mannitol enhanced the tolerance to water deficit stress through osmotic adjustment (Loescher et al., 1992; Valliyodan and Nguyen, 2006).

Oxidative stress and ROS accumulation: Oxidative stress occurs in plants due to excessive accumulation of reactive oxygen species (ROS) in the plant tissues during stress. Drought stress results in the inhibition of photosynthesis, thus leading to production of ROS (Smirnoff, 1993).

ROS production in plants originates mainly in three processes. First, photosystem I reduces molecular oxygen (O_2) in the Mehler reaction to form the primary superoxide radical ($\bullet O_2^-$) that in turn is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) (Apel and Hirt, 2004; Smirnoff, 1993). Second, the hydrogen peroxide can be further reduced to a very harmful hydroxyl radicals (•OH) through Fenton and Haber-Weiss reactions (Hancock et al., 2001). Generation of singlet oxygen under high

light intensities is another known mechanism of ROS generation. Under high light intensities, chlorophyll molecules can transfer the excitation energy to oxygen molecules resulting in the formation of singlet oxygen which can rapidly oxidize amino acids, DNA and lipids (Yoshitaka et al., 2001) Abiotic stress conditions are exacerbated by the effect of ROS accumulation.

Biomembranes are the most susceptible targets of ROS attack due to their high content of polyunsaturated fatty acids in their membrane phospholipids (Grassmann et al., 2002). Lipid peroxidation caused mainly by the hydroxyl radical can lead to loss of membrane fluidity, membrane proteins are affected which in turn disturbs the ion homeostasis and the membranes are finally completely disrupted (Grassmann et al., 2002). Break down products of lipid peroxidation have been shown to increase under drought stress and they have been considered as a reliable indicators of oxidative stress (Moran et al., 1994) (Fig 1).

The hydrogen peroxide formed under stress is broken down to water in the chloroplast by peroxidase (POX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DAR), and glutathione reductase (GR) and require access to reduced ascorbate and glutathione (Apel and Hirt, 2004). Hydrogen peroxide is broken down by catalase in peroxisomes. Antioxidant enzymes such as SOD, CAT, APOX, and GR prevent accumulation of hydroxyl radicals resulting from the overproduction of superoxide and hydrogen peroxide in stressed plants.

Role of osmolytes against oxidative stress: *In vitro* studies have shown that accumulation of compatible solutes can stabilize membranes and protect enzymes against chemical denaturation (Yancey et al., 1982). In 1989 Smirnoff evaluated the hydroxyl

radical scavenging capacity of compatible solutes and confirmed that sorbitol, mannitol and myo-inositol were effective in free radical scavenging (Smirnoff, 1989). Glutathione and hydrogen peroxide are known to cross biological membranes and affect intracellular signaling which helps in achieving stress tolerance to biotic as well as abiotic stresses (Foyer et al., 1997). Several studies on accumulating osmolytes in plant cells point to their role as scavengers of ROS and stabilizers of membranes and proteins (Bohnert and Jensen, 1996; Papageorgiou and Murata, 1995).

Role of mannitol against oxidative stress: Mannitol is known to possess free radical scavenging properties and was reported to scavenge hydroxyl radicals *in vitro* by chelating the iron necessary for the Fenton reaction (Franzini et al., 1994). Yet, the exact role of mannitol in scavenging ROS is unknown. One of the several potential roles include protection of thiol-regulated enzymes, thioredoxin, ferredoxin and glutathione from hydroxyl radicals (Shen et al., 1997). Direct scavenging of hydroxyl radical and prevention of formation of hydroxyl radicals by binding to transition metals necessary for the Fenton reaction have also been proposed (Smirnoff, 1989).

Drought tolerance in wheat: Drought is the dominant abiotic factor limiting the productivity of wheat and other crops. Hence several different approaches have been used to make wheat more drought tolerant. This includes engineering the plants to accumulate different osmolytes such as sugar and sugar alcohols (Abebe et al., 2003; Sivamani et al., 2000; Valliyodan and Nguyen, 2006), over-expressing certain plant proteins, such as, late embryogenesis abundant (LEA) proteins. LEA proteins accumulate during seed desiccation and in vegetative tissues under water deficit stress (Sivamani et al., 2000).

Spring wheat transformed with the barley gene HVA1, which is a member of group 3 LEA protein genes, showed improved biomass productivity and water use efficiency compared to wild type plants under water deficit conditions (Sivamani et al., 2000). A gene for a regulatory enzyme in proline biosynthesis was introduced into wheat, which led to proline accumulation and in turn resulted in tolerance to water deficit stress after 15 days of drought (Vendruscolo, 2007). The amino acid proline protected wheat plants from oxidative damage caused by ROS under drought stress rather than by osmotic adjustment.

Previous experiments in our laboratory: In an attempt to increase the drought tolerance by mannitol accumulation, the spring wheat cultivar Bobwhite was transformed with the *E. coli* gene, *mtlD*, mentioned above and the mannitol accumulation was targeted to cytosol or chloroplast in different lines (Abebe et al., 2003). Presence of a transit peptide sequence in pTA5 lines directs the mannitol-1-phosphate dehydrogenase to the chloroplast which helps in mannitol accumulation in this organelle (Abebe et al., 2003). Absence of the transit peptide sequence in pTA2 lines results in cytoplasmic mannitol accumulation. The T_2 transgenic lines showed increased tolerance to drought and salinity compared with the wild type plants (Abebe et al., 2003). The amount of mannitol accumulated in transgenic lines was too low to account for osmotic adjustment.

The study was continued with T_3 and T_4 generation transgenic plants (Elavarthi, 2005). Various physiological, biochemical and molecular experiments were performed to evaluate drought tolerance in transgenic lines, but they failed to show the same responses that had been observed in T_2 generation transgenic plants (Elavarthi, 2005). The apparent lack of phenotype in the later generations raises the possibility of gene silencing that

needs to be confirmed by determining the quantitative expression of transgenes in different generations of the transgenic lines.

Gene silencing: Inheritance and stable expression of the transgene over generations is important in creating a drought tolerant crop for agricultural purposes. Wheat transformed using the biolistic method with the pathogenesis–related genes, chitinase and β-1,3-glucanase, under the control of the maize ubiquitin promoter showed gene silencing in T_3 generation plants (Anand et al., 2003).

Another study on transgene inheritance and segregation demonstrated that the transgene though inherited as a dominant trait in the T1 generation, did not segregate in a Mendelian fashion (Rooke et al., 2003). A study on transgene inheritance and silencing in spring wheat proposed that high copy number of a transgene could trigger DNA methylation and can cause gene silencing and distortion of segregation ratios (Demeke et al., 1999). In monocots, gene silencing can occur at transcriptional and post-transcriptional levels (Iyer et al., 2000). The biolistic method more frequently results in multiple copies and complex rearranged transgenes as compared to *Agrobacterium* mediated transformation (Hiei et al., 1994). High incidence of transgene silencing has been observed in cases of high copy number or a rearranged transgene (Iyer et al., 2000).

Use of the biolistic method to transform the spring wheat cultivar Bobwhite (Abebe et al., 2003), presence of high copy number of transgene in T_0, T_3 and T_4 generation plants (Elavarthi, 2005), and lower mannitol concentrations in later generations (Elavarthi, 2005) suggest the presence of transcriptional or post-transcriptional gene silencing in transgenic spring wheat. This needs to be confirmed by

quantifying the gene expression, transgene copy number and the mannitol content in different generations of transgenic wheat.

CHAPTER III

METHODOLOGY

Objectives

1. To evaluate the effects of mannitol accumulation on leaf gas exchange and chlorophyll fluorescence in T_4 generation plants transformed with the *mtlD* gene, under well watered and water-deficit stress conditions.
2. To determine activities of antioxidant enzymes in T_4 generation plants transformed with the *mtlD* gene, under well watered and water-deficit stress conditions.
3. To quantify mannitol and major sugars like glucose, sucrose, and fructose in T_4 generation plants transformed with the *mtlD* gene, under well watered and water-deficit-stress conditions.
4. To estimate the lipid peroxidation in T_4 generation plants transformed with the *mtlD* gene, under well watered and watered-deficit-stress conditions.

All experiments were conducted on T_4 generation plants of two chloroplastic (pTA5-108, pTA5-104) and three cytoplasmic (pTA2-110, pTApTA2-115, pTA2-118) lines. The pAHC20 line, containing only the selectable *bar* marker gene, and the wild type Bobwhite were used as controls during the experiments. Experiments I and II included determinations of gas exchange properties, antioxidant enzyme activities, and soluble carbohydrate concentrations. Tissue samples for enzyme and soluble sugar assays were collected 0, 15 and 30 days after discontinuation of watering the plants in the stress treatment. Gas exchange measurements were conducted in the week prior to imposition of stress and at weekly intervals during the following 30-day period.

Seed treatment and growth conditions: Seeds were surface sterilized by washing with 70% ethanol followed by 20% chlorine bleach for five minutes in each solution. The seeds were thoroughly rinsed with deionized water to remove any remaining chemicals. Seeds were distributed in Petri dishes containing filter paper saturated with antibiotic piperacillin (100 mg Kg^{-1}) prepared in 1% dimethyl sulfoxide (DMSO). The Petri dishes were wrapped in aluminium foil and kept at room temperature for 24 h. Petri dishes were moved to $4^{o}C$ for 24 h to overcome seed dormancy. Seeds were next allowed to germinate for a week in Petri dishes, adding water every 2-3 days. After a week, seedlings were planted in small containers in a growth chamber. At 2-3 leaf stage, plants were screened by polymerase chain reaction (PCR) for presence of the transgenes *mtlD* and *bar*. After 2 weeks, positive plants were transplanted into pots and transferred to a greenhouse.

Screening for *mtlD* and *bar* genes: Polymerase chain reaction (PCR) was performed on leaf tissue collected from 2-3 weeks old plants to confirm the presence of *mtlD* and *bar*

genes in transgenic wheat lines. The DNA extraction and PCR analysis were performed using the REDExtract-N-Amp plant PCR kit (Sigma, St. Louis). DNA was extracted from approximately 50 mg of leaf tissue. One hundred microliters of extraction buffer was added to the leaf tissue followed by incubation at 95^{o}C for 10 minutes. After cooling, 100 µl of dilution buffer was added. This extract was used as a DNA template for PCR reactions. The amplified PCR products were subjected to gel electrophoresis using 1.2% agarose (Table 1, 2).

Stress treatment: Experiment I was conducted in a greenhouse in the fall of 2006 and Experiment II in the spring of 2007. The plants were split into two groups, a control group and a group exposed to water-deficit stress. After Day 0, the plants in the stress treatment group received no water until the volumetric soil water content had decreased to 10-15%. This occurred on Day 10 in Experiment I and on Day 8 in Experiment II. From those days until the end of the experiments on Day 30, the stress level of these plants was maintained by addition of 200 ml of water each time this threshold volumetric soil water content was reached. The group of well watered control plants received 500 ml of water on the same days.

Relative water content (RWC): At three time points, leaf tissue was collected in plastic bags and immediately placed on ice and brought to the laboratory. Fresh weight (FW) of an approximately 3-4 cm leaf segment was recorded. Leaf tissue was then transferred to centrifuge tubes filled with 2 ml of cold deionized water. Tubes were kept at 4^{o}C for 3-4 hours to allow full hydration while minimizing metabolic activity. Turgid weights (TFW) were recorded after 3-4 hours following removal of the leaf sections from the

tubes and blotting the excess water off. The samples were then dried in a hot air oven at 60^{o}C for 48 hours. Dry weights (DW) were recorded after the drying (Sharp et al., 1990). The RWC (%) was calculated by using the following formula:

RWC= (FW-DW)/ (TFW-DW)*100

Gas exchange measurements: The rates of CO_2 assimilation (A), transpiration and the stomatal conductance were determined at an irradiance of 1500 µmol m^{-2} s^{-1} PAR, 360 µL CO_2 L^{-1} air, 70% relative humidity and 22^{o}C chamber temperature. In addition to point measurements, CO_2 response curves were generated by measuring CO_2 assimilation rates at a range of CO_2 concentrations. Similarly, light response curves were generated by measuring the rates of CO_2 assimilation at a range of irradiances. These gas exchange measurements were conducted with a LI-6400 (LI-COR, Inc., Lincoln, NE) portable photosynthesis system adapted with a CO_2 mixer and a LED light source. The LI-6400 mixes CO_2 with the air going into the chamber and maintains a particular CO_2 concentration inside the chamber. It also measures the CO_2 exhausted from the chamber and then calculates the A from the difference in the two CO_2 concentrations, the air flow rate (Elavarthi, 2005), and the leaf area in the chamber. Gas exchange experiments were conducted five times starting at well watered condition and ending after 30 days of water-deficit stress, taking measurements at weekly intervals. Different parameters such as net photosynthesis rate, stomatal conductance, and transpiration rate of experimental transgenic and control lines were recorded under well watered and water-deficit stress conditions. Light and CO_2 response curve measurements were conducted only on one transgenic line (pTA2-118) and one control line (pAHC20). These measurements were

recorded only three times namely before the start of the stress, 15 days into the stress and 30 days into the stress.

Chlorophyll fluorescence: The ratio of variable to maximum fluorescence (Fv/Fm) of dark-adapted leaves was measured with the help of the LI-6400-4 leaf chamber fluorometer. Fluorescence measurements were collected along with light response curve measurements at three time points.

Antioxidant enzymes: Activities of antioxidant enzymes were determined at three time points namely before imposition of stress, 15 days into the stress and 30 days into the stress. Approximately 200 mg of leaf tissue was powdered in liquid nitrogen using a precooled mortar and pestle. The powder was then homogenized in extraction buffer and transferred into a precooled centrifuge tube. Phosphate buffer of 0.2 M and containing 2 mM EDTA at a pH of 7.8 was used as an extraction buffer for all assays. Fifty millimolar concentration of the same buffer was used in SOD and GR assays, while 50 mM of the same buffer at pH 7 was used for CAT and APOX assays. The homogenate was then centrifuged at 10,000 g for 20 minutes at 4^{o}C. The supernatant was used in assaying the antioxidant enzymes, APOX and GR. The supernatant was diluted 2X and 200X for SOD and CAT assays, respectively.

Determination of superoxide dismutase (SOD) activity: Total SOD activity of tissue extract was determined from the nitro blue tetrazolium (NBT) to formazon conversion caused by the superoxide radical in the presence of light (Flohe and Otting, 1984). Formation of the blue formazon is inhibited by SOD as it catalyzes the decomposition of the superoxide radical. Formazon formation was followed spectrophotometrically at 560 nm. Final SOD activity in samples was calculated using a standard curve. Final SOD

activity was expressed in units g^{-1} fresh weight. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm (Beauchamp and Fridovich, 1971).

Determination of catalase (CAT) activity: The catalase activity of leaf extracts was measured by following the decrease in absorbance of H_2O_2 at 240 nm caused by the decomposition of H_2O_2 catalyzed by catalase (Beers and Sizer, 1952). Enzyme activity was expressed as µmol of H_2O_2 oxidized min^{-1} g^{-1} fresh weight.

Determination of ascorbate peroxidase (APOX) activity: Leaf extracts were assayed in the presence of H_2O_2 and ascorbic acid (Moran et al., 1994; Nakano and Asada, 1981). The APOX activity was determined spectrophotometrically by following the oxidation of ascorbic acid at 290 nm. Ascorbate oxidase activity in plant samples was not detected. Also, there was no significant oxidation of ascorbate by H_2O_2. Enzyme activity was expressed as µmol of ascorbate oxidized min^{-1} g^{-1} fresh weight.

Determination of glutathione reductase (GR) activity: Leaf extracts were assayed in the presence of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The GR activity was determined spectrophotometrically by following the change in absorbance at 412 nm due to the formation of thionitrobenzoic acid (TNB) (Smith et al., 1988). Enzyme activity was expressed as µmol of TNB formed min^{-1} g^{-1} fresh weight.

Lipid peroxidation assay: The level of lipid peroxidation in leaf tissue was measured by quantifying malondialdehyde (MDA) content determined by the thiobarbituric acid reaction (Dhindsa et al., 1981). Approximately 100 mg of leaf tissue was homogenized in 3 ml 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 g for 5 minutes. Six hundred microliters of the supernatant was transferred to a fresh tube containing 2.4 ml of

20% TCA containing 0.5% TBA (thiobarbutiric acid). The new mixture was heated at 95^{o}C for 30 minutes and quickly cooled on ice. The samples were centrifuged again at 10,000 g for 10 minutes. Finally, the absorbance of the supernatant at 532 nm was read and the non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}.

Soluble carbohydrate analyses: Sugar and sugar alcohol content of the leaf tissue was quantified using a high performance liquid chromatography (HPLC) system coupled with a pulsed amperometric detector (PAD). Pure carbohydrate reagents (sorbitol, mannitol, glucose, fructose, and sucrose) purchased from Sigma (St. Louis, MO) were used as standards. Leaf tissue for HPLC analysis was collected at three time points, before the start of the stress period, 15 days after stress imposition and 30 days after imposition of stress.

Approximately 200 mg of leaf tissue was weighed and powdered using liquid nitrogen and mortar and pestle. The powdered sample was mixed and vortexed in 400 ml of an ethanol/chloroform/water (12:5:3) mixture. An equal volume of water was added and the mixture was centrifuged at 10,000 g for 5 minutes. The upper aqueous phase was transferred to a new tube and the pellet was re-extracted with water at 60^{o}C for 30 minutes followed by another centrifugation. The extracts were then pooled and dried in a speedvac for approximately 3-4 hours. The final pellet was suspended in 300 µl of water. To remove hydrophobic substances, the solution was passed through a preconditioned C_{18} solid phase extraction column (Altech Associates, Inc., IL), following which, 700 µl of water was passed through the column to collect the whole sample. The samples were then diluted 10 times for carbohydrate analysis. A CarboPac PA1 ion exchange column

(Dionex Corporation, Sunnyvale, CA) was used for carbohydrate analysis. Fifty microliters of sample was injected into the sample loop connected to the ion exchange column. Samples were separated isocratically in 40 mM NaOH sparged with helium. The flow rate was set to 2.0 ml min^{-1}. Peak areas of known concentrations of standards were used to calculate carbohydrate concentrations of unknown samples.

Phenotypic measurements: All experimental plants were harvested 30 days after withholding water. Phenotypic measurements such as height and above-ground biomass were recorded for all the transgenic and control lines.

CHAPTER IV

RESULTS

PCR screening for the transgenes: Two cytoplasmic (pTApTA2-115, pTA2-118) and two chloroplastic (pTA5-104, pTA5-108) lines, the empty vector line pAHC20, and wild type Bobwhite were used in this study. As the transgenic seed lots segregated for the transgenes *mtlD* and *bar*, seedlings were screened for presence of the transgenes. Only plants testing positive for transgenes were selected for experimentation (Fig 2).

Volumetric soil water content (VWC): In both experiments, soil VWC of control group plants was maintained in the range of 40-60% while for the stress exposed group the VWC was maintained between 8-18% (Figure 3, 4). The figures indicate a clear difference in VWC between well watered and stressed plants in the two experiments.

Leaf relative water content (RWC): In Experiment I, pTA2-118 and Bobwhite showed approximately 25% and 8% lower leaf RWC under stress treatment compared to the plants under control treatment. In Experiment II all lines except pAHC20 showed significantly lower RWC values. In both experiments, pTA2-118 showed the lowest RWC among the lines on Day 30 in the stress treatment (Table 3, 4).

Phenotypic measurements: In both experiments, a general decrease in height and above-ground biomass was observed in all experimental lines in response to stress (Table

5, 6). The transgenic line pTA2-118 was the tallest line under well watered as well as stressed conditions. In Experiment I, Bobwhite accumulated the greatest biomass under well watered conditions, but under stress, transgenic line pTA2-118 showed slightly greater biomass than the nontransformed Bobwhite. In Experiment II, transgenic line pTA2-118 showed slightly greater biomass than Bobwhite under well watered condition and almost equal amount of biomass under the stress treatment (Table 5, 6).

Gas exchange measurements: In Experiment I, all experimental lines except pTA2-118 and pAHC20 showed significant reduction in net photosynthesis rate (A) 10 days after discontinuation of watering. On Day 24, all lines showed significant reduction in net photosynthesis rate (Table 7). A similar response was not observed in Experiment II (Table 8). Among the lines, there were no significant differences in net photosynthesis rates in Experiment I, but in Experiment II pTA5-104 showed higher net photosynthesis rate than pTA5-108 and Bobwhite on Day 24 of the stress treatment (Table 7, 8).

In Experiment I, stomatal conductance was lowered on Day 10 and 24 in all lines (Table 9). This response was observed only in Bobwhite in Experiment II (Table 10). This suggests that the transgenic lines were no more able to restrict water loss under stress conditions than were the controls (Table 9, 10).

Antioxidant enzyme assays:

Ascorbate peroxidase (APOX) - In Experiment I, the APOX activity of line pTA2-115 on Day 30 was significantly higher in the stress treatment than in the control treatment, but it was significantly lower in pTA2-118 (Table 11). In Experiment II, pTA5-104 showed lower APOX activity on Day 30 in the stress treatment as compared to the control treatment (Table 12). There were no significant differences among the lines in

Experiment I, but in Experiment II, pTA5-104 under well watered condition and pAHC20 after 30 days of stress showed significantly higher APOX activity (Table 11, 12) than the other experimental materials.

Catalase (CAT) - There was no significant change in CAT activity in any of the materials after 15 and 30 days of stress treatment (Table 13, 14). In both experiments, pAHC20 had the highest CAT activity under well watered as well as water-deficit stress conditions.

Glutathione reductase (GR) – In Experiment I on Day 30, the GR activity was significantly lower in stress exposed pTA2-118 plants than in well-watered plants of the same line (Table 15). In Experiment II, only Bobwhite showed significantly lower GR activity on Day 30 in the stressed treatment than in the well-watered treatment (Table 16). The pAHC20 line showed the highest GR activity among the lines on Days 15 and 30 in both water treatments.

Superoxide dismutase (SOD) - In Experiment I on Day 15, the SOD activity was significantly lower in stress-exposed pTA5-104 plants than in well-watered plants of the same line (Table 17). In Experiment II, imposition of stress caused no significant change in SOD activity in all experimental lines. In both experiments, there were no significant differences among the lines in stress and control treatments (Table 17, 18).

Lipid peroxidation assay – In both the experiments**,** there was a significant increase in MDA content after 15 and 30 days of stress treatment. This increase was significantly lower in chloroplastic lines compared to cytoplasmic lines. The transgenic line pTA2-118 showed the highest MDA content among the lines after 15 and 30 days of stress treatment in both the experiments (Table 19, 20).

Light response curves - In Experiment I, quantum efficiency (AQE) and maximum photosynthesis rate (Amax) were lower in the transgenic line pTA2-118 than in the control pAHC20 line 30 days into the stress treatment. The control line showed a significant reduction in light compensation point (LCP), after 15 days of stress treatment. In experiment II, the transgenic line showed significantly lower quantum efficiency than the control line after 30 days of stress treatment (Table 21, 22).

CO_2 response curves- In Experiment I, a significant difference in CO_2 compensation point (CCP) between transgenic and control line was observed on Day 15 of the control treatment (Table 21). In Experiment II on Day 0, the transgenic line showed significantly lower carboxylation efficiency (CE) and CO_2 compensation point (CCP) under control treatment. On Day zero, the control line showed significant differences in CCP from the transgenic line under both treatments (Table 23, 24).

Soluble carbohydrate analyses: In Experiment I, as well as II, there were no significant differences in mannitol concentration between control and stress treatment. Also, there were no significant differences in mannitol concentration among the lines under both the

treatments. On Day 30, lines pTA2-115, pTA2-118, pTA5-104 and non-transformed Bobwhite in Experiment I, and pTA2-115, pTA5-104 and Bobwhite in Experiment II, showed a significant increase in total soluble sugars (TSS) in the stress treatment compared to the control treatment (Table 25, 26). In Experiments I and II, among lines, pTA2-118 and Bobwhite, respectively, showed the highest TSS concentration after 30 days of stress treatment. In Experiment I, there were no significant differences in TSS concentration after 15 days of stress treatment but in Experiment II, pTA5-104 showed a significantly higher TSS concentration compared to other lines under both the treatments.

CHAPTER V

DISCUSSION

Previous experiments conducted on calli and T_2 generation plants in our lab have shown that wheat plants transformed with the bacterial *mtlD* gene accumulated mannitol under water deficit stress and showed increased tolerance to drought and salinity. When the study here was extended to T_4 generation plants, substantially lower concentrations of mannitol were observed, and little effect on drought tolerance was noted.

In T_2 generation plants exposed to drought stress, mannitol accumulated to between 0.6 and 2.0 µmol g^{-1} fresh weight in the mature fifth leaf (Abebe et al., 2003). In our Experiments I and II, mannitol accumulation ranged from 0.17 to 0.64 and 0.09 to 0.63 µmol g^{-1} fresh weight, respectively. There was no significant increase in mannitol concentration upon stress. Thus, the mannitol concentration under stress in our experiments on T_4 wheat was far lower than in T_2 generation wheat (Abebe et al., 2003), tobacco (Tarczynski et al., 1993) and Arabidopsis (Thomas et al., 1995). The difference in mannitol concentrations between T_2 and T_4 generation transgenic plants may explain the differences in drought tolerance observed in our experiments and in the previous

ones. The cause of the lower mannitol concentration in T_4 plants remains unanswered though.

A slightly different method of stress imposition was followed in our experiments compared to that used previously for T_2 generation plants. The T_2 generation transgenic plants in the earlier stress treatment received 1/3 of the amount of water given to plants in the control treatment, while in our experiments T_4 plants in the stress treatment received 40% of the amount given to plants in the control treatment. Although the soil volumetric water content was measured only for the T_4 plants, the difference in watering protocols suggests that the T_4 plants may not have experienced the same magnitude of stress as the T_2 plants.

Yet, our data on soil volumetric water content (VWC) show a clear difference in the amount of water available to T_4 plants in the control and the stress treatments. Also, the leaf relative water content (RWC) was reduced under stress in pTA2-118 and Bobwhite in Experiment I, which indicates the presence of stress. The rest of the transgenic lines did not show a reduction in RWC under stress, however, which shows that these lines were able to maintain their RWC even with less water available than in the control treatment. However, a similar response was not observed in Experiment II, where all the experimental lines except pAHC20 showed significant reduction in RWC after 30 days of stress treatment. The above results suggest that the plants in Experiment II were stressed to a greater magnitude than those in Experiment I. Our soil water data show that the stress exposed group in both experiments was maintained between 10-15% VWC, which was much lower than the control group.

The above data show that with our method of stress imposition, and for the transgenic lines used in our experiments, it took approximately 30 days for a significant reduction in RWC to appear and for stress to develop.

Studies on wheat (Abebe et al., 2003; Kerepesi and Galiba, 2000) and tobacco (Karakas et al., 1997) transformed with the *mtlD* gene have shown an increase in total soluble sugars upon stress imposition. Our data is in agreement with these findings. A significant increase in total soluble sugars was observed in transgenic as well as control lines, but first after 30 days of stress treatment. The presence of mannitol did not affect the stress-induced accumulation of other soluble sugars. The fact that the increase in total soluble sugar content was observed first after 30 days of water stress supports the above conclusion, based on RWC and VWC, that the experimental plants were significantly stressed first toward the end of the 30 days of stress treatment.

It should also be mentioned that pTA2-118 developed and senesced faster than the other experimental materials. This could have contributed to its low RWC. In Experiments I and II, the transgenic line pTA2-118 was the tallest line under well-watered, as well as stressed conditions. Also, it accumulated slightly greater biomass than Bobwhite under stress in Experiment I and an almost equal amount of biomass in Experiment II. These results show that under stress the transgenic line pTA2-118 performed better than the Bobwhite in terms of height and biomass. The possible role of mannitol in better performance of the transgenic line pTA2-118 is not clear.

The different physiological experiments conducted to evaluate drought tolerance in the transgenic wheat plants included measurements of net photosynthesis rate, transpiration rate, and stomatal conductance. Light and CO_2 response curves were also

generated. Similar measurements have been previously used in wheat for studying the effects of water stress on gas exchange and chlorophyll fluorescence (Hassan, 2006).

In Experiment I, the transpiration rate, net photosynthesis rate and the stomatal conductance were lowered in all experimental materials in the stress treatment, which suggests closure of stomata. In Experiment II, a significant reduction in the transpiration rate and the stomatal conductance was observed only in Bobwhite after 24 days of stress treatment. These results suggest that the presence of the *mtlD* transgene did not alter the pattern of restricting water loss by the leaves, i.e. the presence of the levels of mannitol observed in the transgenic lines did not give an added advantage to these plants under water deficit stress.

Photosynthetic quantum efficiency (AQE) is calculated as the ratio between the number of CO_2 molecules assimilated (or O_2 molecules evolved) and the number of photons absorbed by the photosynthetic system (Zeinalov and Maslenkova, 1999). Environmental stresses like temperature and drought can alter the photosynthetic quantum efficiency (Zobayed et al., 2005). A study conducted on wheat showed about 17% reduction in AQE under drought stress and 12% under heat stress (Hassan, 2006).

In Experiment I, the transgenic line pTA2-118 showed lower maximum photosynthesis rate (Amax) and quantum efficiency (AQE) compared to that of the control line pAHC20 after 30 days of stress treatment while only AQE was lowered in Experiment II. The presence of mannitol did not show any effect on Amax and AQE under stress condition in the transgenic line. Also, presence of mannitol in the transgenic line did not show any effect on carboxylation efficiency (CE) under stress conditions.

The biochemical characterization included determination of activities of the antioxidant enzymes catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase and estimation of lipid peroxidation in transgenic and control lines under well-watered and water-deficit-stress conditions. There was no consistent difference in antioxidant enzyme activities between stressed and control transgenic and nontransgenic lines. The presence of mannitol, which is also a scavenger of reactive oxygen species, did not affect the activities of the antioxidant enzymes in the transgenic plants under both well-watered and water-deficit-stress conditions. This suggests that the regular antioxidant system of nontransgenic plants might have been able by itself to adequately scavenge stress-induced reactive oxygen species. The lipid peroxidation measured in terms of malondialdehyde (MDA) concentration was significantly higher in pTA2-118 compared to the chloroplastic lines after 15 and 30 days of stress treatment. Our data on RWC supports the above finding that pTA2-118 was the most stressed lines as it showed the lowest RWC among the experimental lines in Experiments I and II.

Although there was an increase in lipid peroxidation upon imposition of stress in all the experimental lines, this increase was significantly smaller in chloroplastic lines compared to cytoplasmic lines. This suggests that the mannitol accumulation in chloroplasts provides better protection to plants from oxidative damage by hydroxyl radicals compared to cytoplasmic accumulation. These results are in agreement with a study conducted on tobacco which showed that mannitol accumulation in chloroplasts protects the plant from oxidation by hydroxyl radicals (Shen et al., 1997). The initial stress-induced reactive oxygen species, the superoxide radical, is formed in the chloroplast and is there converted to the very damaging hydroxyl radical. Thus presence

of mannitol at the site of hydroxyl radical formation would be expected to be more effective in the elimination of this radical.

In conclusion, transgenic lines performed better than control lines under stress treatment in terms of height and biomass. The transgenic line pTA2-118 was the tallest and fastest growing line. It also accumulated greater biomass than the wildtype under stress. Presence of mannitol did not show any effect on antioxidant enzyme activities of transgenic lines under both the treatments. Physiological experiments showed that presence of mannitol did not give an added advantage to the transgenic lines as they did not perform better under stress. The mannitol concentration did not increase in transgenic lines after 15 and 30 days of stress treatment, but there was a significant increase in total soluble sugar content in some of the transgenic lines and control lines after 30 days of stress treatment. The accumulation of mannitol in the chloroplasts of the transgenic lines provided better protection to the cell membranes from harmful hydroxyl radicals compared to accumulation of mannitol in the cytoplasm.

FUTURE STUDY: The decrease in mannitol concentration over the generations, use of the biolistic method to transform the spring wheat cultivar Bobwhite (Abebe et al., 2003), and presence of high transgene copy numbers in the transgenic lines (Elavarthi, 2005) suggest the presence of transcriptional or post-transcriptional gene silencing. This needs to be confirmed by quantifying transgene copy number, gene expression, and mannitol content in different generations of transgenic wheat.

REFERENCES

Abebe, T., A.C. Guenzi, B. Martin, and J.C. Cushman. 2003. Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiology. 131:1748-1755.

Alexieva, V., I. Sergiev, S. Mapelli, and E. Karanov. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environment. 24:1337-1344.

Anand, A., H.N. Trick, B.S. Gill, and S. Muthukrishnan. 2003. Stable transgene expression and random gene silencing in wheat. Plant Biotechnology Journal. 1:241-251.

Apel, K., and H. Hirt. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology. 55:373-399.

Araus, J.L., T. Amaro, J. Voltas, H. Nakkoul, and M.M. Nachit. 1998. Chlorophyll fluorescence as a selection criterion for grain yield in durum wheat under Mediterranean conditions. Field Crops Research. 55:209-223.

Arora, A., R.K. Sairam, and G.C. Srivastava. 2002. Oxidative stress and antioxidant system in plants. Current Science. 82:1227-1238.

Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry. 44:276-287.

Beers, R.F., and I.W. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. Journal of Biological Chemistry. 195:133-140.

Bohnert, H.J., and R.G. Jensen. 1996. Strategies for engineering water-stress tolerance in plants. Trends in Biotechnology. 14:89-97.

Boyer, J.S. 1982. Plant productivity and environment. Science. 218:443-448.

Demeke, T., P. Hucl, M. Båga, K. Caswell, N. Leung, and R.N. Chibbar. 1999. Transgene inheritance and silencing in hexaploid spring wheat. Theoretical and Applied Genetics. 99:947-953.

Dhindsa, R.S., P. Plumb-Dhindsa, and T.A. Thorpe. 1981. Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. Journal of Experimental. Botany. 32:93-101.

Easterling, D., T.W.R. Wallis, J.H. Lawrimore, and R.R. Heim, Jr. 2007. Effects of temperature and precipitation trends on U.S. drought. Geophysical Research Letters. 34: L20709.

Elavarthi, S. 2005. Biochemical, physiological and molecular characterization of mannitol accumulating transgenic wheat in response to water deficit stress. Ph.D. dissertation, Oklahoma State University, Stillwater.

Flohe, L., and F. Otting. 1984. Superoxide dismutase assays. Methods in Enzymology. 93-104.

Foyer, C.H., H. Lopez-Delgado, J.F. Dat, and I.M. Scott. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiologia Plantarum. 100:241-254.

Franzini, E., H. Sellak, J. Hakim, and C. Pasquier. 1994. Comparative sugar degradation by (OH). produced by the iron-driven fenton reaction and gamma radiolysis. Archives of Biochemistry and Biophysics. 309:261-265.

Goddijn, O.J.M., and K. van Dun. 1999. Trehalose metabolism in plants. Trends in Plant Science. 4:315-319.

Grassmann, J., S. Hippeli, and E.F. Elstner. 2002. Plant's defense and its benefits for animals and medicine: role of phenolics and terpenoids in avoiding oxygen stress. Plant Physiology and Biochemistry. 40:471-478.

Hancock, J.T., R. Desikan, and S.J. Neill. 2001. Role of reactive oxygen species in cell signaling pathways. Biochemical Society Transactions. 29:345-350.

Hassan, I. 2006. Effects of water stress and high temperature on gas exchange and chlorophyll fluorescence in Triticum aestivum L. Photosynthetica. 44:312-315.

Hiei, Y., S. Ohta, T. Komari, and T. Kumashiro. 1994. Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. The Plant Journal. 6:271-282.

Iyer, L.M., S.P. Kumpatla, M.B. Chandrasekharan, and T.C. Hall. 2000. Transgene silencing in monocots. Plant Molecular Biology. 43:323-346.

Karakas, B., P. Ozias-Akins, C. Stushnoff, M. Suefferheld, and M. Rieger. 1997. Salinity and drought tolerance of mannitol-accumulating transgenic tobacco. Plant, Cell & Environment. 20:609-616.

Kerepesi, I., and G. Galiba. 2000. Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. Crop Science. 40:482-487.

Loescher, W.H., R.H. Tyson, J.D. Everard, R.J. Redgwell, and R.L. Bieleski. 1992. Mannitol synthesis in higher plants: evidence for the role and characterization of a NADPH-dependent mannose 6-phosphate reductase. Plant Physiology. 98:1396-1402.

Matthews, M.A., and J.S. Boyer. 1984. Acclimation of photosynthesis to low leaf water potentials. Plant Physiology. 74:161-166.

McCue, K.F., and A.D. Hanson. 1990. Drought and salt tolerance: towards understanding and application. Trends in Biotechnology. 8:358-362.

Moran, J.F., M. Becana, I. Iturbe-Ormaetxe, S. Frechilla, R.V. Klucas, and P. Aparicio-Tejo. 1994. Drought induces oxidative stress in pea plants. Planta. 194:346-352.

Nakano, Y., and K. Asada. 1981. Hydrogen Peroxide is scavenged by sscorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiology. 22:867-880.

Papageorgiou, G.C., and N. Murata. 1995. The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. Photosynthesis Research. 44:243-252.

Pathan, M.S., P.K. Subudhi, B. Courtois, and H.T. Nguyen. 2004. Molecular dissection of abiotic stress tolerance in sorghum and rice. Physiology and Biotechnology Integration for Plant Breeding. A case study, 525-569.

Pilon-Smits, E.A.H., M.J.M. Ebskamp, M.J. Paul, M.J.W. Jeuken, P.J. Weisbeek, and S.C.M. Smeekens. 1995. Improved performance of transgenic fructan-accumulating tobacco under drought stress. Plant Physiology. 107:125-130.

Rathinasabapathi, B., K.F. McCue, D.A. Gage, and A.D. Hanson. 1994. Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. Planta. 193:155-162.

Rhodes, D., and A.D. Hanson. 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology. 44:357-384.

Romero, C., J.M. Bellés, J.L. Vayá, R. Serrano, and F.A. Culiáñez-Macià. 1997. Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta. 201:293-297.

Rontein, D., G. Basset, and A.D. Hanson. 2002. Metabolic enginneing of odmoprotectant accumulation in plants. Metabolic Engineering. 4:49-56.

Rooke, L., S. Steele, P. Barcelo, P. Shewry, and P. Lazzeri. 2003. Transgene inheritance, segregation and expression in bread wheat. Euphytica. 129:301-309.

Sakamoto, A., and N. Murata. 2001. The use of bacterial choline oxidase, a glycinebetaine-synthesizing enzyme, to create stress-resistant transgenic plants. Plant Physiology. 125:180-188.

Serraj, R., and T.R. Sinclair. 2002. Osmolyte accumulation: can it really help increase crop yield under drought conditions. Plant, Cell & Environment. 25:333-341.

Sharp, R.E., T.C. Hsiao, and W.K. Silk. 1990. Growth of the Maize Primary Root at Low Water Potentials : II. Role of Growth and Deposition of Hexose and Potassium in Osmotic Adjustment. Plant Physiology. 93:1337-1346.

Shen, B., R.G. Jensen, and H.J. Bohnert. 1997. Mannitol protects against oxidation by hydroxyl radicals. Plant Physiology. 115:527-532.

Sheveleva, E., W. Chmara, H.J. Bohnert, and R.C. Jensen. 1997. lncreased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum*. Plant Physiology. 115:1211-1219.

Sivamani, E., A. Bahieldin, J.M. Wraith, T. Al-Niemi, W.E. Dyer, T.-H.D. Ho, and R. Qu. 2000. Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. Plant Science. 155:1-9.

Sivamani E, B.A., Wraith JM, Al-Niemi T, Dyer WE, Ho TD, Qu R. 2000. Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. Plant Science. 12:1-9.

Smirnoff, N. 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry. 28:1057.

Smirnoff, N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytologist. 125:27-58.

Smith, I.K., T.L. Vierheller, and C.A. Thorne. 1988. Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithiobis(2-nitrobenzoic acid). Analytical Biochemistry. 175:408-413.

Souza, R.P., E.C. Machado, J.A.B. Silva, A.M.M.A. Lagôa, and J.A.G. Silveira. 2004. Photosynthetic gas exchange, chlorophyll fluorescence and some associated

metabolic changes in cowpea (Vigna unguiculata) during water stress and recovery. Environmental and Experimental Botany. 51:45-56.

Tarczynski, M.C., R.G. Jensen, and H.J. Bohnert. 1993. Stress Protection of Transgenic Tobacco by Production of the Osmolyte Mannitol. Science. 259:508-510.

Thomas, J.C., M. Sepahi, B. Arendall, and H.J. Bohnert. 1995. Enhancement of seed germination in high salinity by engineering mannitol expression in Arabidopsis thaliana. Plant, Cell & Environment. 18:801-806.

Valliyodan, B., and H.T. Nguyen. 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Current Opinion in Plant Biology. 9:189-195.

Wardlaw, I.F. 1971. The early stages of grain development in wheat: response to water stress in a single variety. Australian Journal of Biological Sciences. 24:1047-1055.

Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus, and G.N. Somero. 1982. Living with water stress: evolution of osmolyte systems. Science. 217:1214-1222.

Yoshitaka, N., Y. Hiroshi, I. Suleyman, Allakhverdiev, I. Masami, Y. Akiho, and M. Norio. 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. The EMBO Journal. 20:5587-5594.

Zeinalov, Y., and L. Maslenkova. 1999. Estimation of the quantum efficiency of photosynthesis. I. Theoretical ground and experimental approaches. Bulgarian Journal of Plant Physiology. 25(1-2):26-38.

Zhang, S., B. Yang, C. Feng, and H. Tang. 2005. Genetic Transformation of Tobacco with the Trehalose Synthase Gene from Grifola frondosa Fr. Enhances the

Resistance to Drought and Salt in Tobacco. Journal of Integrative Plant Biology. 47:579-587.

Zobayed, S.M.A., F. Afreen, and T. Kozai. 2005. Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in St. John's wort. Plant Physiology and Biochemistry. 43:977-984.

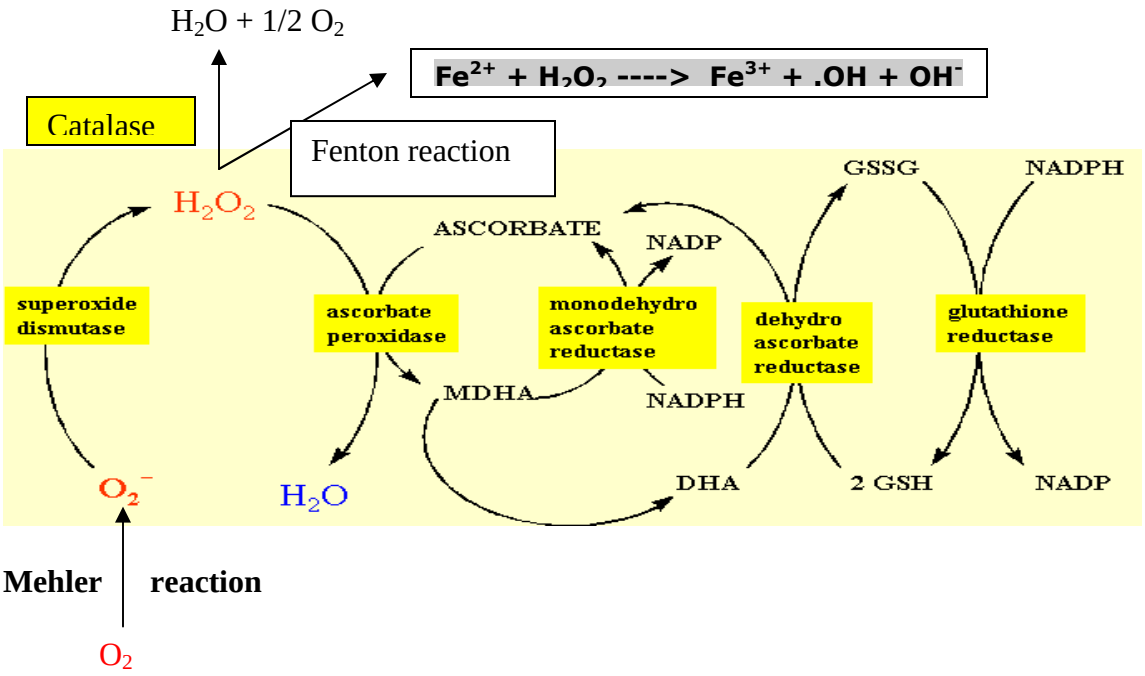

Figure 1: **Role of antioxidant enzymes under oxidative stress:** Superoxide radicals ($^{\cdot}O_2^-$) are produced in chloroplasts in the Mehler's reaction. The superoxide dismutase enzyme, which is an important enzyme of plant's antioxidant system, converts this superoxide radical into hydrogen peroxide which can be degraded by the enzyme catalase. An alternative path is through the ascorbate-glutathione cycle consisting of various antioxidant enzymes, where it finally gets converted to water. The hydrogen peroxide formed can form harmful hydroxyl radicals in the Fenton reaction (Arora et al., 2002).

L 2 3 4 5 N P 8 9 10 11 12 N P

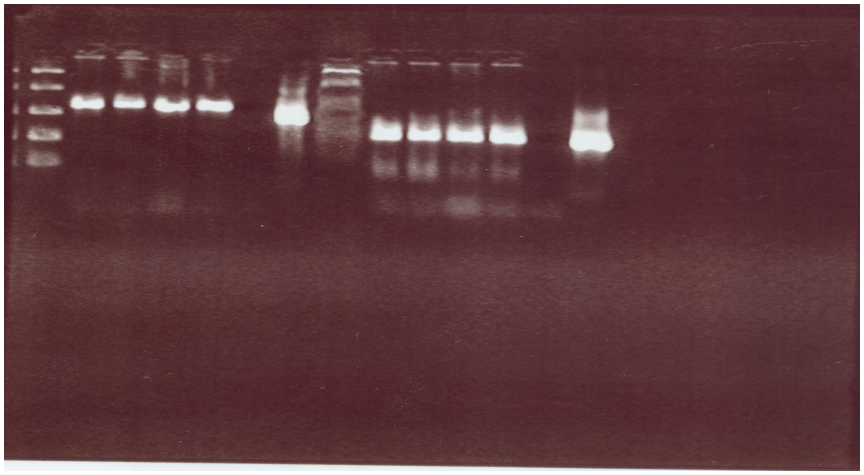

Figure 2: PCR screening for the presence of transgenes *mtlD* and *bar*.

L- 1 Kb ladder

2-5- 600bp band for the *mtlD* gene in the transgenic line pTA2-115

N. Negative control for the *mtlD* gene

P. Positive control for the *mtlD* gene

8. 1 Kb ladder

9-12. 300bp band for the *bar* gene in the transgenic line pTA2-115

13. Negative control for the *bar* gene

14. Positive control for the *bar* gene

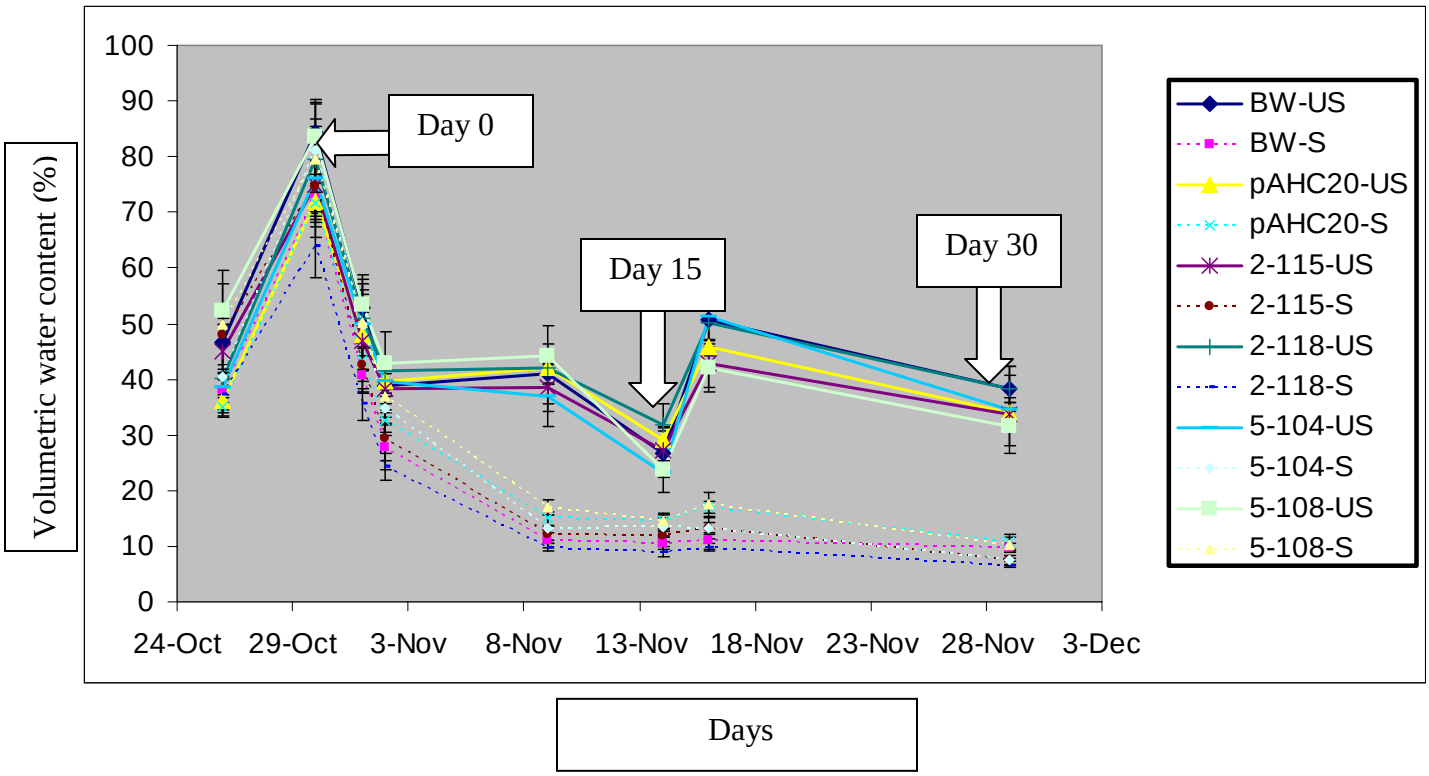

Figure 3: Soil volumetric water content (%) of the experimental pots in the Experiment I (Fall 2006) measured using Time Domain Reflectometry (bars are ± SE, n= 4). Days 0, 15 and 30 refer to withholding of water in the stress treatment. S and US following line names refer to the stressed and unstressed treatments

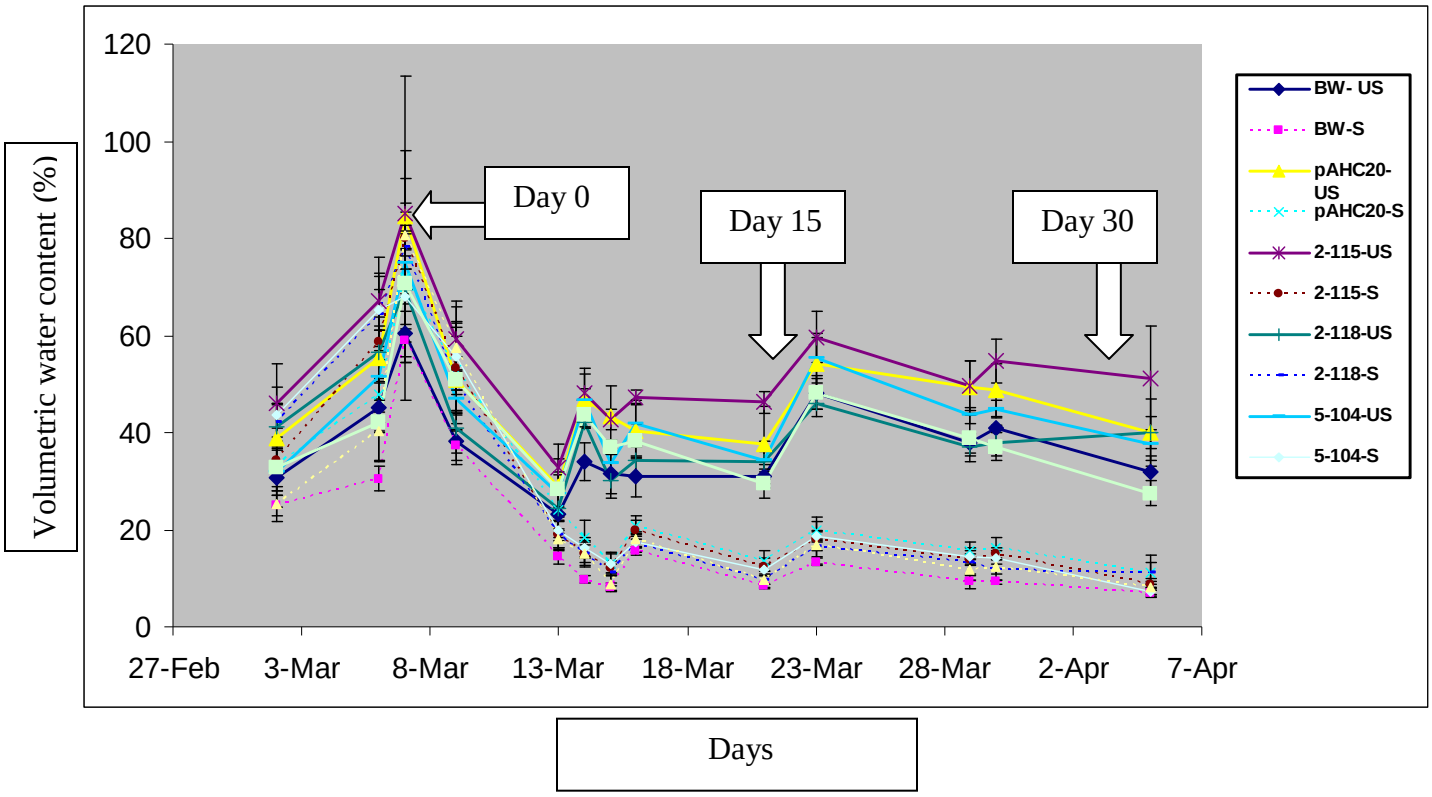

Figure 4: Soil volumetric water content (%) of the experimental pots in the Experiment II (Spring 2007) measured using Time Domain Reflectometry (bars are ± SE, n= 4). Days 0, 15 and 30 refer to withholding of water in the stress treatment. S and US following line names refer to the stressed and unstressed treatments

Table 1: Nucleotide primer sequences and amplicon size of the target genes *mtlD* and *bar* used for screening transgenic wheat plants.

Target Gene		***Primer Sequence (5'-3')***	***Amplicon Size (bp)***
mtlD	Forward Primer	5'-CGG GTA TCC AAC TGA CGT TT-3'	600
	Reverse Primer	5'-CCG TGT TCA GGG TGA AGA GT-3'	
bar	Forward Primer	5'-CAT CGA GAC AAG CAC GGT CAA CTT C-3'	300
	Reverse Primer	5'-CTC TTG AAG CCC TGT GCC TCC AG-3'	

Table 2: PCR parameters used for screening transgenic wheat plants

Step	***Temperature***	***Time/ Duration***	***Cycles***
Initial Denaturation	95^{o}C	3 min	1
Denaturation	95^{o}C	1 min	30
Annealing	52^{o}C	1 min	
Extension	72^{o}C	1 min	
Final Extension	72^{o}C	5 min	1
Hold	4^{o}C		

Table 3: Relative water content (%) of leaf tissue in Experiment I (Fall 2006)

Stress level	Days	pTA2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	92.6± 1.70a	92.5± 1.70	90.7± 2.70	90.5± 2.00	91.3 ± 1.30	91.3± 2.10
	15	93.2± 0.40	93.2± 1.00	91.4± 2.50	92.5± 1.00	92. 6± 0.50	91.6± 1.60
	30	94.2± 0.70	96.3± 0.40a	92.3± 3.90	94.4± 0.65	93.8± 3.00a	94.4± 1.03
Stress Treatment	0	86.7± 2.30b	93.4± 0.60	87.7± 1.70	93.8± 0.80	90.8 ± 1.30	87.9± 2.90
	15	91.0± 1.50	89.8± 0.64	89.0± 2.30	91.4± 0.60	91. 6± 0.80	91.3± 1.20
	30	89.6± 0.80 1	77.0± 3.09b2	88.7± 0.80 1	91.0± 0.80 1	86.6± 3.90b1	90.8± 0.92 1

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a row are significantly different at P < 0.05 among the lines

Table 4: Relative water content (%) of leaf tissue in Experiment II (Spring 2007)

Stress level	Days	pTA2-115	2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	94.68±0.44	92.85±1.51	94.10±0.98	96.56±3.63	94.14±1.68	95.58±1.28
	15	97.22±0.51	95.75±0.58	96.81±1.27	93.81±1.18	95. 39±0.40	94.14±1.34
	30	93.08±1.08 a	92.27±1.10 a	92.96±0.95 a	92.74±1.39 a	94.18±0.95 a	91.25±0.31
Stress Treatment	0	94.50±0.48	93.91±1.15	94.28±0.87	94.90±1.35	97.09±1.53	94.30±1.21
	15	96.40±0.95	95.45±0.87	97.01±0.64	95.26±0.86	94. 63±0.49	93.98±0.65
	30	87.48±1.78 b 12	80.15±3.33 b 3	84.93±2.66 b 2	84.64±1.26 b 2	89.62±2.31 b 1	87.45±2.81 12

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 5: Plant height and dry weight of the above ground biomass, recorded at the end of the 30 days of stress period in Experiment I (Fall 2006)

Stress level	Plant ID	Height (cm)	Biomass (Dry wt in grams)
Control treatment	pTA2-115	56.00± 0.41 5	16.55± 2.20 2
	pTA2-118	75.50± 2.10 a 1	14.40± 1.72 2
	pTA5-104	61.00± 1.73 a 4	16.68± 2.08 a2
	pTA5-108	68.50 ±2.50 a 2	17.39± 0.39 2
	BW	68.00± 0.71 23	22.14 ±1.30 a 1
	pAHC20	65.75± 4.09 234	15.12 ±1.85 2
Stress treatment	pTA2-115	51.50± 2.72 2	12.42± 0.35
	pTA2-118	63.25± 2.59 b 1	15.19± 0.57
	pTA5-104	51.50± 1.55 b 2	12.47± 0.41 b
	pTA5-108	61.50± 1.44 b 1	14.31± 0.59
	BW	62.75± 2.25 1	13.67± 1.10 b
	pAHC20	59.50 ±1.92 1	11.95± 0.79

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a column in each treatment are significantly different at P < 0.05 among the lines.

Table 6: Plant height and dry weight of the above ground biomass, recorded at the end of the 30 days of stress period in Experiment II (Spring 2007)

Stress level	Plant ID	Height (cm)	Biomass (Dry weight in grams)
Control treatment	pTA2-115	54.00±3.87 3	11.05±1.37 23
	pTA2-118	71.75±2.32 1	16.53±0.93 a 1
	pTA5-104	61.25±4.05 23	11.01±1.84 23
	pTA5-108	63.00±2.48 2	13.23±1.81 123
	BW	65.50±1.50 12	13.49±2.01 12
	pAHC20	58.00±3.08 23	9.97±0.55 3
Stress treatment	2-115	49.75±3.71 3	9.23±1.32 12
	pTA2-118	67.50±3.50 1	11.74±1.66 b 12
	pTA5-104	59.00±1.35 23	10.07±0.27 12
	pTA5-108	57.25±2.63 23	10.57±0.83 12
	BW	64.75±1.44 12	12.43±0.50 1
	pAHC20	53.25±1.25 3	8.72±0.40 2

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers among in a column in each treatment are significantly different at P < 0.05 among the lines.

Table 7: Net photosynthesis rate (µmol m^{-2} s^{-1}) measured five times in Experiment I (Fall 2006)

Stress level	Days	pTA2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	25.28± 0.62	19.18± 2.20	23.03± 2.10	28.33± 1.00 a	20.80± 1.50	14.35± 2.10
	3	22.78 ±1.50	26 70± 4.20	26.85± 2.40	26.80± 1.20	25.10 ±4.00	23.80± 2.00
	10	29.32± 0.90 a	27.15± 2.30	28.08± 1.56 a	24.07± 2.11 a	26.55± 0.42 a	28.50 ±1.59
	17	22.55± 1.99	22.12± 2.40 a	21.23± 1.96	21.65± 1.54	23.55± 1.05	24.12± 1.55
	24	23.53± 2.60 a	24.82± 0.69 a	23.55± 2.50 a	23.62± 3.17 a	21.75± 0.27 a	26.35± 1.48 a
Stress Treatment	0	27.58 ±1.80	23.68± 1.80	22.60± 0.24	21.60± 4.00 b	21.78± 3.30	20.05 ±2.40
	3	22.68± 1.45	27.18± 3.10	28.32± 1.60	24.10 ±3.01	27.00± 4.40	24.97± 2.80
	10	16.34± 2.72 b	22.50± 3.20	17.83± 3.80 b	15.40± 1.03 b	19.80± 2.60 b	23.35± 1.74
	17	16.92± 2.80	15.40± 2.60 b	19.55 ±1.01	17.80± 1.74	18.62± 0.75	20.67± 1.29
	24	12.13 ±1.90 b	6.24± 0.90 b	9.98± 1.31 b	12.90 ±2.40 b	9.31± 1.61 b	15.49± 2.10 b

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments.

Table 8: Net photosynthesis rate (µmol m^{-2} s^{-1}) measured five times in Experiment II (Spring 2007)

Stress level	Days	pTA2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	20.80±3.30	19.63±1.72	22.45±2.18	14.62±2.42	18.38 ±2.58	20.20±1.87
	3	22.33±1.08	26.55±1.62	25.45±1.22	21.68±2.89	28. 10±2.85	24.50±0.92
	10	20.5±0.66	21.08±1.55	20.30±1.71	20.93±0.68	18. 50±0.90	19.60±1.33
	17	24.80±1.50	25.23±2.88	26.70±1.21	23.15±0.88	27 .03±2.08	27.80±0.98
	24	22.85±2.35	21.95±2.54	21.05±2.53	19.08±0.81	20 .36±1.19	17.15±2.29
Stress Treatment	0	16.48±0.81	19.50±1.19	20.03±2.43	19.08±2.08	19.20 ±1.45	15.93±4.08
	3	22.35±0.77	23.18±2.19	22.78±0.39	24.48±0.57	25. 75±2.17	22.33±1.31
	10	17.68±1.67	17.78±1.99	18.15±2.26	19.65±0.98	18 .28±1.71	21.40±2.07
	17	19.54±4.48	23.38±3.28	22.00±3.46	21.88±2.43	21 .80±2.99	26.43±1.51
	24	20.25±1.09 12	20.35±1.99 12	23.83±1.59 1	16.10±2.00 2	15.72±2.39 2	20.07±2.91 12

Values are means ± SE of four replications. Means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 9: Stomatal conductance (mmol m^{-2} s^{-1}) measured five times in Experiment I (Fall 2006)

Stress level	Days	pTA2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	-4	0.49± 0.02	0.35± 0.09	0.45± 0.06	0.54± 0.05	0.31± 0.02	0.36± 0.05
	3	0.24± 0.03	0.39± 0.11	0.36± 0.05	0.34± 0.02	0.34 ± 0.09	0.32± 0.10
	10	0.63± 0.13a	0.65± 0.07a	0.58± 0.07a	0.54± 0.11a	0.57± 0.04a	0.69± 0.10a
	17	0.43± 0.06a	0.42± 0.09a	0.39± 0.06	0.41± 0.06a	0.45± 0.04a	0.57± 0.30a
	24	0.32± 0.05a	0.54± 0.03a	0.42± 0.11a	0.35± 0.08a	0.32± 0.05a	0.42± 0.05a
Stress Treatment	-4	0.52± 0.07	0.34± 0.03	0.49± 0.04	0.42± 0.12	0.44± 0.08	0.32± 0.05
	3	0.20± 0.03	0.29± 0.07	0.39± 0.07	0.24± 0.03	0.33 ± 0.08	0.29± 0.09
	10	0.20± 0.05b	0.29± 0.04b	0.26± 0.06b	0.20± 0.04b	0.23± 0.05b	0.31± 0.06b
	17	0.22± 0.07b	0.15± 0.04b	0.26± 0.04	0.21± 0.04b	0.19± 0.01b	0.32± 0.05b
	24	0.09± 0.02b	0.05± 0.01b	0.07± 0.01b	0.11± 0.03b	0.07± 0.02b	0.16± 0.04b

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments.

Table 10: Stomatal conductance (mmol m^{-2} s^{-1}) measured five times in Experiment II (Spring 2007)

Stress level	Days	pTA2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	-4	0.42±0.09	0.35±0.06	0.30±0.01	0.21±0.04	0.25±0.07	0.30±0.03
	3	0.62±0.04	0.63±0.10	0.59±0.04	0.49±0.08	0.72±0. 10	0.59±0.10
	10	0.75±0.04	0.67±0.03	0.68±0.03	0.68±0.11	0.75±0 .04 a	0.64±0.10
	17	0.85±0.02 a	0.78±0.03	0.84±0.05	0.79±0.08	0.85±0.04 a	0.86±0.03
	24	0.64±0.09	0.53±0.08	0.63±0.06	0.57±0.01 a	0.61±0.10 a	0.55±0.12
Stress Treatment	-4	0.37±0.04	0.38±0.02	0.27±0.02	0 .32±0.04	0.31±0.04	0.23±0.6
	3	0.62±0.11	0.56±0.13	0.56±0.07	0.59±0.05	0.55±0. 06	0.47±0.07
	10	0.66±0.10	0.56±0.13	0.72±0.03	0.63±0.05	0.52±0 .30 b	0.55±0.10
	17	0.65±0.18 b	0.69±0.11	0.73±0.09	0.72±0.06	0.57±0.06 b	0.74±0.07
	24	0.63±0.12 1	0.57±0.08 1	0.68±0.09 1	0.36±0.08 b 2	0.35±0.05 b 2	0.60±0.11 1

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 11: Ascorbate peroxidase activity (µmol min^{-1} g^{-1} fwt) recorded at three time points in Experiment I (Fall 2006)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	16.00±3.29	16.94±0.69	17.14±1.26	15.73±1.38	21.60 ±2.15	18.61±1.22
	15	17.02±0.60	22.18±3.62	20.71±2.38	23.85±2.62	20.2 1±1.79	23.93±1.28
	30	21.76±2.29 a	33.54±2.91 a	25.45±2.21	23.99±2.17	28.50±3.65	28.11±6.45
Stress Treatment	0	17.37±1.36	20.20±2.35	15.37±2.11	19.02±1.98	19.94 ±2.25	16.20±1.22
	15	19.33±1.53	23.24±2.0	17.98±1.03	22.72±2.33	24.08 ±1.37	21.49±2.26
	30	32.50±8.36 b	25.57±4.79 b	22.82±1.20	26.30±4.04	27.23±2.46	25.57±2.33

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at $P < 0.05$ between the two treatments.

Table 12: Ascorbate peroxidase activity (µmol min^{-1} g^{-1} fwt) recorded at three time points in Experiment II (Spring 2007)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	14.45±1.29	16.10±3.16	15.37±1.63	16.38±0.29	17.09 ±2.07	15.27±1.04
	15	14.47±1.94	11.80±0.36	14.20±2.23	15.91±1.48	13.6 6±0.83	16.81±0.97
	30	20.48±2.29 23	21.08±0.90 23	31.39±7.13 a 1	24.66±4.10 23	22.98±4.14 23	26.86±2.04 12
Stress Treatment	0	16.06±1.19	14.83±1.40	15.92±1.15	15.30±0.66	17.06 ±0.90	16.83±2.20
	15	12.37±0.95	13.18±1.21	13.26±1.03	12.19±0.68	17.4 6±1.45	13.50±0.47
	30	18.47±2.72 2	25.93±5.78 1	21.20±3.00 b 12	22.27±2.19 12	18.47±1.74 2	26.19±3.04 1

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 13: Catalase activity (mmol m^{-1} g^{-1} fwt) recorded at three time points in Experiment I (Fall 2006)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	5.58± 0.60 2	5.53 ±0.21 2	5.95± 0.33 2	5.00± 0.71 2	6.33 ±0.36 2	8.11± 0.26 1
	15	5.15± 0.29	6.73 ±0.19	7.00± 0.30	7.04 ±0.88	6.20± 0.17	7.10± 0.77
	30	6.30 ±0.32 23	6.80± 0.90 23	7.30 ±0.97 23	7.01± 0.49 23	7.80± 0.90 12	9.01± 1.05 1
Stress Treatment	0	5.90± 0.50	6.51± 0.71	5.81± 0.46	6.20 ±0.69	6.80± 0.80	7.40 ±0.65
	15	5.46 ±0.51	6.28± 0.44	6.21 ±0.77	6.50 ±0.52	6.10± 0.43	7.80± 0.32
	30	6.80± 0.42 2	5.82± 0.80 2	6.95± 0.31 2	6.83 ±0.30 2	6.60 ±0.14 2	9.70± 0.61 1

Values are means ± SE of four replications. Means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 14: Catalase activity (µmol m^{-1} g^{-1} fwt) recorded at three time points in Experiment II (Spring 2007)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	4.35±0.33 23	4.82±0.35 12	5.09±0.51 12	5.81±0.31 1	4.62±0.22 2	5.87±0.38 1
	15	3.90±0.39 23	3.82±0.30 23	4.31±0.79 23	4.91±0.41 2	3.48±0.27 a 3	6.37±0.32 1
	30	4.53±0.53 2	4.13±0.55 2	4.81±0.73 2	4.90±0.53 2	4.20±0.71 2	6.98±0.68 1
Stress Treatment	0	4.67±0.26 2	4.47±0.25 2	5.00±0.20 2	5.22±0.28 2	5.18±0.45 2	6.75±0.43 1
	15	3.35±0.28 3	3.12±0.30 3	4.23±0.36 23	3.88±0.09 23	4.86±0.17 b 12	5.46±0.26 1
	30	3.93±0.89 2	3.60±0.58 2	4.01±0.41 2	4.16±0.68 2	3.69±0.33 2	6.87±0.40 1

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 15: Glutathione reductase (mmoles min^{-1} g^{-1} fwt) activity recorded at three time points in Experiment I (Fall 2006)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	3.59± 0.74	3.97± 0.61	4.32 ±1.30	4.07± 0.90	4.74± 0.45	5.38 ±0.25
	15	4.68± 0.12	6.13± 0.34	6.03± 0.45	5.83± 1.05	5.02± 0.23	5.84± 0.97
	30	4.47 ±0.40	6.35± 0.26 a	4.96± 0.55	4.77± 0.52	4.60± 0.28	5.16± 0.52
Stress Treatment	0	4.28± 0.59	4.92± 1.02	4.28± 1.05	3.91± 1.22	5.24± 0.95	5.38 ±0.90
	15	4.27 ±0.69 3	5.10± 0.62 123	5.62± 0.94 12	5.22± 0.83 123	4.80± 0.47 23	6.37 ±1.20 1
	30	4.79± 0.85	4.70± 0.82 b	4.67± 0.35	4.75± 0.73	4.65± 0.40	5.41± 0.48

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 16: Glutathione reductase (mmoles min^{-1} g^{-1} fwt) activity recorded at three time points in Experiment II (Spring 2006)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	1.42±0.16	1.90±0.19	1.87±0.05	1.88±0.36	1.89±0.13	2.08±0.16
	15	3.55±0.27	3.57±0.28	3.37±0.48	4.23±0.37	3.44±0. 32	4.72±0.32
	30	3.83±0.36 2	4.66±0.69 2	4.30±0.68 2	4.51±0.59 2	4.82±1.44 a 2	6.78±0.45 1
Stress Treatment	0	1.80±0.08	1.82±0.06	1.72±0.12	1.98±0.15	1.99±0.13	2.34±0.06
	15	3.11±0.21	3.39±0.24	3.71±0.17	3.34±0.25	4.29±0. 19	4.03±0.52
	30	3.41±0.45 3	4.96±1.13 2	4.33±0.56 23	3.86±0.49 23	3.46±0.35 b 3	6.21±0.62 1

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

.

Table 17: Superoxide dismutase activity (units g^{-1} fwt) recorded at three time points in Experiment I (Fall 2006)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	1106 ±239	972 ±95	1083 ±94	943 ±126	985 ±100	1003 ±198
	15	537 ±60	916 ±95	1134 ±185 a	896 ±114	830 ±28	782 ±105
	30	981 ±39	1537 ±141	1338 ±103	1131 ±153	1070 ±162	1205 ±228
Stress Treatment	0	1038 ±42	841 ±67	929 ±112	1257 ±198	1011 ±242	1220 ±196
	15	511 ±62	667 ±125	639 ±162 b	785 ±149	818 ±49	935 ±112
	30	1078 ±266	1117 ±352	1049 ±148	1258 ±187	1187 ±113	1151 ±210

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at $P < 0.05$ between the two treatments.

Table 18: Superoxide dismutase activity (units g^{-1} fwt) recorded at three time points in Experiment II (Spring 2007)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control Treatment	0	858±89.13	1050±131.18	749±110.89	909±64.93	1026±139.65	888±124.76
	15	957±124.34	905±107.01	958±112.58	1066±121.59	1038±254.83	1115±96.76
	30	1219±136.02	1435±201.50	1756±464.98	1328±55.89	1670±326.19	1204±141.08
Stress Treatment	0	1057±70.34	1016±203.74	994±50.50	1109±132.40	992±89.06	927±50.46
	15	794±47.11	776±99.28	827±102.62	1005±48.37	660±284.62	1045±104.95
	30	1643±520.35	1753±516.91	1358±341.84	1620±267.48	1378±337.12	1515±104.17

Table 19: Lipid peroxidation estimated by malondialdehyde (MDA) concentration (nmol g^{-1} fwt) in Experiment I (Fall 2006)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control treatment	15	28.5± 4.5	30.9± 4.2	24.0± 1.5	22.1± 3.5	26.6± 2 .5	22.3± 4.8
	30	36.7± 5.0	44.0± 6.5	40.2± 3.4	34.5± 4.2	40.2± 3.3	32.5± 6.2
Stress treatment	15	72.1± 8.4 12	85.1± 7.4 1	61.4± 6.0 2	55.6± 7.4 2	70.6± 11.2 12	65.6± 10.5 12
	30	148.4± 12.2 12	165.0± 14.5 1	126.4± 10.9 23	118.7± 9.0 3	154.0± 14.5 12	132.3± 16.7 123

Values are means ± SE from four replications followed by numbers. The means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 20: Lipid peroxidation estimated by malondialdehyde (MDA) concentration (nmol g^{-1} fwt) in Experiment I (Spring 2007)

Stress level	Days	2-115	pTA2-118	pTA5-104	pTA5-108	BW	pAHC20
Control treatment	15	23.1± 4.3	21.4± 5.6	26.9± 3.4	22.4± 4.0	20.1± 3 .5	25.9± 4.1
	30	30.7± 5.6	34.5± 7.2	30.1± 6.2	26.3± 6.2	28.5± 5.5	32.0± 6.6
Stress treatment	15	54.0± 9.2 12	72.3± 11.5 1	58.5± 8.5 12	48.3± 7.5 2	55.4± 9.0 12	60.1± 10.2 12
	30	118.4± 20.2 12	148.3± 18.0 1	94.5± 12.8 23	87.5± 10.8 3	122.5± 14.5 12	99.7± 13.5 23

Values are means ± SE from four replications followed by numbers. The means followed by different numbers in a row are significantly different at P < 0.05 among the lines.

Table 21: Light response curve measurements were recorded at two time points in Experiment I (Fall 2006)

Stress level	Lines	AQE		Amax (µmol m^{-2} s^{-1})		LCP (µmol m^{-2} s^{-1})	
		Days		Days		Days	
		15	30	15	30	15	30
Control treatment	pAHC20	0.060± 0.004	0.061± 0.005	31.5 ±2.37	27.3 ±0.57	33.7 ±1.0 1	22.2± 6.6
	pTA2-118	0.062 ±0.002	0.055± 0.004	28.9 ±1.42	16.2 ±3.41	26.5± 5.6	25.3± 1.4
Stress treatment	pAHC20	0.054± 0.003	0.063± 0.002 1	29.8 ±1.27	25.2 ±0.92 1	19.3± 4.3 2	18.1± 2.1
	pTA2-118	0.052± 0.004	0.047± 0.001 2	30.7 ±3.19	14.6 ±1.55 2	21.9± 4.8	18.6± 2.4

Values are means ± SE of four replications. Means followed by different numbers in a column are significantly different at P < 0.05 among the lines.

AQE= quantum efficiency
Amax= maximum photosynthesis rate
LCP= light compensation point

Table 22: Light response curve measurements were recorded at two time points in Experiment II (Spring 2007)

Stress level	Lines	AQE		Amax (µmol m^{-2} s^{-1})		LCP (µmol m^{-2} s^{-1})	
		Days		Days		Days	
		15	30	15	30	15	30
Control treatment	pAHC20	0.065± 0.001	0.066± 0.007	32.8± 0.90	27.8± 0 .4	23.7± 1.6	19.2± 1.3
	pTA2-118	0.064± 0.003	0.068± 0.003	28.7± 0.65	27.2± 2.2	21.9± 3.4	24.0± 1.4
Stress treatment	pAHC20	0.062± 0.003	0.075± 0.004 1	29.9± 2.3	25.4± 2.5	21.4± 0.79	24.9± 1.7
	pTA2-118	0.056± 0.005	0.057± 0.007 2	27.4± 3.3	23.5± 6.1	22.8± 7.3	16.6± 3.2

Values are means ± SE of four replications. Means followed by different numbers in a row are significantly different at P < 0.05 among the lines.
AQE= quantum efficiency
Amax= maximum photosynthesis rate
LCP= light compensation point

Table 23: CO_2 response curves measurements were recorded at three time points in Experiment I (Fall 2006)

Stress level	Lines	Carboxylation efficiency (µmol m^{-2} s^{-1})			CO_2 compensation point (µmol m^{-2} s^{-1})		
		Days			Days		
		0	15	30	0	15	30
Control treatment	pAHC20	1.42± 0.02	1.20± 0.15	1.13± 0.05	47.4± 9.6	46.5± 9.2 1	46.3± 8.3
	pTA2-118	1.21± 0.09	1.07± 0.10	1.27± 0.13	46.9± 10 .1	38.1± 7.2 2	46.5± 9.3
Stress treatment	pAHC20	1.36± 0.03	1.26± 0.04	1.28± 0.05	48.8± 9.7	37.5± 5.4	47.4± 8.7
	pTA2-118	1.29± 0.05	1.19± 0.02	1.35± 0.12	45.1± 9 .4	42.5± 6.7	54.4± 9.9

Values are means ± SE of four replications. Means followed by different numbers in a column are significantly different at P < 0.05 between the two treatments.

.

Table 24: CO_2 response curves measurements were recorded at three time points in Experiment II (Spring 2007

Stress level	Lines	Carboxylation efficiency (µmol m^{-2} s^{-1})			CO_2 compensation point (µmol m^{-2} s^{-1})		
		Days			Days		
		0	15	30	0	15	30
Control treatment	pAHC20	1.20± 0.13	1.41± 0.06	1.00± 0.04	37.4± 041 b	44.3± 1.9	36.8± 6.5
	pTA2-118	1.27± 0.10	1.27± 0.10	0.97± 0.15	39.9± 00 9	42.0± 3.8	35.7± 5.8
Stress treatment	pAHC20	1.05± 0.15 2	1.21± 0.03	1.08± 0.07	50.0± 054 a1	42.3± 2.8	45.2± 1.9
	pTA2-118	1.38± 0.15 1	1.29± 0.09	1.09± 0.19	38.3± 041 2	45.9± 2.1	46.8± 3.8

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a column are significantly different at P < 0.05 among the lines.

Table 25: Carbohydrate concentrations (µmol g^{-1} fwt) recorded at three time points in Experiment I (Fall 2006)

Stress Level	Lines	Time Points					
		Days					
		0		15		30	
		Mannitol	Total soluble sugar	Mannitol	Total soluble sugar	Mannitol	Total soluble sugar
Control treatment	pTA2-115	0.15 ±0.07	22.79 ±2.73	0.37 ±0.22	29.75 ±3 .81	0.48 ±0.25	31.21 ±3.26 a
	pTA2-118	0.09 ±0.05	17.49 ±4.22	0.22 ±0.15	33.68 ±18.55	0.42 ±0.25	43.53 ±6.04 a
	pTA5-104	0.06 ±0.05	18.26 ±1.90	0.34 ±0.14	31.46 ±3.40	0.52 ±0.28	32.40 ±11.59 a
	pTA5-108	0.07 ±0.04	14.97 ±2.24	0.11 ±0.06	27.80 ±3.27	0.27 ±0.17	65.82 ±19.40
	BW	0	19.34 ±1.82	0	23.73 ±2.46	0	23.29 ±4.44 a
	pAHC20	0	22.42 ±4.37	0	23.95 ±4.52	0	29.17 ±2.46
Stress treatment	2-115	0.12 ±0.06	20.19 ±3.27	0.35 ±0.22	29.43 ±2.28	0.47 ±0.22	77.63 ±24.70 b 23
	pTA2-118	0.09 ±0.06	18.22 ±2.15	0.36 ±0.17	36.76 ±6.43	0.64 ±0.23	110.06 ±40.05 b 1
	pTA5-104	0.12 ±0.06	18.86 ±3.63	0.17 ±0.12	23.29 ±3.15	0.45 ±0.22	86.80 ±8.38 b 12
	pTA5-108	0.07± 0.04	14.33 ±2.91	0.23 ±0.10	41.03 ±6.67	0.34 ±0.20	42.43 ±14.58 4
	BW	0	13.30 ±2.31	0	26.80 ±3.05	0	87.57 ±22.01 b 12
	pAHC20	0	14.27 ±3.18	0	22.11 ±5.86	0	50.42 ±17.88 34

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a column within each treatment are significantly different at P < 0.05 among the lines.

Table 26: Carbohydrate concentrations (µmol g^{-1} fwt) recorded at three time points in Experiment II (Spring 2007)

Stress Level	Lines	Time Points					
		Days					
		0		15		30	
		Mannitol	Total soluble sugar	Mannitol	Total soluble sugar	Mannitol	Total soluble sugar
Control treatment	pTA2-115	0.38 ±0.14	33.78 ±5.26	0.17 ±0.16	12.23 ±2 .97 2	0.32 ±0.22	39.55 ±3.42 b
	pTA2-118	0.47 ±0.24	26.55 ±3.30	0.28 ±0.10	18.00 ±5.24 2	0.52 ±0.25	42.14 ±7.18
	pTA5-104	0.16 ±0.06	40.87 ±9.58	0.21 ±0.12	46.03 ±9.37 1	0.39 ±0.23	37.65 ±4.42 b
	pTA5-108	0.22 ±0.08	28.49 ±7.14	0.13 ±0.09	18.83 ±5.14 2	0.18 ±0.11	26.45 ±2.61
	BW	0	35.87 ±13.44	0	12.84 ±1.32 2	0	51.33 ±15.03 b
	pAHC20	0	24.53 ±2.61	0	11.22 ±1.97 2	0	41.65 ±8.06 b
Stress treatment	2-115	0.59 ±0.21	27.86 ±2.45	0.21 ±0.13	9.78 ±2.22 2	0.52 ±0.22	100.25 ±23.92 a 1
	pTA2-118	0.15 ±0.08	20.36 ±6.88	0.21 ±0.07	17.57 ±5.24 2	0.63 ±0.29	50.63 ±7.23 23
	pTA5-104	0.44 ±0.27	21.10 ±3.63	0.08 ±0.07	48.25 ±13.53 1	0.35 ±0.21	65.33 ±10.02 a 2
	pTA5-108	0.25 ±0.12	28.40 ±3.54	0.09 ±0.08	17.23 ±4.77 2	0.19 ±0.11	41.27 ±6.10 3
	BW	0	27.85 ±5.81	0	15.42 ±3.96 2	0	105.81 ±5.13 a 1
	pAHC20	0	21.78 ±3.80	0	9.62 ±2.74 2	0	85.42 ±20.24 a 2

Values are means ± SE of four replications. Means followed by different letters in a column are significantly different at P < 0.05 between the two treatments, and means followed by different numbers in a column within each treatment are significantly different at P < 0.05 among the lines.

VITA

Shraddha Shriram Vadvalkar

Candidate for the Degree of

Master of Science

Thesis: CHARACTERIZATION OF MANNITOL ACCMULATING T4 GENERATION TRANSGENIC WHEAT EXPOSED TO DROUGHT STRESS

Major Field: Plant and Soil Sciences

Biographical:

Personal Data: Born in Nasik, Maharashtra, India, on March 29, 1981, to Mr. Shriram Vadvalkar and Mrs. Anjali Vadvalkar and married to Mr. Vijay Muthukumar.

Education: Received Bachelor of Science degree in Microbiology from Pune University, Pune, India in May 2001; received Master of Science degree in Microbiology from Pune University, Pune, India in May 2003. Completed the requirements for the Master of Science in Plant and Soil Sciences at Oklahoma State University, Stillwater, Oklahoma in December, 2008.

Experience: Graduate research assistant, Department of Plant and Soil Sciences, Oklahoma State University, January 2006 to December 2008. Mentor for critical thinking in biological sciences, May 2007 to August 2008.

.

Name: Shraddha Shriram Vadvalkar **Date of Degree**: December, 2008

Institution: Oklahoma State University **Location**: Stillwater, Oklahoma

Title of Study: CHARACTERIZATION OF MANNITOL ACCUMULATING T4 GENERATION TRANSGENIC WHEAT EXPOSED TO DROUGHT STRESS

Pages in Study: 69 Candidate for the Degree of Master of Science

Major Field: Plant and Soil Sciences

Findings and Conclusions:

Wheat is an important crop grown worldwide, and it is severely affected by the drought stress. Sugars and sugar alcohols are known for protecting plants from drought stress. Among these, the sugar alcohol mannitol that is not normally found in wheat is known to scavenge free radicals. In this study, the mannitol accumulating transgenic T4 generation of wheat was characterized by measuring different properties such as biomass, height, net photosynthesis rate, stomatal conductance, light and CO_2 response curves, and antioxidant enzyme activities. The T4 generation wheat lines did not show the drought and salt tolerance previously observed in the T1 generation. The mannitol concentration found in the T4 generation was also far lower than in the T1 generation, and it did not increase under stress. The transgenic lines performed better than the wild type Bobwhite in terms of only height and biomass. Accumulation of mannitol in the chloroplasts of the transgenic lines provided better protection from membrane lipid peroxidation caused by harmful hydroxyl radicals compared to accumulation of mannitol in the cytoplasm.

ADVISER'S APPROVAL: Dr. Bjorn Martin