TREHALOSE TRANSGENIC LINES IN *ORYZA SATIVA* L. HAVE ALTERED CARBOHYDRATE PARTITIONING AND UTILIZATION IN RESPONSE TO WATER DEFICIT AND RECOVERY

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Tomohiro Kurai

August 2007

ABSTRACT

In plants, there is evidence that trehalose, a non-reducing disaccharide, has roles in water stress responses, but its functions in this response are incompletely understood. Previously, investigators created transgenic rice (*Oryza sativa*, L, cv. Pusa Basmanti) with tissue-specific and stress-dependent promoters that overexpress trehalose synthesis genes (Garg et al. 2002). The current study tested the hypothesis that these transformed genotypes have altered carbohydrate partitioning under water stress. In response to water deficit and recovery treatments, the time course of changes in transpiration rate and leaf abscisic acid concentration were not significantly different between transgenic and non-transgenic plants. Glucose content per g DW was not affected by water stress in leaf blades, upper and lower leaf sheaths in all genotypes except during recovery in the A-line where the stressed plants had lower glucose levels in leaf sheaths. However; sucrose contents per g DW were higher in water stressed transgenic lines compared to their well-watered controls, whereas after rewatering and recovery transgenics had slightly lower sucrose levels than well-watered controls. In contrast, the nontransformed line had minimal or no change in these constituents in response to stress and recovery. Starch levels were decreased 25 to 50% in the upper and lower sheaths of trehalose transformants, but were unaffected by stress in the non-transformed controls. Upon rewatering, starch levels remained at less than 65% of well-watered controls in the three organs of trehalose overexpressing lines, whereas starch levels in the non-transformed line were the same as in well-watered controls. These results are consistent with a role of trehalose in modulating sugar sensing and carbohydrate partitioning among sucrose and starch pools in response to water deficit.

BIOGRAPHICAL SKETCH

The author was born in Japan and received his Bachelor of Science degree in Environmental Biology from Queen's University, ON Canada in the year of 2002. In 2003, the author pursued his Master of Science degree at the Department of Crop and Soil Sciences, Cornell University under the guidance of Professor Timothy L. Setter, and completed his Master's research in Summer 2005.

ACKNOWLEDGMENTS

I would like to thank Professor Timothy L. Setter for accepting me as his graduate student. I also appreciate his guidance, support, understanding, and patience. His behavior, attitude, assistance, and knowledge were always unconditional, and understand my circumstance as an International Student more than anybody. I would also like to thank Professor Ray Wu for his comprehension and support as a special committee member. I also thank Ajay Garg for his assistance and providing me materials for experiments.

I would like to show my appreciation to the members of Setter's Lab and friends, especially Luis Duque, Kanishika De Silva, and Dr. Long-Xi Yu.

More than anyone else, my greatest appreciation is to my parents and family members for giving me such an opportunity to study at Cornell University. It was impossible to pursue a Masters Degree without their love and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Rice (*Oryza sativa* and related species) is the most widely consumed food in the world, and many people eat rice as the main component of their diet. Rice is grown world-wide; it is cultivated on approximately 13 billion ha of land area; in 2004 about 600 million tons of rice grain was produced worldwide, placing it first in terms of biomass and energy among the food crops (IRRI, 2006). There are three main genetic groups of rice, two subspecies of the *Oryza sativa* species, known as Japonica, Indica, and African Rice (*Oryza glaberrima*). Based on the way/location farmers grow the crop, these rice types are further divided into five categories; upland rice, semi-upland-rice, irrigated-rice, wet-land rice, and low-land rice. Amongst all, the upland rice and semi-upland rice are known to have higher drought tolerance.

Drought and subsequent desertification is one of the most severe environmental problems to be resolved. In fact, the arid-land area is more than 6 billion ha, which is more than 40% of earth's total land area (UNEP, 1991). Furthermore, desertification of land occurs at the rate of approximately 6 million ha/year (UNCOD, 1978). Although the world population expands at a high rate, this desertification decreases cultivable land area, which limits our ability to supply sufficient food to meet human needs. Abiotic stresses such as drought, freezing and salinity are known as major causes for loss in potential crop yield (Hare *et al.*, 1996). Therefore, there is a large potential to increase crop yield by eliminating or reducing the yield losses due to these stresses.

Abscisic acid (ABA) is a plant hormone which plays a central role in regulating both physiological and molecular responses in plants under abiotic stress (Zhang *et al.*,

2006). Under drought, ABA regulates numerous responses, including guard cell closing of stomata, expression of genes that produce substances that stabilize macromolecular structure and growth. When plants are exposed to water stress and plant tissues reach to zero turgor, ABA is synthesized in roots and shoots, and then functions as a signaling molecule (Chaerle *et al.*, 2005). One example of ABA's effect on stress response is that ABA induces sequential events such as nitric oxide synthesis, inositol-tri-phosphate synthesis (Rolland *et al.*, 2006), increase in calcium ion concentration, and ion channel alteration, which subsequently leads to guard-cell regulation and stomatal closure (Chaerle *et al.*, 2005; Desikan *et al.*, 2004; Raghavendra and Reddy, 2006, Schroeder *et al.*, 2001). ABA is also known to induce and suppress many genes involved in abiotic stress tolerance (Luan, 2002; Zhu, 2002). This ABA-mediated signal transduction pathway includes *AREB1/ABF2, AREB2/ABF4* (Choi *et al*., 2000; Uno *et al*., 2000), and *rd22* (Abe *et al.*, 1997). For instance, ABF3 and ABF4 overexpression in *Arabidopthis thaliana* resulted in ABA hypersensitivity, and the plants showed reduced transpiration and increased stress tolerance (Youn *et al.*, 2002). Similarily, ABF3 and ABF4 mutants exhibited defects in ABA and stress tolerance (SunMi *et al.*, 2004). Furthermore, ABA is also found to affect sugar status within plants. It is reported that ABI4 transcriptionally controls the expression of *APL3, RBCS* and *CAB* by binding the promoter regions, and consequently regulates starch biosynthesis and photosynthesis, respectively (Rook *et al.*, 2006).

Trehalose is the α,1-1-linked non reducing glucose disaccharide. In bacteria and yeast, it is synthesized in two steps from UDP-glucose and glucose-6-phosphate, catalyzed by trehalose phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). Trehalose can be found in species in many kingdoms from bacteria, fungi to plants (Elbein, 1974; Wingler, 2002). Recently, genes homologous with

bacterial genes for TPS and TPP were found in *Arabidopsis thaliana* and other plants, which suggests the existence of a similar trehalose biosynthesis pathway in plants as well (Goddijn and van Dun, 1999). This finding is further supported by the fact that plant TPS genes can complement Δtps1 mutant yeast (Zentella *et al.*, 1999).

Several potential functions of trehalose in abiotic stress tolerance have been reported. The most well known function of trehalose is as a compatible solute; that is, a highly soluble compound that does not interfere with metabolic functions even at a very high concentration. When plants or other organisms are subjected to water stress, one of the typical responses is the accumulation of sugars and compatible solutes such as mannitol, sorbitol, raffinose, proline and glycine betaine (Hare *et al.*, 1998). These solutes increase internal solute concentration, which increases cellular osmolarity and maintains turgor pressure (Bohnert and Jensen 1996; Ingram and Bartlet, 1996; Serrano 1996). The higher internal solute concentration is also helpful to allow plant water potential to decrease (without cellular injury) to enhance water uptake. In fact, yeasts and fungi are known to accumulate trehalose at very high concentration (up to 15-20% of dry mass) under abiotic stresses (Arguelles, 2000; Elbein, 1974). Contrary to this, the concentrations of trehalose in plants are very small (Goddijn *et al.*, 1997). Among plants, highly drought-tolerant "resurrection plants" whose vegetative organs can survive severe desiccation, such like *Selaginella lepidophylla*, accumulate large amounts of trehalose (up to 20% of dry mass) (Goddijin and Smeekens, 1998, Wingler, 2002). But in higher plants, trehalose is hardly detectable (Goddijn *et al.*, 1997) except in a few dessication–tolerant angiosperms such as *M. flabellifolius* which accumulates trehalose up to 3% of its dry weight (Goddijn and van Dun, 1999).

In addition to its potential role as an osmotically active solute *per se*, another

reported function of trehalose is to increase the capability of maintaining or stabilizing membrane and protein 3-dimentional integrity under abiotic stresses. Trehalose plays an important role in stabilizing proteins and membrane structure even in low concentrations in microorganisms (Colaco *et al.* 1992; Crowe *et al.*, 1984; Iwahashi *et al.* 1995; Jang *et al.* 2003; Lee *et al.*, 2003; Schellenbaum *et al.* 1998; Wiekman 1990). The way trehalose achieves this is that trehalose gives the macromolecular structures greater flexibility and chemical stability (Colaco *et al.*, 1995). Another opinion is that trehalose stabilizes intrinsic membrane proteins by maintaining membrane fluidity under water deficit (Lesile *et al.* 1995). Since it is shown that plant TPS and TPP are homologous to yeast equivalents, plant trehalose is expected to function similarly to yeast homologs as an osmolyte and/or signaling molecule regulating hexokinase (Pramanik and Imai, 2005). Interestingly, in tobacco, accumulation of trehalose into the chloroplast, where thylakoid membranes are at high density, was shown to increase dehydration tolerance more than accumulation in cytosol (Lee *et al.*, 2003).

In addition to their roles in metabolism, sugars sometimes function as signaling molecules. Recent research has identified signal transduction pathways by which gene expression is regulated by glucose and sucrose concentration (Rolland *et al.*, 2006). A similar signalling role has been proposed for trehalose. This thought has arisen from the fact that trehalose is wide-spread in all major taxonomic groups and its concentration is minute, yet it has a strong impact on growth and/or metabolism in plants. Evidence for the impact of small amounts of trehalose have been from studies where trehalose synthesis is transgenically manipulated to increase its accumulation, and when its synthesis is blocked due to mutation (Grennan, 2007; Penna, 2003). It is also based on the observation in yeast that TPS1 controls glycolysis by regulating hexokinase (Thevelein and Hohmann, 1995). This is further supported by Blazquez *et al.* (1993)

that T6P is an inhibitor of hexokinase, especially hexokinase II in *Sachharomyces cerevisiae*. Although neither trehalose, nor its precursor, trehalose-6-phosphate (T6P) regulate hexokinase in plants (Eastmond *et al.*, 2002), significant effects of them on plant growth, development and flowering have been reported (Avonce *et al.*, 2004; Schluepmann *et al.*, 2003; Schluepmann *et al.*, 2004; van Dijken *et al.*, 2004). In many cases the regulation by trehalose involves changes in carbohydrate contents by affecting expression of carbohydrate-pathway enzymes (Grennan, 2007). An example of this is that trehalose induces *APL3* gene, which encodes ADP-glucose pyrophosphorylase (AGPase), involved in starch biosynthesis, and increases subsequent AGPase activity (Wingler *et al.*, 2000).

There have been several studies focusing on altering trehalose concentrations to increase abiotic stress tolerance, such as some of the examples listed above (Penne, 2003). This type of research was carried out mainly on dicots such as *Arabidopsis* though monocots have shown better performance upon exogeneous trehalose treatment (Garcia *et al.*, 1997). Moreover, given that crop drought tolerance is important to world agriculture and rice is among the most consumed crops, it is important to determine the effects of trehalose on rice drought responses. Previously, Garg *et al.* (2002) reported the transformation of rice to transgenically overexpress trehalose- synthesizing genes as a TPSP fusion protein from bacterial TPS and TPP genes. These transgenic plants accumulate trehalose and have greater tolerance to drought and salinity. The objective of the present study was to determine whether the underlying bases of the trehalose effects on stress tolerance in three transgenic lines include effects on stomatal closure, ABA accumulation, or carbohydrate partitioning. These studies show that transgenic plants accumulate significantly more sucrose than corresponding well-watered controls while starch content of transgenic plants was substantially decreased in response to

water-stress treatment. In contrast, transpiration rate, ABA concentration and glucose amounts did not differ significantly between well-watered and water stressed plants on any of the tested genotypes. After a week of recovery, transgenic plants showed statistically lower content per g DW in both sucrose and starch. These results suggest that trehalose effects are involved in sugar metabolism and partitioning.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant material and growth conditions

Rice (*Oryza sativa*, L, cv. Pusa Basmanti (PB-1); Indica subspecies) was the parental genotype for the transformants used in this study. Seeds of trehalose-synthesis transformants were kindly provided by AJ Garg and Ray Wu who have described their DNA-construct and transformation previously (Garg *et al.* 2002). DNA constructs for transforming plants contained trehalose-synthesis genes, TPS and TPP, from *Escherichia coli* with expression driven by either the Rubisco small subunit promoter (R-line) or the abscisic acid response element complex (A-line). Three seeds from each genotype of the T_5 generation were sown into 3 liter pots with drain-holes (Poly-tainer #1, Nursery Supplies Inc., Chambersburg, PA, USA) containing Cornell Plant Breeding rooting medium (vermiculite:sphagnum peat moss [3:2 volume ratio], 4 g L^{-1} powdered dolomitic limestone and 3.2 g L^{-1} of Peter's Unimix Plus III 10-5-10 fertilizer [Scotts Company, Marysville, OH, USA]) and watered daily with a solution containing 0.4 g L^{-1} Peter's Excel 15-5-15 Cal-Mag (Scotts Company). The plants were grown in a glass house controlled at mean day/night temperatures of 25 /20°C. Ambient solar illumination was supplemented with 400W high pressure sodium lamps.

2.2 Treatments and statistical design

Six replicate batches of plants were used in this study, arranged as a randomized complete block design (batches representing blocks). For each batch of plants, control and water stress treatments were randomly assigned to 3 seedlings from

each genotype. There were 18 plants used in a batch; 9 plants were subjected to water-stress and 9 plants were well-watered. Amongst 9 plants of each treatment, there were 3 plants for each genotype, A05, R80, and NT. Plants at the age of 60 to 70 days from sowing were selected for uniformity and fully watered right before experiments were initiated. Pots of the well watered (WW) treatment were watered every two hours. Water stress (WS) treatments were imposed by withholding water supply for 7 days after stomatal closure followed by rewatering and 7 days of recovery. During the stress period, the pre-dawn leaf water potential was approximately -2.2 MPa, on average, as measured with the pressure chamber on leaf blades.

2.3 Transpiration assessment

Transpiration rates of all plants were measured gravimetrically. Four balances were connected to a computer and weights on each balance were monitored continuously and recorded to the computer every 2 minutes. Three pots subjected to the water-stress and one well-watered control pot were placed on the balances. The amounts of transpiration were calculated from the changes in the mass of water lost by each pot. Based on a preliminary experiment, the threshold lethal water content was determined as 64% of relative-pot-water-content by considering fully-saturated weights as 100%. These threshold weights were gravimetrically maintained by the automated computer-based watering system with which, on an hourly frequency, sufficient water was added to return each pot to its set-point. Based on the recorded data on the computer, whole day transpiration was calculated by the sum of the weight losses within a day.

2.4 Sample collection

After 10 days of water stress treatment, one plant out of three from each pot was harvested by cutting stems at the soil surface level. The remaining two plants were re-watered and subjected to 7 days of recovery. Another plant from each pot was harvested after the recovery period. The third plant of each pot was used for the daily leaf sample collection for ABA analysis. Each harvested plant was immediately separated into leaf blades, upper sheath and lower sheath. In this experiment, the lower sheath is defined as 8cm from the bottom of the stem and it includes sheaths and stems. The remaining sheath was classified as the upper sheath. The separated plants were dried in an 80ºC oven, weighed, and ground with a Udy Mill to a fine powder. Fifty mg dry weight of the ground material was assayed for sugars and starch content. Besides, 5 leaf disks (diameter = 0.635 cm each) of fully expanded leaf were sampled for ABA measurement beginning the first day of water withholding until 4 days after recovery period for all treatments and genotypes. Sampled leaf disks were immediately immersed in 300 µL of cold (0ºC) 80% methanol, and samples were stored with solvent at -20ºC. ABA concentration was calculated on an area basis.

2.5 Abscisic acid extraction and analysis

For ABA extraction, leaf disks were homogenized in 300 µL of 80% methanol (v/v) and incubated at least for 24 h at 4°C. Supernatants were removed to 96-well plates, and then air-dried. ABA was separated with C18 reverse-phase chromatography using a modification of the method of Setter *et al.* (2001). The C18 columns in 96 wells (model: DSC- 18, 25 mg packing material, Supelco, Belleionte, PA, USA) were initially wetted with 400 µL of 95% (v/v) ethanol:water per well, followed by equilibration to

initial conditions with 600 µL of 30% (v/v) acidified methanol solution (30% methanol, 69% distilled water, 1% glacial acetic acid). Extracts were reconstituted in 100 µL of 30% acidified methanol solution and 8 µg bromecresol green was added as a chromatograph tracer. Extracts were loaded and solvents were drawn through the columns under vacuum to provide flow rates of about 50 µL/min or less. Columns were washed with 280 µL of 30% acidified methanol solution to remove additional hydrophilic compounds from samples and ABA was eluted with 200 µL of 65% methanol plus 1% glacial acetic acid. ABA fractions were dried at room temperature and stored at -20 °C. Samples were re-dissolved in 100 µL distilled water and 10 µL aliquots were analyzed by enzyme-linked immunosorbant assay (ELISA) for ABA according to the procedure of Setter *et al*. (2001).

2.6 Sugar assay

Glucose and sucrose content per g DW were determined in reconstituted 80% methanol extracts by a peroxidase/glucose oxidase method (PGO) (Setter *et al.*, 2001) with modifications. Glucose content was determined by adding 180 µL PGO reagent, and after reaction was completed by 1 hour of incubation at room temperature, absorbance at 490 nm was read using a plate reader (Cambridge 750). PGO reagent (constituents purchased from Sigma Chemical Company, St. Louis, MO, USA) consisted of 22 mM p-aminohydroxybenzoic acid, 0.5 mM 4-aminoantipyrene, 7.3 IU per mL glucose oxidase type V, 2 IU per mL peroxidase type VI, in 50 mM KH_2PO_4 [pH 7.0]. Sucrose was hydrolyzed to glucose and fructose by adding invertase solution to the sample aliquots. The invertase solution consists of 825 IU per mL invertase, grade VII, in 45 mM acetate buffer [pH 4.6]. The mix was incubated at 35 °C for 1 h, and its glucose content was measured by the same plate reader (Cambridge 750) at 490 nm.

The sucrose content was determined by subtracting the amount of free glucose from the total glucose in the hydrolyzed samples, as described above. For starch determination, leaf disk debris after removal of extracts was autoclaved in 200 μL H_2O at 121 °C for 20 minutes. After cooling, starch was completely hydrolyzed to glucose with α-amylase and amyloglucosidase (#208469, Gibco BRL) as described by Ober *et al.* (1991) and Setter *et al.* (2001). Aliquots were assayed for glucose by PGO based analysis using the plate reader at 490 nm.

2.7 Statistical analysis

Analysis of variance was conducted to compare plants under WW and WS treatments as well as between genotypes of the same treatment using statistical software SPSS ver.13.0 (SPSS Inc.). Using the procedure given from SPSS software called "one-way ANOVA repeated measure", variance due to batches was removed from the error term and precision for detecting differences due to genotype and watering treatment was improved for all data on glucose, sucrose and starch content between genotypes and treatments.

CHAPTER THREE

RESULTS

3.1 Transpiration rate

A potential mechanism by which the overexpression of genes for trehalose biosynthesis could affect tolerance of water deficit is by causing stomatal closure. If stomata are closed earlier or more completely during a water deficit episode, soil water could be depleted slower and plant tissues could avoid becoming exposed to low water potentials that cause cellular damage. To test this possibility, irrigation water was withheld from potted plants, while changes in transpiration rate were measured by monitoring plant and pot system weight with computer-interfaced balances. At the beginning all plants had the same transpiration rate. The transpiration rate gradually decreased as the water was depleted from soil of the drought stress treatment (Fig. 1). The rate of drop in transpiration as well as the timing of stomatal closure were not significantly different between genotypes. In water stressed plants, after they reached the point when stomata were fully closed (Day 0), they showed almost no transpiration, whereas control plants in all genotypes maintained transpiration at high levels. There were no significant differences between genotypes under the same treatment while water stress was imposed. When water was re-supplied after 7 days of stress treatment, both transgenic and non-transgenic plants subjected to water stress gradually started to re-transpire similarly. After two days of recovery, the transpiration rates recovered close to the ones at the beginning. No significant differences were observed on the rates of recovery between genotypes as well.

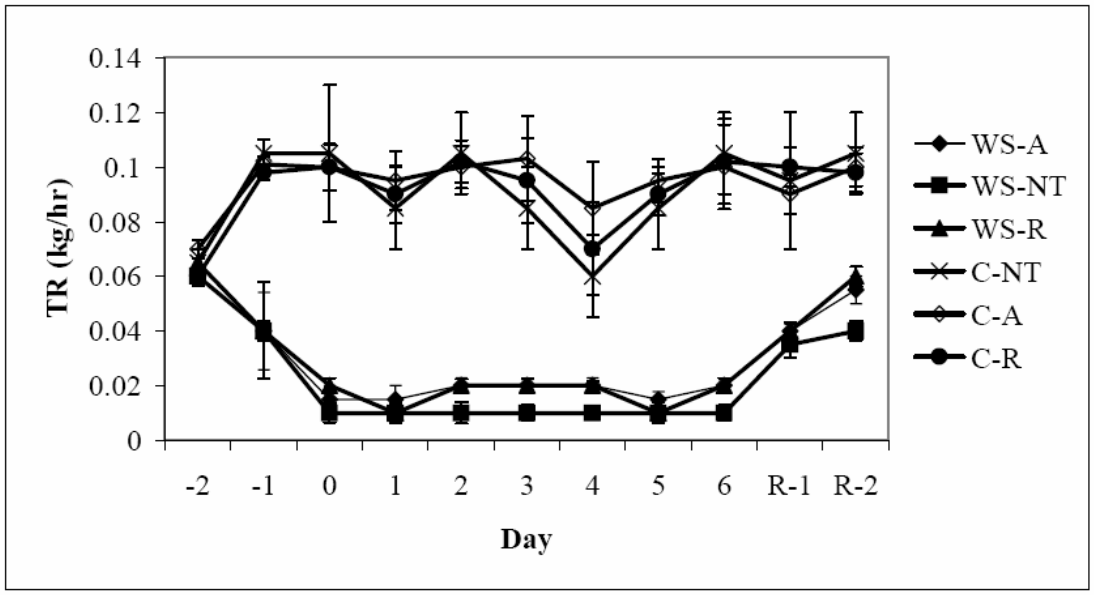


Figure 1. Comparisons of changes in transpiration rates (kg /hour) between three genotypes (A=A05, NT=Wild Type, R=R80) and two treatments (WS=Water Stress, C=Well-Watered Control). Day -2 indicates the first day of experiment when plants were fully saturated and the water stress imposition was begun. Day 0 indicates when the stomata were fully closed. Day R -1 and R -2 indicate the number of days after plants are under recovery period. Bars indicate standard errors of mean (SEM), and each points represent combined data of six replicate blocks.

3.2 Abscisic acid concentrations

Abscisic acid (ABA) concentrations at different time points including before and after stress treatment were measured and compared. All the water-stressed plants exhibited a sudden increase in leaf ABA concentration at Day 0, coinciding with the time when stomata first closed. In the following days of water stress, leaf ABA in all genotypes showed a gradual decrease in ABA concentrations through the stress period.

The ABA concentrations returned to the same level as control plants upon recovery. There were no significant differences in ABA concentrations before the stress imposition and after the recovery period between the 3 genotypes. There was also no obvious difference observed between genotypes in the time course over which ABA concentrations were changed.

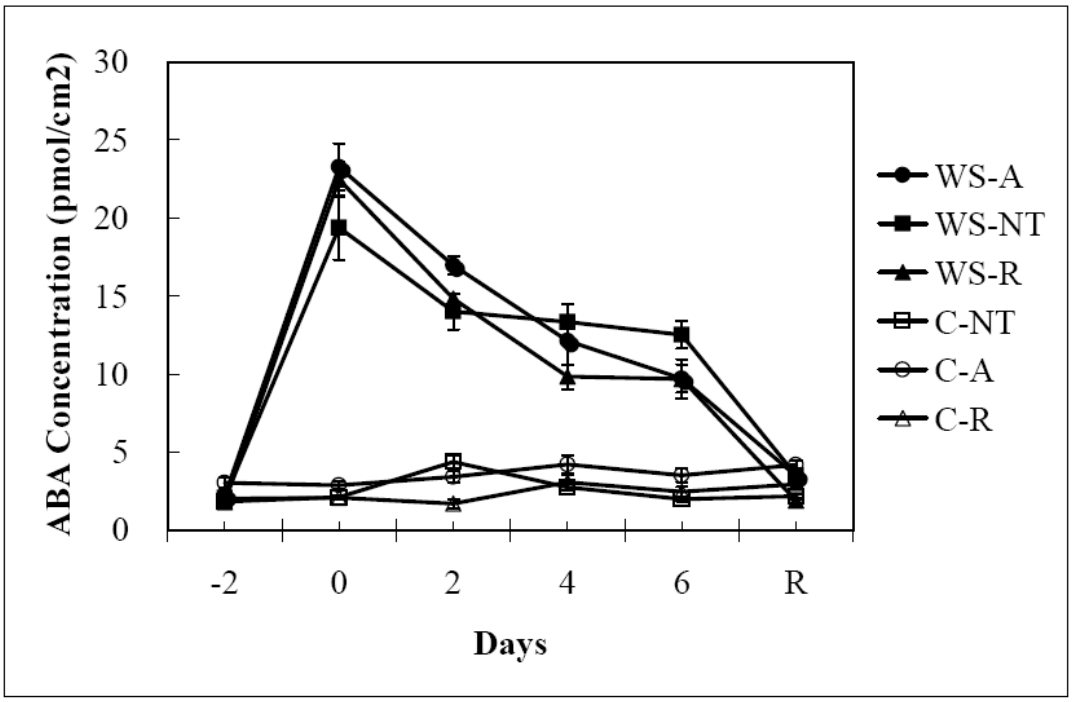


Figure 2. Comparisons of changes in ABA concentrations (pmol / cm^2) in leaf between three genotypes (A=A05, NT=Wild Type, R=R80) and two treatments (WS=Water Stress, C=Well-Watered Control). Day -2 shows when plants were fully saturated and the water stress imposition began. Day 0 indicates when the stomata first fully closed. Day R indicates 2 days after watering was resumed. Bars indicate SEM, and each point represents combined data of six blocks.

3.3 Glucose content

The concentration of sugars can reflect the metabolic and carbohydrate status of a tissue, which could become limiting during stress due to the accompanying decrease of photosynthesis as a result of stomatal closure. Sugars also serve as osmotica, which is an important role for the maintenance of cell turgor, volume, and stability during water deficit.

Under the water-stress treatment, glucose content per g DW in leaf blades were significantly ($P<0.05$) decreased by water stress in the NT control line, whereas they were not decreased in the A-line and R-line overexpression transformants (Fig.3 A - C). The content per g DW of glucose were not significantly affected by water stress in upper and lower leaf sheaths in any of the genotypes (Fig.3 D - I).

After rewatering and 7 days of recovery, glucose content per g DW in leaf blades and sheaths of the R-line were not significantly affected (Fig.3 C, F, and I), whereas it was significantly ($P<0.05$) lowered in upper and lower sheaths in the A transformant (Fig.3 E and H), and lowered in leaf blades of the NT control (Fig.3 A).

Figure 3. Comparisons of glucose content per g DW in leaf blades and leaf sheaths of three genotypes (A=A05 transformed, NT=non-transformed, R=R80 transformed) between two treatments (WS=Water Stress, C=Well-Watered Control) in the following tissues and genotypes; A) Leaf blades of NT, B) Leaf blades of A-line, C) Leaf blades of R-line, D) Upper sheath of NT, E) Upper sheath of A-line, F) Upper sheath of R-line G) Lower sheath of NT, H) Lower sheath of A-line, I) Lower sheath of R-line. Bars indicate pooled SEM, and each average represents combined data of six blocks. Asterisks indicate significant difference compared to the well-watered control of the same genotypes at $P<0.05$ level.

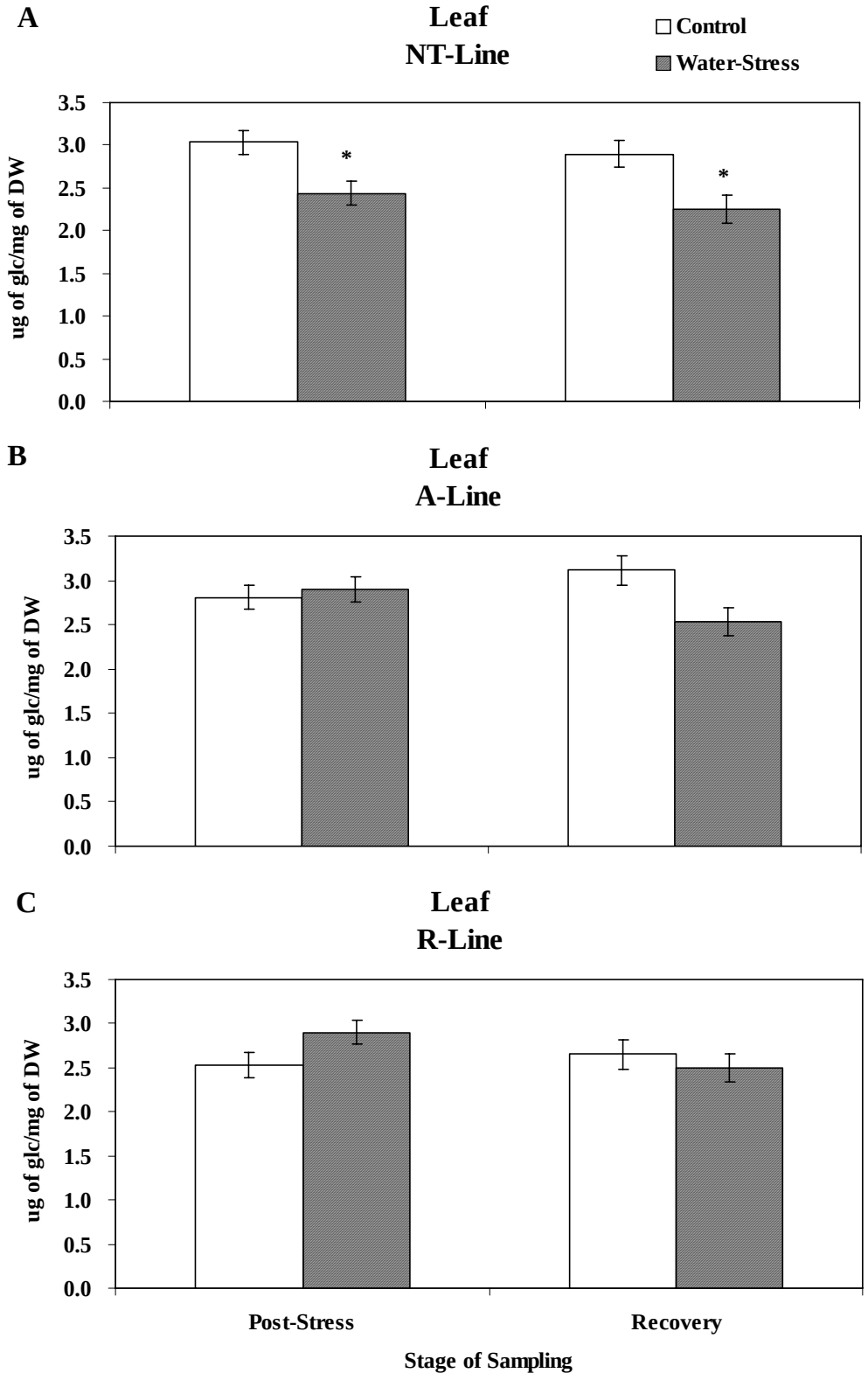

A
Leaf
NT-Line
Control
Water-Stress
ug of glc/mg of DW
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
B
Leaf
A-Line
ug of glc/mg of DW
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C
Leaf
R-Line
ug of glc/mg of DW
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Post-Stress
Recovery
Stage of Sampling

Figure 3. (Continued)

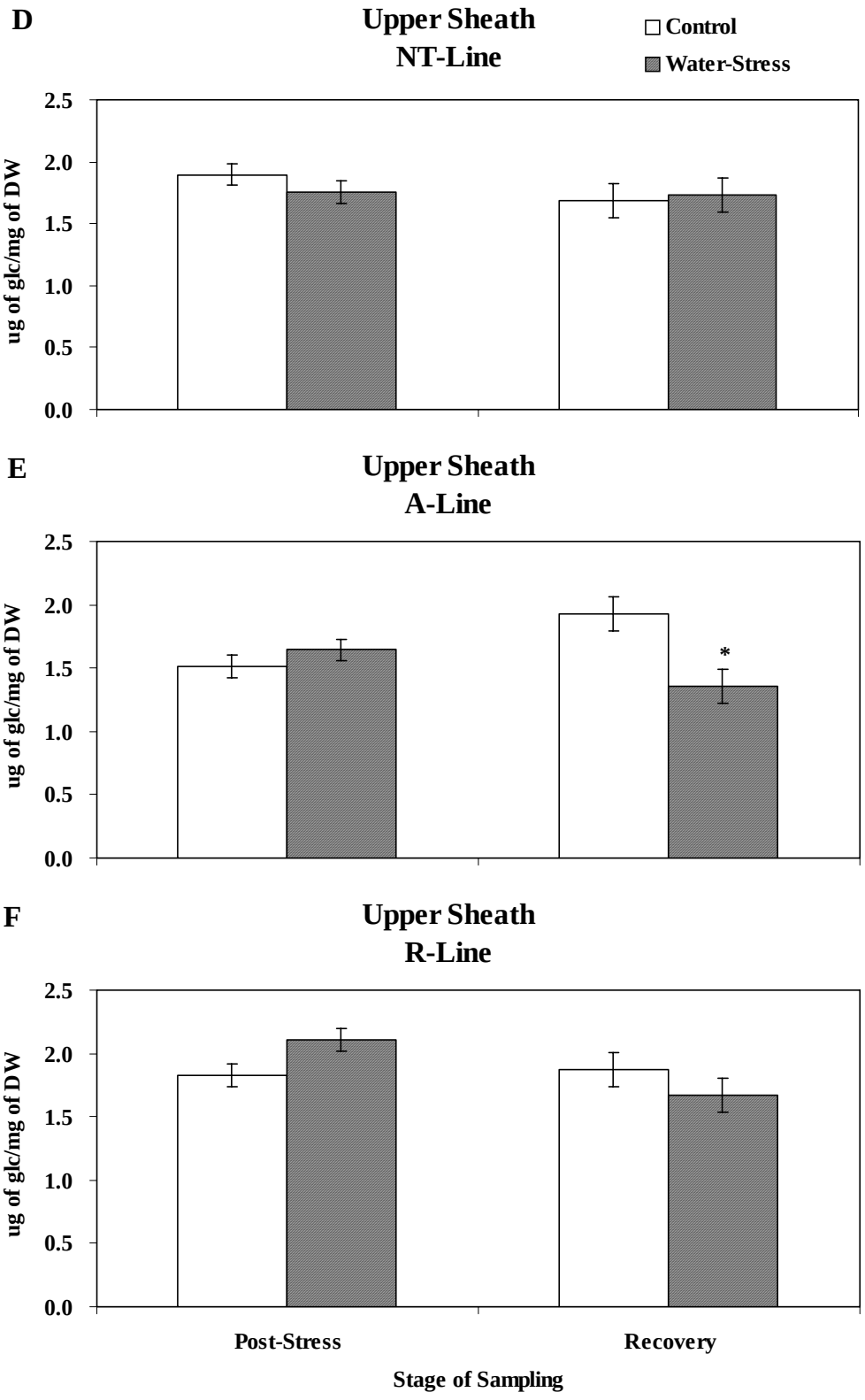
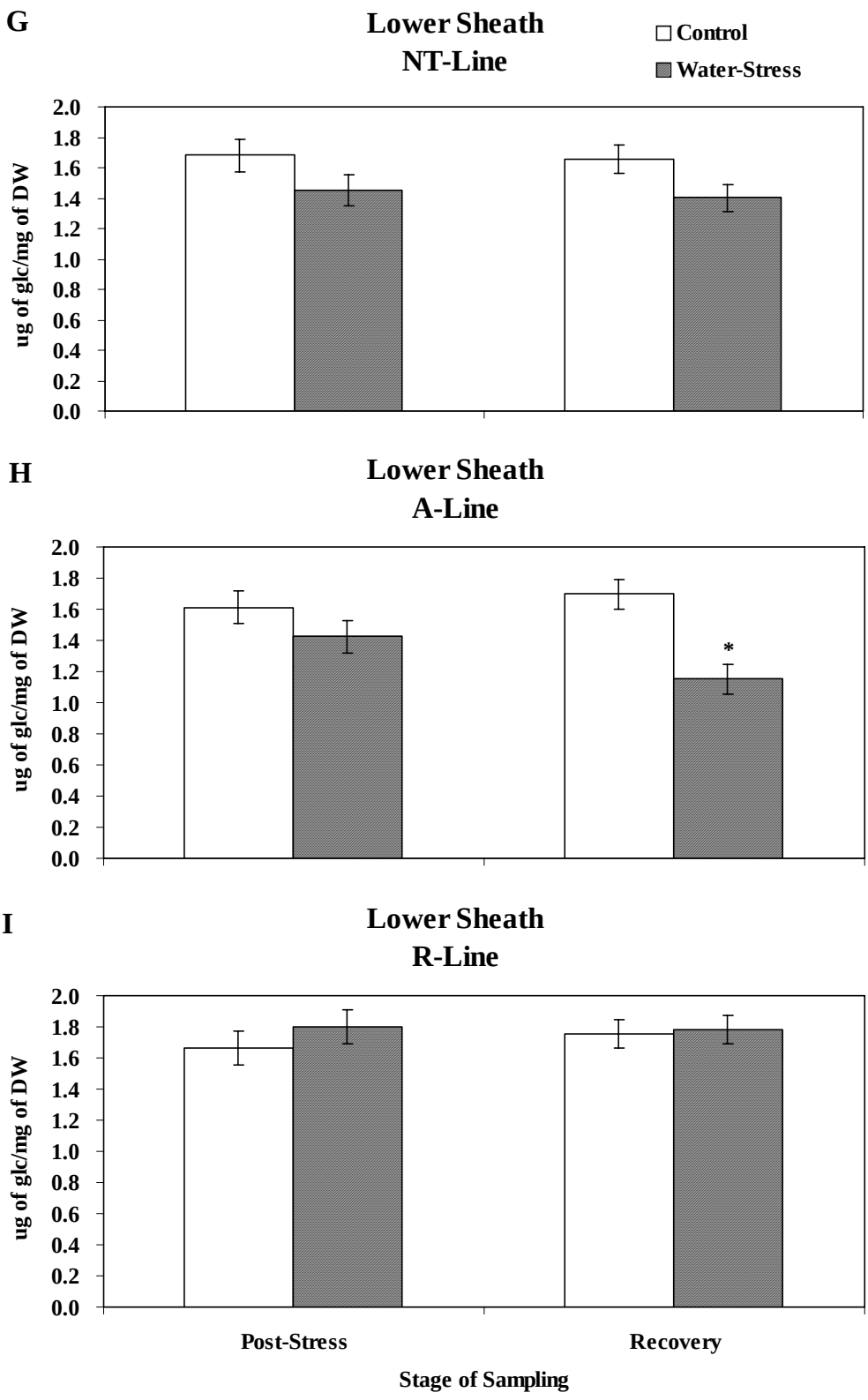


Figure 3. (Continued)



3.3 Sucrose content

Sucrose concentrations were measured to see metabolic and carbohydrate status within plants, which could be limited due to significant decrease in photosynthesis during water deficit. It also indicates osmotic adjustments of plants since sucrose is one of the most important sugars serving as an osmolyte to maintain cell turgor and stability under water stress.

Under water-stress treatment, sucrose content per g DW of both A- line and R-line were significantly ($P<0.05$) greater than corresponding well-watered controls in all of three tissues (leaf blades, upper sheaths and lower sheaths) of each genotype (Fig.4 B, C, E, F, H, and I). Water stressed NT plants also showed significant increases in sucrose level in leaf blades and lower sheaths, but to a lesser degree, and no difference was observed in upper sheaths (Fig.4 A, D, and G).

During the developmental period between the conclusion of stress to the sampling 7 days after rewatering, well-watered plants of the A- and R-lines increased their sucrose content per g DW in upper and lower sheath organs. However, sucrose contents per g DW in water stressed plants of R- and A-lines did not increase further during this period and after recovery were significantly ($P<0.05$) less than corresponding well-watered controls in all three tissues (leaf blades, upper sheaths and lower sheaths) (Fig.4 C, F, and I; Fig.4 B, E, and H). On the other hand, sucrose content per g DW in leaf blades and upper sheaths of water stressed NT plants remained the same as well-watered controls (Fig.4 A and D), or even showed significant increase ($P<0.05$) in the sucrose content per g DW in the lower sheaths (Fig.4 G).

Figure 4. Comparisons of sucrose content per g DW in blades and leaf sheath of three genotypes (A=A05 transformed, NT=non-transformed, R=R80 transformed) between two treatments (WS=Water Stress, C=Well-Watered Control) in the following tissues and genotypes; A) Leaf blades of NT, B) Leaf blades of A-line, C) Leaf blades of R-line, D) Upper sheath of NT, E) Upper sheath of A-line, F) Upper sheath of R-line G) Lower sheath of NT, H) Lower sheath of A-line, I) Lower sheath of R-line. Bars indicate pooled SEM, and each data represents combined data of six blocks. Asterisks indicate significant difference compared to the well-watered control of the same genotypes at $P<0.05$ level.

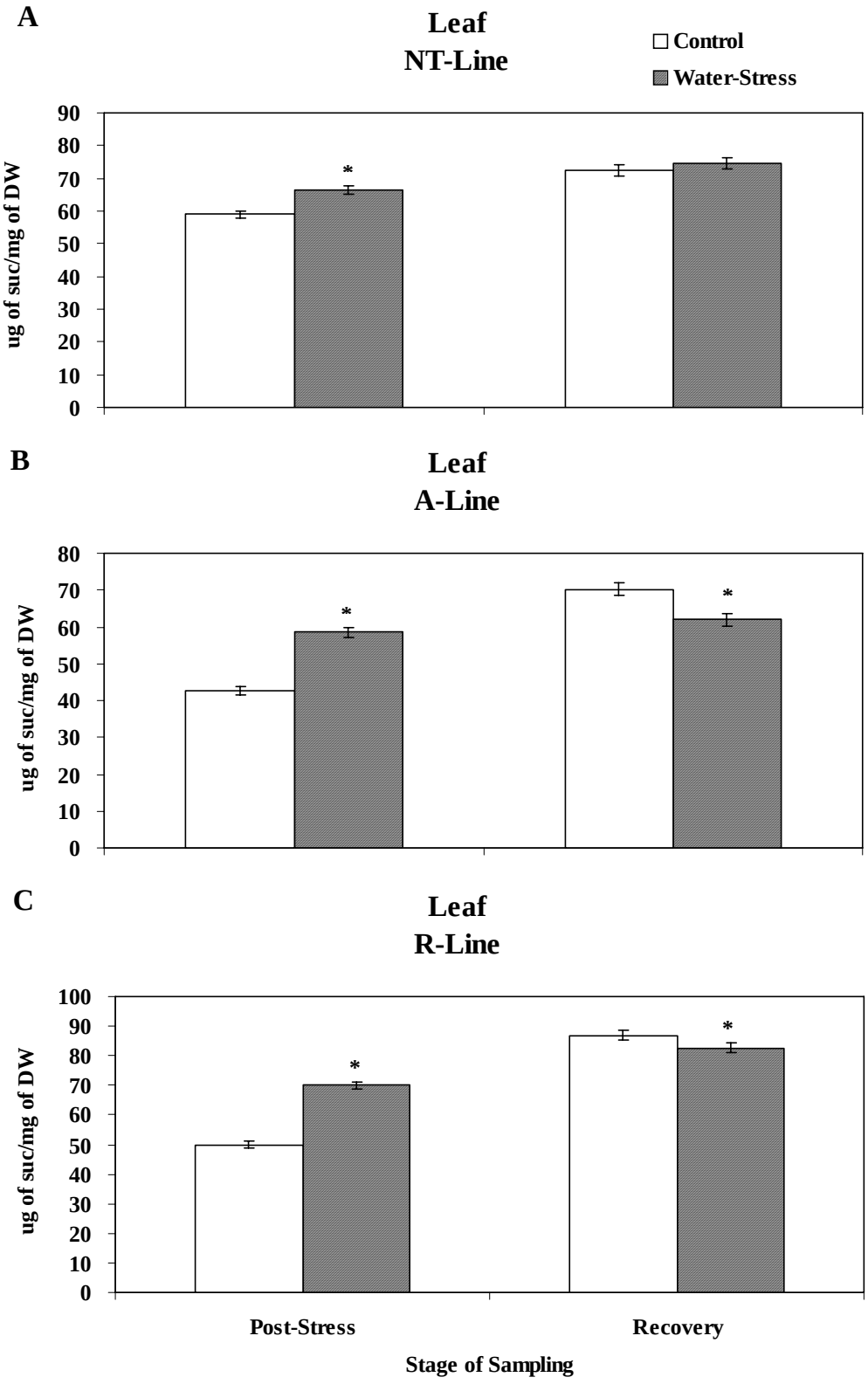

A
Leaf
NT-Line
Control
Water-Stress
ug of suc/mg of DW
90
80
70
60
50
40
30
20
10
0
*
B
Leaf
A-Line
ug of suc/mg of DW
80
70
60
50
40
30
20
10
0
*
*
C
Leaf
R-Line
ug of suc/mg of DW
100
90
80
70
60
50
40
30
20
10
0
*
*
Post-Stress
Recovery
Stage of Sampling

Figure 4. (Continued)

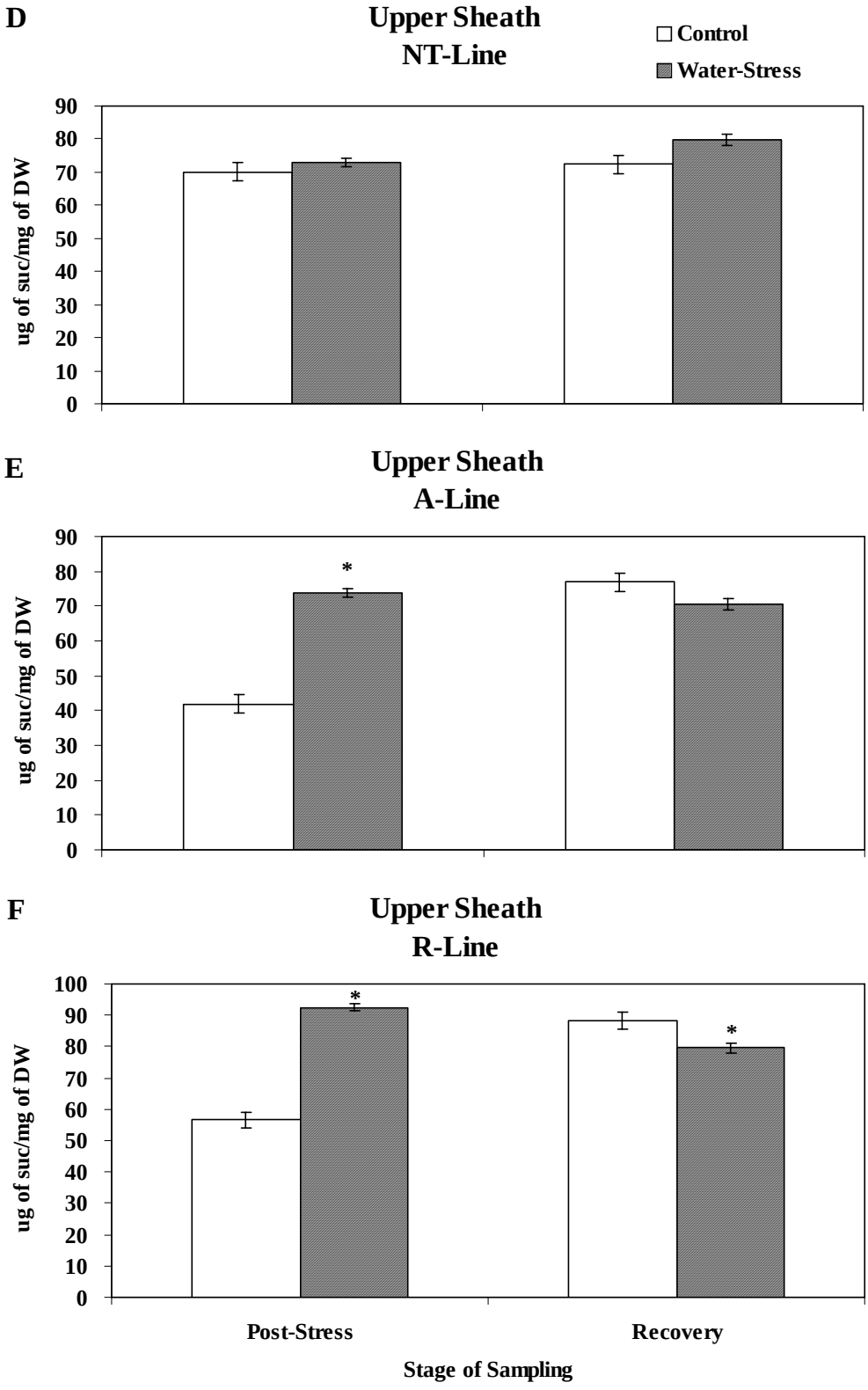
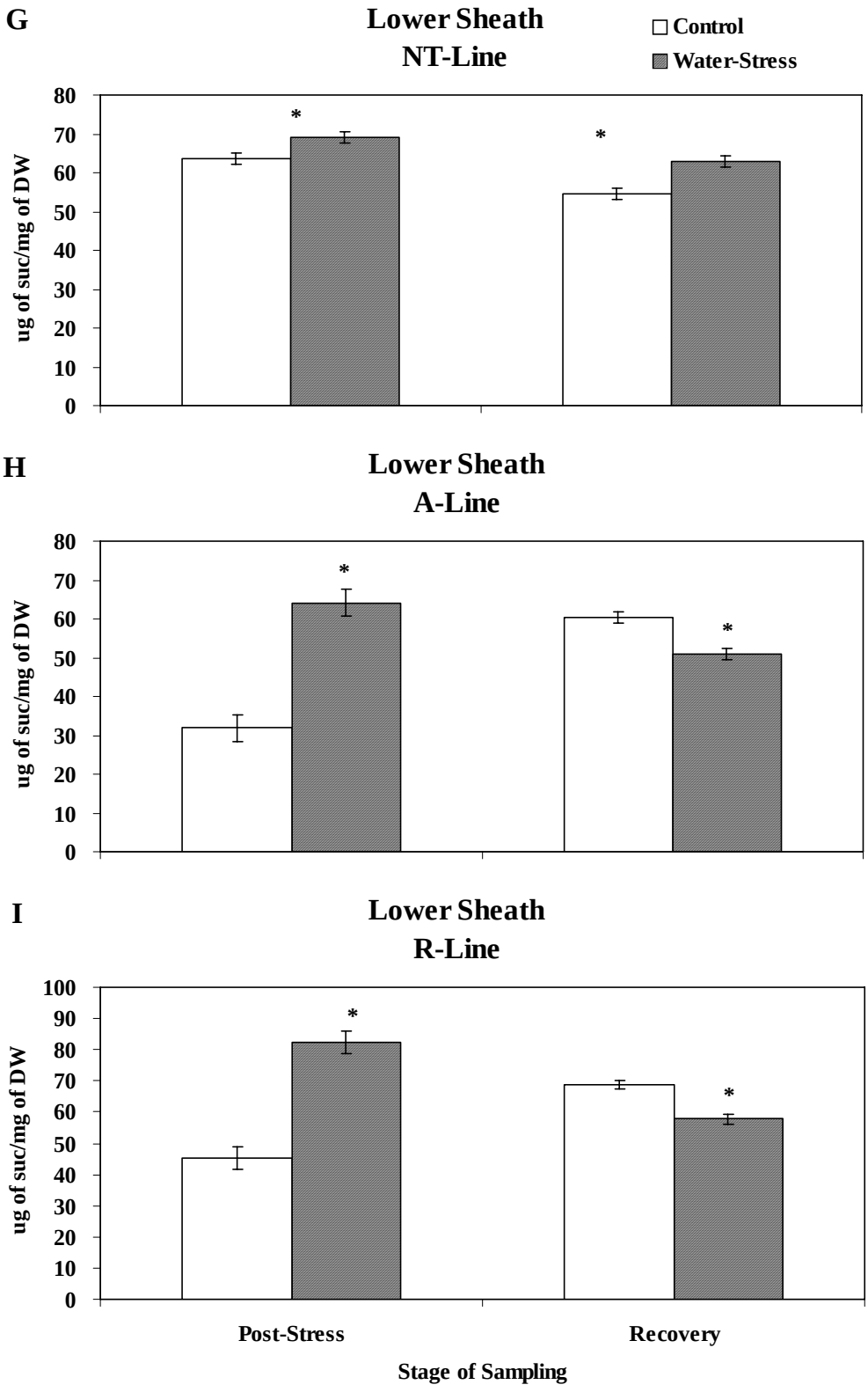


Figure 4. (Continued)



3.3 Starch content

Starch levels were measured as a potential indicator in leaf blades of the balance between starch synthesis and phloem export of sugar. In leaf sheaths, starch concentration is a possible indicator of the extent to which a plant utilized starch to supply carbon for respiratory and maintenance needs during photosynthate scarcity.

In leaf blades, starch content per g DW remained almost the same level in all of the three genotypes, NT control, R-line, and A-line (Fig.5 A, B, and C). Contrary to this, in upper and lower leaf sheaths, the transgenic A-line showed about 35% and 25%, and R-line, showed about a 50% and 40% reduction in starch content per g DW respectively in response to water stress (Fig. 5 E, F, H, and I), whereas starch content per g DW was relatively unaffected by water stress in the NT control (Fig.5 D and G). After a week of recovery, starch content per g DW remained unaffected in all three tissues of the NT line (Fig.5 A, D, and G); however, in the A and R transformed plants starch content per g DW remained low, or declined further (<50%) in all three tissues (Fig.5 B, C, E, F, H, and I).

The three tissues examined differed considerably in the range of starch they accumulated. Whereas leaf blades had only about 3 to 4 μg/mg DW (well-watered controls), upper leaf sheaths had about 60 to 85 μg/mg DW (well-watered controls), and lower leaf sheaths had about 160 to 195 μg/mg DW (well-watered controls).

Figure 5. Comparisons of starch content per g DW in blades and leaf sheath of three genotypes (A=A05 transformed, NT=non-transformed, R=R80 transformed) between two treatments (WS=Water Stress, C=Well-Watered Control) in the following tissues and genotypes; A) Leaf blades of NT, B) Leaf blades of A-line, C) Leaf blades of R-line, D) Upper sheath of NT, E) Upper sheath of A-line, F) Upper sheath of R-line G) Lower sheath of NT, H) Lower sheath of A-line, I) Lower sheath of R-line. Bars indicate pooled SEM, and each data represents combined data of six blocks. Asterisks indicate significant difference compared to the well-watered control of the same genotypes at $P<0.05$ level.

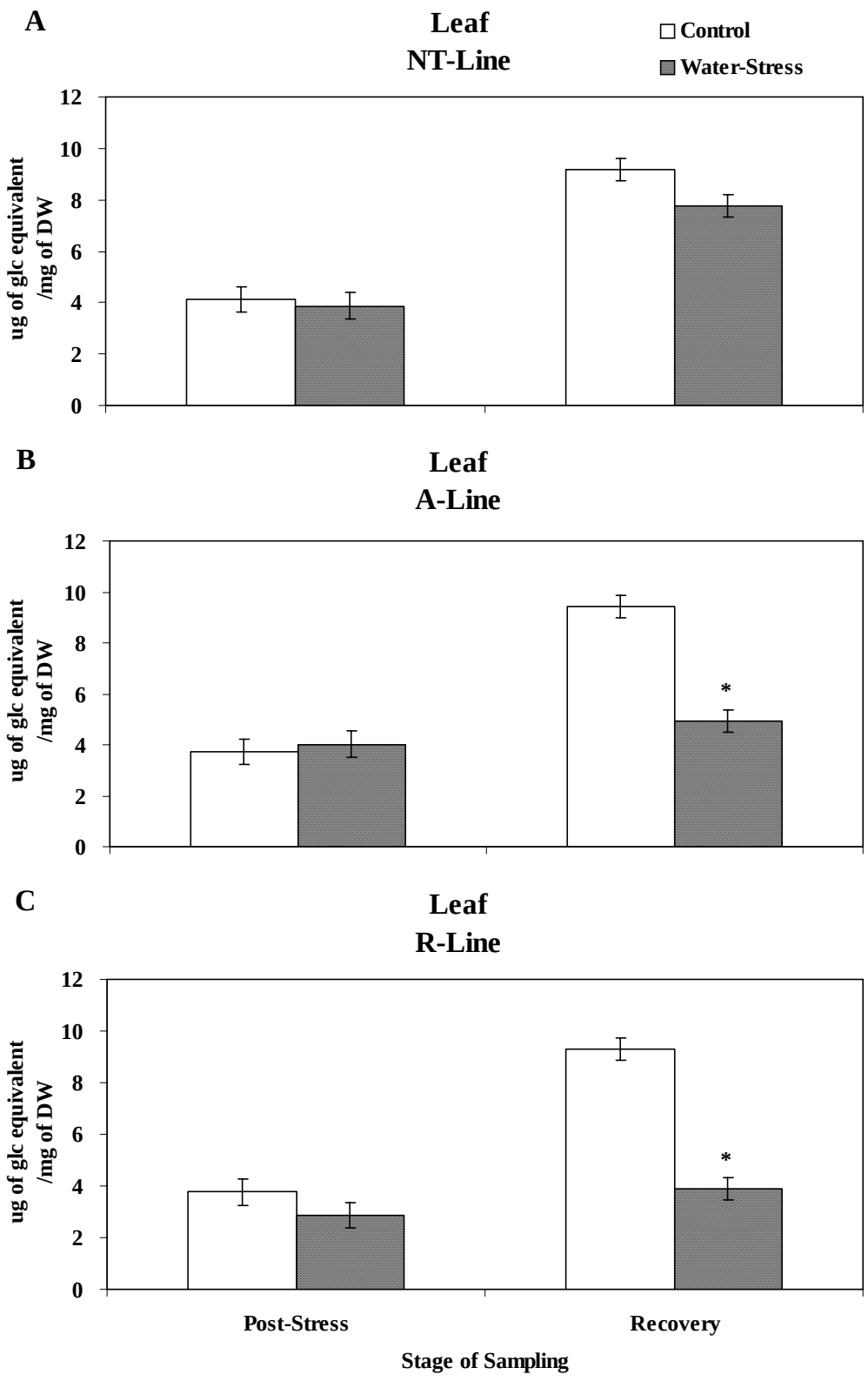

A
Leaf
NT-Line
Control
Water-Stress
ug of glc equivalent /mg of DW
12
10
8
6
4
2
0
B
Leaf
A-Line
ug of glc equivalent /mg of DW
12
10
8
6
4
2
0
*
C
Leaf
R-Line
ug of glc equivalent /mg of DW
12
10
8
6
4
2
0
*
Post-Stress
Recovery
Stage of Sampling

Figure 5. (Continued)

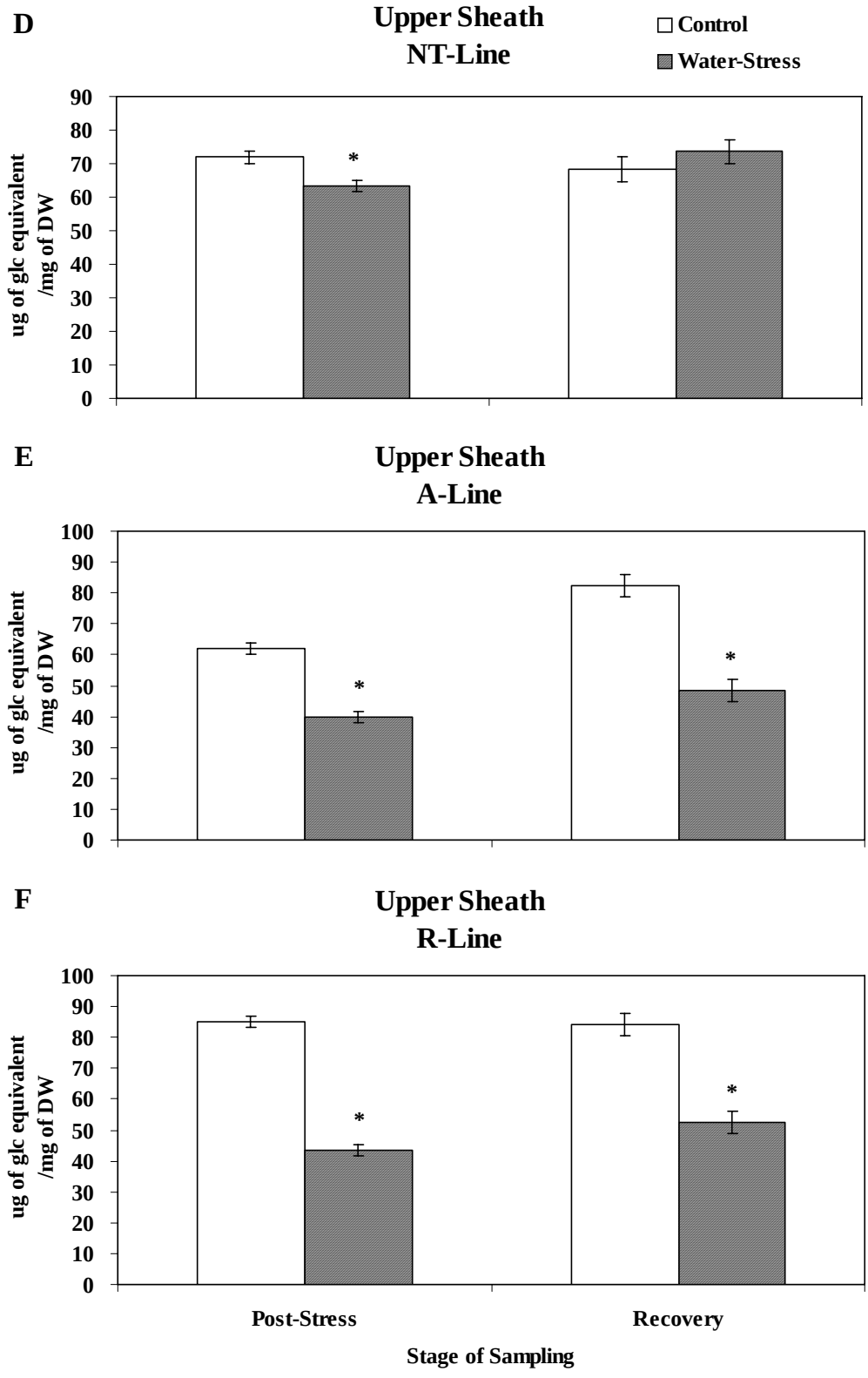


Figure 5. (Continued)

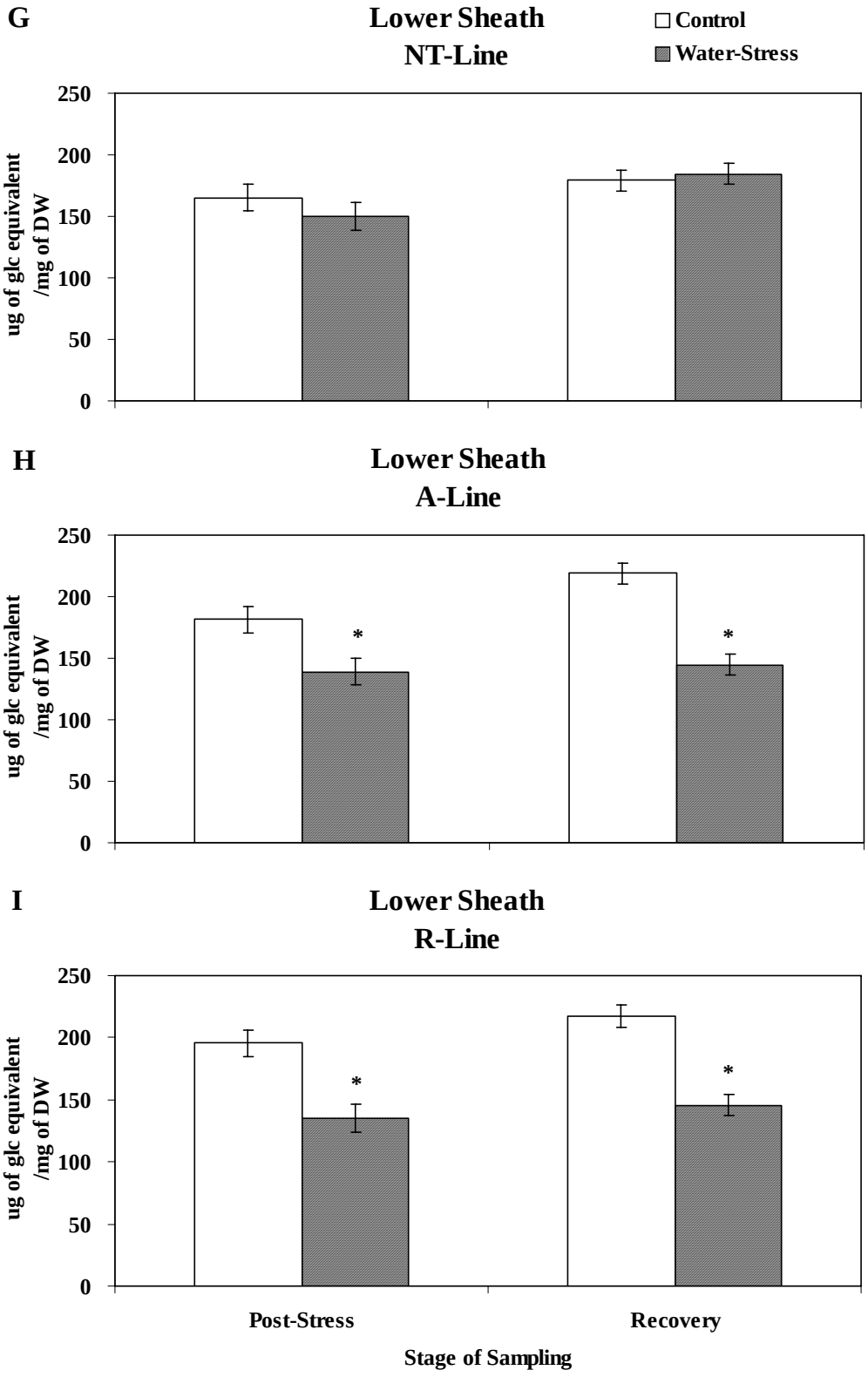


Table 1. A) Comparisons of sucrose:starch ratio after the stress period in leaf blades, upper and lower sheaths in three genotypes (NT=non-transformed, A=A05 transformed, R=R80 transformed) and two treatments (WS=Water Stress, C=Well-Watered Control) after 7 days of water-stress treatment. B) Relative sucrose:starch ratio by dividing sucrose:starch ratio in water-stressed plants by sucrose:starch ratio in well-watered control plants.

A

Treatment/Genotype	Leaf Blades	Upper Sheath	Lower Sheath
WS-NT	17.15	1.36	0.46
C-NT	14.31	0.97	0.32
WS-A	13.54	1.85	0.46
C-A	11.41	0.68	0.18
WS-R	24.34	2.13	0.61
C-R	13.25	0.67	0.23

B

WS-NT/C-NT	1.20	1.40	1.42
WS-A/C-A	1.19	2.74	2.62
WS-R/C-R	1.84	3.21	2.64

Table 2. A) Comparisons of sucrose:starch ratio after the recovery period in leaf blades, upper and lower sheaths in three genotypes (NT=non-transformed, A=A05 transformed, R=R80 transformed) and two treatments (WS=Water Stress, C=Well-Watered Control) after 7 days of recovery period. B) Relative sucrose:starch ratio by dividing sucrose:starch ratio in WS-recovery plants by sucrose:starch ratio in C-recovery plants.

A

Treatment/Genotype	Leaf Blades	Upper Sheath	Lower Sheath
WS-NT	9.62	1.08	0.34
C-NT	7.89	1.06	0.30
WS-A	12.58	1.45	0.35
C-A	7.44	0.93	0.28
WS-R	16.09	1.32	0.40
C-R	9.36	1.05	0.32

B

WS-NT/C-NT	1.22	1.02	1.13
WS-A/C-A	1.69	1.56	1.28
WS-R/C-R	1.72	1.26	1.25

CHAPTER FOUR

DISCUSSION

4.1 Transpiration and Stomatal Closure

Stomatal opening and closing is tightly regulated in response to several environmental factors such as light, photosynthetic rate, atmospheric humidity, and leaf water status (Outlaw, 2003). Rice is very sensitive to water status and it physiologically responds to slight decreases in water potential by closing its stomata and rolling its leaves. In this experiment, transpiration rates were calculated from weight loss monitored and logged every two minutes and displayed as graphs (Fig. 1). Since cuticular conductance to water vapor diffusion is quite low, when the stomata close, transpiration shuts down nearly completely. The stomata closure point was determined as the time transpiration rate goes to nearly zero. Since transpiration rate was determined continuously every 2 minutes from whole plant weight loss, it represents a robust time- and plant-averaged estimate of stomata. In fact, identification of the stomatal closure point was easy and obvious on the computer monitor since the changes in transpiration rates were drastic on the graphs.

The phytohormone ABA plays a pivotal role in stomatal responses to environmental factors, particularly those involving tissue water deficit (Marten *et al.*, 2007). In the present study, I examined the differences in response to water deficit in rice transformants that overexpress genes for trehalose under the control of either a Rubisco small-subunit (cytoplasmic) promoter (R-line) or an ABA response promoter complex (A-line). One of the hypotheses of trehalose function was that it exerts effects via control of stomata, to slow the rate of water depletion from the soil and avoid low water potentials that damage tissues. In line with this hypothesis, previous studies have

shown that the trehalose-synthesizing enzyme, OsTPS1, interacts with ABA in the response of drought, salt and cold stress in rice (Kim *et al.,* 2005).

In the current study, however, transgenic and NT lines did not differ in transpiration and stomatal behavior (Fig. 1). No significant differences were found between transgenic and NT plants in transpiration rate, timing of stomatal closure, and pot water content at the point of stomatal closure. This finding suggests that trehalose is not involved in regulation of stomatal closure and subsequent transpiration, and the transgenic plants did not acquire abiotic stress tolerance by regulating stomata and subsequent transpiration.

4.2 ABA concentrations

ABA is widely studied in plant stress physiology since it is one of the key molecules regulating a wide range of stress responses (Luan, 2002; Zhu, 2002). This includes stomatal closure (Schroeder *et al.*, 2001), increase in the expression of LEA (Late Embryogenesis-Abundant) genes, which encode osmoprotectant LEA proteins (Wang *et al.*, 2007), osmolyte accumulation such as proline (Abraham *et al.,* 2003), and plant growth and development such like shoot and root elongation (Sharp *et al.*, 2000; Spollen *et al.*, 2000) Regarding the relationship between trehalose and ABA signaling, Avonce *et al.* (2004) reported that trehalose up-regulates the ABI4 gene. Ramon *et al.* (2007) also showed that exogeneous trehalose regulates ABI4 expression in *Arabidopsis*. ABI4 (ABA INSENSITIVE 4) in *Arabidopsis* encodes an AP2-type transcription factor that when mutated blocks ABA signaling for numerous stress responses, including osmotic stress (Arroyo *et al.*, 2003). Also, feedback upregulation of ABA synthesis is known to involve such ABA signaling pathways (Xiong et al. 2003). Therefore, I hypothesized that trehalose overexpressing plants might increase their ABA synthesis

during stress, and by this effect, boost ABA signaling and their level of stress tolerance.

However; in this experiment, no significant differences were observed in ABA concentrations between transgenic and non-transgenic controls at any time point throughout the study (Fig. 2). This indicates that trehalose signaling was not involved in regulating ABA synthesis or turnover. Although there are no previously published studies that have examined trehalose effects on ABA synthesis or turnover, there are several studies showing ABA-mediated trehalose accumulation. Kim *et al.* (2005) showed that in rice, *OsTPS1* is induced by ABA, which leads to subsequent accumulation of T6P and trehalose. Furthermore, *OsTPP1*, whose gene-product catalyzes the second step in trehalose biosynthesis, is also suggested to be regulated by ABA (Pramanik *et al.*, 2005). These findings suggest that trehalose is involved in ABA signaling cascades, and located downstream of ABA. The current observation that ABA concentration did not differ in transgenic lines with altered trehalose synthesis is therefore consistent with this. At the same time, the results of this experiment suggest that trehalose is not involved in feedback regulation of ABA signaling pathway.

4.3 Glucose content

In Arabidopsis, ABI4 is known to be involved in the hexokinase1 (HXK1) signaling pathway, and ABI4 and trehalose are located downstream of ABA as discussed above (Rolland *et al.*, 2006). Furthermore, trehalose is also known to regulate hexokinase in yeast (Mulet *et al.,* 2004). Though some studies have found that trehalose does not regulate HXK1 in plants (Gonzali *et al*., 2002), there are other studies that have been interpreted as evidence for trehalose-hexokinase interaction (Avonce *et al.*, 2004). Therefore, it is possible that trehalose signaling could affect hexose accumulation, in particular during stress when the ABI4 signaling pathway is active.

It appeared in this study that glucose content per g DW was not altered significantly by accumulation of trehalose (Fig.3). In all tissues, transgenic plants and non-transgenic plants showed similar glucose content per g DW, and it did not differ much before and after the water-stress application (Fig. 3). In some parts, significantly lowered glucose content per g DW were observed (Fig. A, E, H), but this was probably due to experimental errors since glucose content in this experiment was generally low, and so small errors may have had large relative effects. In general, glucose content per g DW was only about 10% of the sucrose content per g DW, in all three tissues examined. This result agrees with previous study in *Arabidopsis thaliana* that exogeneous trehalose did not significantly change glucose and fructose concentration (Bae *et al.*, 2005). Furthermore, a recent study reported that the relationship between trehalose and hexokinase signaling is through the precursor T6P instead of trehalose itself (Avonce *et al.*, 2005). The results in the current study support the current widely accepted idea that trehalose does not regulate carbon metabolism by modulating hexokinase activity since glucose content per g DW was not significantly different in any cases.

4.4 Sucrose content

Sucrose is commonly accumulated in cells under drought and other abiotic stresses that involve dehydration (freezing, salinity) in a diverse range of organisms (Cioni *et al.*, 2005; Wang *et al.*, 2005). Sucrose is known to stabilize membranes to maintain cellular structure by its molecular interactions with membrane lipids and intrinsic proteins (Hincha, 2006) and its contribution to the osmotic solute component of water potential, which retains water to keep turgor pressure from collapsing and/or cells from shrinking during episodes of low water potential (Cushman, 2001; Johansson *et al.*, 1998). There are several reasons to expect that factors influencing sucrose and trehalose

might interact. Trehalose and sucrose are structurally quite similar as they are the only two carbohydrates known to have non-reducing ends. Also, exogeneous sucrose alters TPS and TPP expression in *Arabidopsis thaliana* seedlings, suggesting these two disaccharides might have similar or overlapping functions in signaling (Lunn *et al.*, 2006).

Sucrose accumulation is commonly found in response to water stress, contributing to osmotic solute concentration and serving as a compatible solute (Sanchez *et al.*, 1998). The current study revealed that whereas tissues of the NT line had only a small increase in sucrose in response to water stress (Fig.4 A, D, and G), all three tissues of both A-line and R-line responded substantially to water stress by accumulating 35 to 100% more sucrose in water stress compared to the well-watered treatments (Fig.4 B, C, E, F, H, and I). This suggests that trehalose overexpressing lines respond to a greater extent to stresses, possibly by amplifying signal strength for stress tolerance responses. This finding is supported by previous reports that exogeneous trehalose in *Arrabidopsis* increases sucrose concentration (Bae et al. 2005). Wang *et al*. (2005) also showed that water stress alters sucrose metabolism by regulating enzymes such as sucrose-synthase and sucrose-phosphate-synthase, and drives the plants to convert stored carbon into sucrose. These results suggest that one of the possible functions of trehalose is in signaling carbon status within plants so that plants allocate carbohydrate toward sucrose accumulation.

In this experiment, the starch content per g DW was significantly lowered, especially in transgenic plants (Fig. 5). Since during water stress current photosynthesis is expected to be very low (due to stomatal closure and associated regulation), starch hydrolysis was likely used to synthesize sucrose and supply other metabolic reactions. In addition, Table 1 showed that both A-line and R-line transgenic plants had a higher relative ratio of sucrose:starch in WS:Control than NT-line plants. This suggests that

transgenic plants were operating more in the direction of sucrose synthesis and accumulation than toward starch storage. Sucrose: starch ratio is known to be regulated by several factors; such as stage of organ development, time of day, light-intensity, and surrounding environmental condition; and this ratio is often used to gauge photo-assimilate allocation (Sahrawy *et al.*, 2004). In upper and lower sheaths, where carbohydrate is mainly stored as starch, relative sucrose:starch ratio in WS:Control was much higher in transgenic than non-transgenic plants (Table 1 Bottom). This result suggests that additional accumulation of sucrose in both A-line and R-line compared to NT under water stress was primarily due to conversion of starch into sucrose. Previous studies showed that trehalose is related to this sucrose accumulation and partitioning. Wingler *et al.* (2000) reported that trehalose upregulates expression of the sucrose transporter *AtSUC2* and alters carbon allocation.

At the recovery stage of sampling in transgenic plants, well-watered control plants continued to increase their accumulation of sucrose in all three tissues, whereas sucrose content per g DW in water-stressed plants remained the same or declined slightly. In contrast, NT-line did not alter sucrose content per g DW very much in response to water stress or rewatering (Fig. 4). In addition, relative sucrose: starch ratio of WS:Control upon recovery was decreased to about 1.6 to 1.2, and became close to the one of NT (Table 2). This further suggests that plants released from stress by rewatering return to normal status in carbohydrate.

4.5 Starch content

In rice, reserve carbohydrate is stored mainly in the form of starch. In this experiment, starch content per g DW in upper and lower sheaths were substantially decreased in response to drought stress in both of the trehalose overexpression

genotypes (A-line, R-line), while it was decreased only slightly in the NT line (Fig. 5 D - I). These losses in starch content were probably due to hydrolysis to sugars and remobilization via phloem to sinks. Under drought condition, photosynthesis is limited due to stomatal closure and downregulation, which diminishes a plant's ability to create necessary energy and carbon sources for metabolism. Breakdown of starch during stress provides carbon supply to sustain respiration and synthesis of osmolytes such as sucrose. Consistent with previous reports on rice, the major storage tissue for starch was in the lower sheaths (Aoki *et al.*, 2003; Togari and Sato, 1952), which in the present study was a composite tissue including the young unexpanded stem. The amounts of starch in lower sheaths were the highest of any tissues (overall, the percentage of shoot starch was distributed 70% in lower sheaths, 27% in upper sheaths, and 3% in leaf blades). This result is consistent with the hypothesis that trehalose acts in regulating starch metabolism in response to plant carbohydrate status. Although starch remobilization from leaves and stems is known to occur as a drought stress response in rice and other grass species (Wang *et al.*, 2005; Yang *et al.*, 2001), transgenic plants with elevated trehalose-pathway enzymes could be more sensitive to sugar status within plants and respond by quickly altering expression of starch-pathway enzymes, so that stored carbohydrate would be more rapidly utilized, whereas NT plants were less sensitive and did not utilize starch substantially under the imposed stress. It has been reported that water stress induces the expression of α-amylase, which is a key enzyme of starch catabolism (Wang *et al.*, 2004). It has also been found that the expression of *APL3*, a gene encoding ADP-glucose pyrophosphorylase large subunit, a key enzyme for starch biosynthesis, is down-regulated when trehalose and T6P are increased by over-expressing *TPS1* (Avonce *et al.* 2004). These previous studies are consistent with the results obtained in this study.

After a week of recovery, starch content per g DW of water stressed/recovered

NT controls were at the same level as well-watered control plants (Fig.5 A, D, and G), whereas the starch content per g DW of both A-line and R-line remained significantly lowered in both the well-watered plants of the same genotypes and NT plants (Fig. 5 B, C, E, F, H and I). One possible explanation for this phenomenon is that transgenic plants night resume regrowth and utilize stored starch after rewatering and therefore not resume storage of starch. In contrast to transgenic plants, NT plants might not have been able to respond as quickly or to as large an extent, and so starch remained largely intact in storage tissues. Non-transgenic plants appear to retain starch which may improve their carbon status in longer-term stress episodes than the 7-day stress applied here. Leaves may be severely damaged and incapable for high photosynthesis right after a stress period, and so energy necessary for regeneration may be supplied from stored carbohydrate.

Contrary to the results in this experiment however, other previous studies have reported that trehalose induces starch biosynthesis. For example, exogeneous trehalose increased expression of *APL3*, encoding the starch synthesis enzyme AGPase (Fritzius *et al*., 2001; Muller *et al*., 2000), and increased starch concentration in the shoots of *Arabidopsis thaliana* seedlings (Bae *et al*., 2005; Winger *et al*., 2000). Furthermore, T6P was found to induce redox activation of the starch synthesis enzyme AGPase and convert AGPase into monomeric form, which has higher affinity for its substrates (Kolbe *et al*., 2005; Lunn *et al.*, 2006; Tiessen *et al.*, 2002). The changes in carbohydrate partitioning due to trehalose alone is perhaps overridden by the changes caused by water stress. In fact, it is reported that the activation of AGPase is closely related to sucrose concentration, thus this increase in AGPase and subsequent starch concentration may not be only due to trehalose, but to sucrose as well. (Kolbe *et al.*, 2005; Tiessen *et al.*, 2003). This suggestion is supported by the findings that sucrose activates AGPase via SNRK-1 (Hendriks *et al.*, 2003; Kolbe *et al.*, 2005). Under water

stress, sucrose may be depleted in the cytosol since photosynthesis is limited. Therefore, AGPase may not be activated during water deficit. In this experiment, however, sucrose content per g DW in transgenic lines was increased, and this sucrose may have been synthesized from the hydrolysis of starch. So, another possible interpretation is that the activation of AGPase is overridden when enzymes for starch break-down are active. Alternatively, there might be a microsite in each organ where sucrose concentration is low, and sucrose signals are sent out to other cells to alter metabolism. It is also possible that trehalose plays a signaling role in this process as the regulatory metabolite Fructose-2, 6-*bis*phosphate does in relation to sugar-phosphate metabolism (Wu *et al.*, 2006).

In summery, by overexpressing trehalose synthesis pathway, plants may have been sensitized such that they respond more strongly and quantitatively to water stress and recovery and altered carbohydrate metabolism in favor of sucrose synthesis and starch breakdown. For further understanding of the effects of trehalose on stress response, the measurement of related enzymes such like sucrose transporter and AGPase, or the expression of corresponding genes, *AtSUC1* and *APL3* respectively will be helpful.

BIBLIOGRAPHY

Abe H., Yamaguchi-Shinozaki K., Urao T., Iwasaki T., Hosokawa D., and Shinozaki K. (1997) Role of *Arabidopsis* MYC and MYB homologs in drought- and ABA-regulated gene expression. *Plant Cell.* **9**: 1859-1868.

Abraham E., Rigo G., Szekely G., Nagy R., Koncz C., and Szabados L. (2003) Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassionosteroid in Arabidopsis. *Plant Molecular Biology.* **51** (3): 363-372.

Aoki N., Ono K., Sasaki H., Seneweera S.P., Sakai H., Kobayashi K., and Ishimaru K. (2003) Effects of Elevated CO2 Concentration on Photosynthetic Carbon Metabolism in Flag-Leaf Blades of Rice before and after Heading. *Plant Production Science*. **6** (1): 52-58.

Arguelles J.C. (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. *Archive of Microbiology.* **174** (4): 217-224.

Arroyo A., Bossi F., Finkelstein R.R., and Leon P. (2003) Three genes that affect sugar sensing (*abscisic acid insensitive 4*, *abscisic acid insensitive 5*, and *constitutive triple response 1*) are differentially regulated by glucose in Arabidopsis. *Plant Physiology.* **133** (1): 231-242.

Avonce N., Leyman B., Mascorro-Gallardo J.O., van Dijck P., Thevelein J.M., and Iturriaga G. (2004) The Arabidopsis Trehalose-6-P Synthase *AtTPS1* Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signaling. *Plant Physiology* **136**: 3649-3659.

Avonce N., Leyman B., Thevelein J.M., and Iturriaga G. (2005) Trehalose metabolism and glucose sensing in plants. *Biochemical Society of Transactions.* **33** (1): 276-279.

Bae H., Herman E.M., and Sicher Jr R.C. (2005) Exogenous trehalose induces chemical detoxification and stress response proteins and promotes nonstructural carbohydrate accumulation in *Arabidopsis thaliana* grown in liquid culture. *Plant Science*. **168**: 1293-1301.

Blazquez M.A., Langunas R., Gancedo C, and Gancedo J.M. (1993) Trehalose-6-phosphate, a new regulator of yeast gycolysis that inhibits hexokinases. *FEBS Letters* **329**: 51-54.

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

Chaerle L., Saibo N., Van-der Straeten D. (2005) Tuning the pores: towards engineering plants for improved water use efficiency. *Trends in Biotechnology.* **23**: 308-315.

Choi H., Hong J., Ha J., Kang J., and Kim S.Y. (2000) ABFs, a family of ABA-responsive element binding factors. *Journal of Biological Chemistry* **275**:1723–30

Cioni P., Bramanti E., and Strambini G.B. (2005) Effects of sucrose on the internal dynamics of azurin. *Biophysical Journal* **88**(6): 4213-4222.

Colaco C., Sen S., Thangavelu M., Pinder S., and Roser B. (1992) Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. *Biotechnology* **10**: 1007-1011.

Colaco K., Kampinga J., and Roser B. (1995) Amorphous stability and trehalose. *Science* **268**: 788-789.

Crowe J.H., Crowe L.M., and Chapman D. (1984) Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. *Archives of Bilochemistry and Biophysics.* **232** (1): 400-407.

Cushman J.C. (2001) Osmoregulation in Plants: Implications for Agriculture. *American Zoologist.* **41** (4): 758-769.

Davies WJ and Kozlowski TT. (1977) Variations among Woody Plants in Stomatal Conductance and Photosynthesis During and after Drought. *Plant and Soil.* **46**: 435-444.

Desikan R., Cheung M.K., Bright J., Henson D., Hancock J.T., and Neill S.J. (2004) ABA, hydrogen peroxide and nitric oxide signaling in stomatal guard cells. *Journal of Experimental Botany.* **55**: 205-212.

Eastmond P.J., van Dijken A.J.H., Spielman M., Kerr A., Tissier A.F., Dickinson H.G., Jones J.D.G., Smeekens S.C., and Graham I.A. (2002) Trehalose-6-phosphate synthase1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo mutation. *The Plant Journal* **29** (2): 225-235.

Elbein A.D. (1974) The metabolism of α, α-trehalose. *Advances in Carbohydrate Chemistry and Biochemistry* **30**: 227-256.

Fritzius T., Aeschbacher R., Wiemken A., and Wingler A. (2001) Induction of *ApL3* expression by trehalose complements the starch-deficient Arabidopsis mutant *adg2-1* lacking ApL1, the large subunit of ADP-glucose pyrophosphorylase. *Plant Physiology.* **126** (2):883-889.

Garcia A.B., Engler J. deA., Iyer S., Gerats T., Van Montague M., and Caplan A.B. (1997) Effects of osmoprotectants upon NaCl stress in rice. *Plant Physiology.* **155**: 159-169.

Garg A.K., Kim J.-K., Owens T.G., Ranwala A.P., Choi Y.D., Kochian L.V., and Wu R.J. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *The Proceedings of National Academy of Science* **99** (25): 15898-15903.

Goddijn O. and Smeekens S. (1998) Sensing trehalose biosynthesis in plants. *The Plant Journal.* **14** (2): 143-146.

Goddijn O.J.M. and van Dun K. (1999) Trehalose metabolism in plants. *Trends in Plant Science* **4**, (8): 315-319

Goddijn O.J.M., Verwoerd T.C., Voogd E., Krutwagen R.W., de Graaf P.T., van Dun K., Poels J., Ponstein A.S., Damm B., and Pen J. (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. *Plant Physiology.* **113** (1): 181-190.

Gonzali S., Alpi A., Blando F., and de Bellis L. (2002) *Arabidopsis* (HXK1 and HXK2) and yeast (HXK2) hexokinases overexpressed in transgenic lines are characterized by different catalytic properties. *Plant Science*. **163**: 943-954.

Grennan A.K. (2007) The Role of Trehalose Biosynthesis in Plants. *Plant Physiology.* **144**: 3-5.

Hare P.D., Cress W.A., and van Staden J. (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell and Environment* **21**: 535-553.

Hare P.D., du Plessis S., Cress W.A. & van Staden J. (1996) Stress induced changes in plant gene expression: prospects for enhancing agricultural productivity in South Africa. *South African Journal of Science* **92,** 431–439.

Hendriks J.H.M., Kolbe A., Gibon Y., Stitt M. and Geigenberger P. (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. *Plant Physiology*. **133**: 838–849.

Hincha D.K. (2006) High concentrations of the compatible solute glycinebetaine destabilize model membranes under stress conditions. *Cryobiology.* **53** (1): 58-68.

Ingram J. and Bartles D. (1996) The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 377-403.

International Rice Research Institute (IRRI) 2006. Electronic access: http://www.irri.org

Iwahashi H., Obuchi K., Fujii S., and Komatsu Y. (1995) The correlative evidence suggesting that trehalose stabilizes membrane-structure in the yeast *Saccharomyces cerevisiae*. *Cell and Molecular Biology* **41**: 763-769.

Jang I.C., Oh S.-J., Seo J.-S., Choi W.-B., Song S.-I., Kim C.-H., Kim Y.-S., Seo H.-S., Choi Y.-D., Nahm B.-H., and Kim J.-K. (2003) Expression of Bifunctional Fusion of the *Escherichia coli* Genes for Trehalose-6-Phosphate Synthase and Trehalose-6-Phosphate Phosphatase in Transgenic Rice Plants Increases Trehalose Accumulation and Abiotic Stress Tolerance without Stunting Growth. *Plant Physiology* **131**: 516-524.

Johansson I., Karlsson M., Shunkla V.K., Chrispeels M.J., Larsson C., and Kjellbom P. (1998) Water transport activity of the plasma membrane aquaporin pm28a is regulated by phosphorylation. *Plant Cell*. **10**: 451-459.

Kim S.-J., Jeong D.-H., An G., and Kim S.-R. (2005) Characterization of a drought-responsive, OSTPS1, identified by the T-DNA gene-trap system in rice. *Journal of Plant Biology.* **48** (4): 371-379.

Kolbe A., Tiessen A., Schluepmann H., Paul M., Ulrich S. and Geigenberger P. (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. *The Proceedings of the National Academy of Sciences of the United States of America*. **102**: 11118–11123.

Lee S.-B., Kwon H.-B., Kwon S.-J., Park S.-C., Jeong M.-J., Han S.-E., Byun M.-O., and Daniel H. (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. *Molecular Breeding* **11**: 1-13.

Lesile S.B., Israeli E., Lighthart B., Crowe J.H., and Crowe L.M. (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology* **61**: 3592-3597.

Lopez FB, Setter TL, McDavid CR. 1987. Carbon Dioxide and Light Responses of Photosynthesis in Cowpea and Pigeonpea During Water Deficit and Recovery. *Plant Physiology* **85**, 990-995.

Luan S. (2002) Signaling drought in guard cells. *Plant Cell and Environment.* **25**: 229-237.

Lunn J.E., Feil R., Hendriks J.H.M., Gibon Y., Morcuende R., Osuna D., Scheible W.-R., Carillo P., Hajirezae M.-R., and Stitt M. (2006) Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *The Biochemical Journal*. **397**: 139-148.

Marten H., Konrad K.R., Dietrich P, Roelfsema M.R., and Herich R. (2007) Ca^{2+}-dependent and -independent abscisic acid activation of plasma membrane anion channels in guard cells of Nicotiana tabacum. *Plant Physiology.* **143** (1): 28-37.

Mulet J.M., Alejandro S., Romero C., and Serrano R. (2004) The trehalose pathway and intracellular glucose phosphates as modulators of potassium transport and general cation homeostasis in yeast. *Yeast.* **21** (7): 569-582.

Muller J., Aeschbacher R.A., Sprenger N., Boller T., and Wiemken A. (2000) Disaccharide-mediated regulation of sucrose:fructan-6-fructosyltransferase, a key enzyme of fructan synthesis in barley leaves. *Plant Physiology.* **123:** 265-273.

Ober E.S., Setter T.L., Madison J.T., Thompson J.F., and Shapiro P.S. (1991) Influence of Water Deficit on Maize Endosperm Development: Enzyme Activities and RNA Transcripts of Starch and Zein Synthesis, Abscisic Acid, and Cell Division. *Plant Physiology.* **97** (1): 154-164.

Outlaw Jr. W.H. (2003) Integration of cellular and physiological functions of guard cells. *Critical Review in Plant Sciences.* **22**: 503-529.

Penna S. (2003) Building stress tolerance through over-producing trehalose in transgenic plants. *Trends in Plant Science.* **8**: 355-357.

Pramanik M.H., and Imai R. (2005) Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transit inducton of trehalose biosynthesis during chilling stress in rice. *Plant Molecular Biology.* **58** (6): 751-762.

Raghavendra A.S. and Reddy A.R. (2006) Signal transduction in guard cells during stomatal closure by abscisic acid. *Journal of Plant Biology.* : 51-67.

Ramon M., Rolland F., Thevelein J.M., van Dijck P., and Leyman B. (2007) ABI4 mediates the effects of exogeneous trehalose on Arabidopsis growth and starch breakdown. *Plant Molecular Biology.* **63** (2): 195-206.

Rolland F., Baena-Gonzalez E., and Sheen J. (2006) Sugar Sensing and Signaling in Plants: Conserved and Novel Mechanisms. *Annual Review of Plant Biology* **57**: 675-709.

Rook F., Handingham S.A., Li Y., and Bevan M.W. (2006) Sugar and ABA response pathways and the control of gene expression. *Plant, Cell and Environment.* **29**: 426-434.

Sahrawy M., Avila C., Chueca A., Canovas F.M., and Lopez-Gorge J. (2004) Increased sucrose level and altered nitrogen metabolism in *Arabidopsis thaliana* transgenic plants expressing antisense chloroplastic fructose-1, 6-*bis*phosphatase. *Journal of Experimental Botany.* **55** (408): 2495-2503.

Sanchez F.J., Manzanares M., de Andres E. F., Tenorio J.L., and Ayerbe L. (1998) Turgor maintenance, osmotic adjustment and soluble sugar and proline accumulation in 49 pea cultivars in response to water stress. *Field Crops Research.* **59**: 225-235.

Schellenbaum L., Muller J., Boller T., Wiemken A., and Schuepp H. (1998) Effects of drought on non-mycorrhizal and mycorrhizal maize: changes in the pools of non-structural carbohydrates, in the activities of invertase and trehalose, and in the pools of amino acids and imino acids. *New Phytology.* **138**: 59-66.

Schluepmann H., Pellny T., van Dijken A.J.H., Smeekens S., and Paul M. (2003) Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. *The Proceedings of National Academy of Science* **100** (11): 6849-6854.

Schluepmann H., van Dijken A.J.H., Aghdasi M., Wobbes B., Paul M., and Smeekens S. (2004) Trehalose Mediated Growth Inhibition of Arabidopsis Seedlings Is Due to Trehalose-6-Phosphate Accumulation. *Plant Physiology* **135**: 879-890.

Schroeder J. I., Kwak J. M., Allen G. J. (2001) Guard cell abscisic acid signaling and engineering of drought hardiness in plants. *Nature.* **410**: 327–330.

Serraro R. (1996) Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *International Review of Cytology* **165**: 1-52.

Setter T.L., Flannigan B.A., and Melkonian J. (2001) Loss of Kernel Set Due to Water Deficit and Shade in Maize: Carbohydrate Supplies, Abscisic Acid, and Cytokinins. *Crop Science* **41**: 1530-1540.

Sharp R. E., LeNoble M. E., Else M. A., Thorne E. T., Gherardi F. (2000) Endogenous ABA maintains shoot growth in tomato independently of effects on plant water balance: evidence for an interaction with ethylene. *Journal of Experimental Botany*. **51**: 1575–1584.

SpollenW. G., LeNoble M. E., Sammuels T. D., Bernstein N., Sharp R. E. (2000) Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production. *Plant Physiology* **122:** 967–976.

SunMi K., Youn K.-J., Im C.-D., Hye P.-J., and Young K.-S. (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *Plant Journal.* **40** (1): 75-87.

Thevelein J.M., and Hohmann S. (1995) Trehalose synthase: guard to the date of glycolysis in yeast? *Trends in Biochemical Science* **20**: 3-10.

Tiessen A., Hendriks J.H.M., Stitt M., Branscheid A., Gibon Y., Farre E. M. and Geigenberger P. (2002) Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: a novel regulatory mechanism linking starch synthesis to the sucrose supply. *Plant Cell* **14**: 2191–2213.

Tiessen A., Prescha K., Bransceid A., Pacios N., McKibbin R., Halford N.G. and Geigenberger P. (2003) Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. *The Plant Journal* **35**: 490–500.

Togari Y. and Sato K. (1952) Studies on the production and behavior of carbohydrates in rice plants II. On the accumulation and distribution of starches in the organs of rice plant with its development of growth. *Proceedings of the Crop Science Society of Japan.* **22**: 98-99.

United Nation Conference of Desertification (UNCOD). 1978. *Round-up, plan of action and resolutions.* New York: United Nations.

United Nation Environmental Programme (UNEP). 1991. *Status of desertification and implementation of the United Nations plan of action to combat desertification*. New York. United Nations.

Uno Y., Furihata T., Abe H., Yoshida R., Shinozaki K., and Yamaguchi-Shinozaki K. (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Science of the United States of America* **97** (21):11632–11637.

Van Dijken A.J., Schluepmann H., and Smeekens S.C. (2004) Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. *Plant Physiology.* **135** (2): 969-977.

Wang W., Cai Y.X., Cai K.Z., Zhang J.H., Yang J.C., and Zhu Q.S. (2005) Regulation of soil water deficits on stem-stored carbohydrate remobilization to grains of rice. *Acta Agronomica Sinica* **29**(5): 819-828.

Wang W., Zhang J.H., Yang J.C., and Zhu Q.S. (2004) Effect of water stress on metabolism of stored carbohydrate of stem and yield in rice grown under unfavorable-delayed senescence. *Acta Agronomica Sinica* **30**(3): 196-204.

Wang X.-S., Zhu H.-B., Jin G.-L., Liu H.-L., Wu W.-R., Zhu J. (2007) Genome-scale identification and analysis of *LEA* genes in rice (*Oryza sativa* L.) *Plant Science.* **172**: 414-420.

Wiemkmen A. (1990) Trehalose in yeast, stress protectant rather than reserve carbohydrate. *J. Gen.Microbiol.* **58**: 209-217.

Wingler A. (2002) The function of trehalose biosynthesis in plants. *Phytochemistry* **60**: 437-440.

Wingler A., Fritzius T., Wiemken A., Boller T., and Aeschbacher R.A. (2000) Trehalose induces the ADP-glucose pyrophosphorylase gene, APL3, and starch synthesis in Arabidopsis. *Plant Physiology.* **124**: 105-114.

Wu C., Khan S.A., Peng L.J., and Lange A.J. (2006) Roles for fructose-2, 6-bisphosphate in the control of fuel metabolism: beyond its allosteric effects on glycolytic and gluconeogenic enzymes. *Advances in Enzyme Regulation.* **46**: 72-88.

Xiong, L. and Zhu, J.K. (2003) Regulation of abscisic acid biosynthesis. *Plant Physiology* **133:** 29-36.

Yang J., Zhang J., Wang Z., and Zhu Q. (2001) Activities of starch hydrolytic enzymes and sucrose-phosphate synthase in the stems of rice subjected to water stress during grain filling. *Journal of Experimental Botany.* **52** (364): 2169-2174.

Youn K.-J., In C.-Y., Young I.-M., Young K.-S. (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell.* **14** (2) :343-357.

Zentella R.**,** Mascorro-Gallardo J.O.**,** van Dijck P.**,** Folch-Mallol J.**,** Bonini B.**,** van Vaeck C.**,** Gaxiola R.**,** Covarrubias A.A.**,** Nieto-Sotelo J.**,** Thevelein J.M.**,** and Iturriaga G. (1999) A *Selaginella lepidophylla* Trehalose-6-Phosphate Synthase Complements Growth and Stress-Tolerance Defects in a Yeast *tps1* Mutant. *Plant Physiol* **119**: 1473-1482.

Zhang J., Jia W., Yang J., and Ismail A.M. (2006) Role of ABA in integrating plant responses to drought and salt stress. *Field Crops Research.* **97**: 11-119.

Zhu J.K. (2002) Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology.* **53**: 247-273.

van Dijken A.J.H., Schluepmann H., and Smeekens S.C.M. (2004) Arabidopsis Trehalose-6-Phosophate Synthase 1 is Essential for Normal Vegetative Growth and Transition to Flowering. *Plant Physiology* **135**: 969-977.