Identification of Drought-Responsive Genes and Validation for Drought Resistance in Rice

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ABSTRACT

Drought stress was studied in rice (*Oryza sativa*) and maize (*Zea mays*) to identify drought-responsive genes and associated biological processes. One experiment with rice examined drought responses in vegetative and reproductive tissues and identified drought-responsive genes in each tissue type. The results showed that brief periods of acute drought stress at or near anthesis reduced photosynthetic efficiency and ultimately lowered grain yield. Yield was reduced as a result both of fewer spikelets developed and of lower spikelet fertility. Affymetrix arrays were used to analyze global gene expression in the transcriptomes of rice vegetative and reproductive tissue. Comparative analysis of the expressed genes indicated that the vegetative and reproductive tissues responded differently to drought stress.

An experiment was conducted with maize, using GS-FLX pyrosequencing to identify differentially expressed genes in vegetative and reproductive tissues; and these results were compared with those from the just-described rice transcriptome. Some of the drought-responsive genes in the maize reproductive tissue were validated by quantitative real time polymerase chain reaction (qRT-PCR). The differentially expressed genes common to both maize and rice were further analyzed by gene ontology analysis to reveal core biological processes involved in drought responses. In both species, drought caused a transition from protein synthesis to degradation, and photosynthesis was one of the most severely affected metabolic pathways.

In a validating experiment, a drought-responsive transcription factor found in rice and dubbed HIGHER YIELD RICE (HYR) was constitutively expressed in rice, and the transgenic HYR plants were studied. Under well-watered conditions, the HYR plants developed higher rates

of photosynthesis, greater levels of soluble sugars (glucose, fructose, and sucrose), more biomass, and higher yield. They also exhibited a drought-resistant phenotype, with higher water use efficiency, photosynthesis, and relative leaf water content under drought stress. Taken together, these studies demonstrate the potential value of newer technologies for identifying genes that might impart drought resistance and for using such genes to make crops more productive either in the presence or in the absence of drought stress.

Dedications

To my family Oaitse and Gaone Batlang, my parents Ngwanyana and Batlang Modikwa, and my siblings and their children.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction and Literature Review

1.1. Introduction, Rationale, and Objectives

Drought, also known as water deficit, can result from insufficient moisture for a plant to grow adequately and complete its life cycle. Insufficient moisture can be the consequence of a shortage in rainfall, coarse textured soils that retain little water in the root zone, or drying winds (Swindale and Bidinger, 1981). Drought stress is one of the most important environmental factors limiting the growth and productivity of economically important plants (Tuberosa and Salvi, 2006; Yue et al., 2005). The demand for water in non-agricultural sectors is increasing, likely meaning that there will be less opportunity to increase crop productivity through more irrigation. In addition, nearly all land suitable for agriculture is currently in use or protected, and irrigation has rendered some soils saline.

In crop production, genetic improvement for drought adaptation is addressed implicitly, or indirectly, by selecting for yield stability over locations and years (Nguyen et al., 1997). Despite efforts to improve major crops for drought resistance by traditional breeding, success has been limited. However, advances in molecular approaches hold promise for physiological and breeding research on drought avoidance and tolerance in crop plants, and it is now possible to use these approaches to understand drought sensitivity and improve drought resistance of important crops.

In this study, transcriptome analyses and other molecular techniques have been used to identify drought-response mechanisms in rice (*Oryza sativa*) and maize (*Zea mays*). A novel drought-responsive gene has been identified and characterized by overexpression in rice. These experiments were done to test the overall hypothesis that drought-responsive genes in rice and

maize can be manipulated to play an increased role in drought resistance. Specific objectives were to:

1. Examine drought responses in rice vegetative and reproductive tissues and identify and characterize some drought-responsive genes. (Chapter 2)

2. Compare the genomic responses of rice and maize to drought stress. (Chapter 3)

3. Overexpress a candidate drought-responsive gene in rice and evaluate its effects on drought resistance and other plant properties. (Chapter 4)

1.2. Literature Review

1.2.1. Effects of Drought Stress on Plants

The ultimate detrimental effect of drought stress is reduction in yield, as reported in crops such as rice (Brevedan and Egli, 2003), wheat (*Triticum aestivum*) (Cabuslay et al., 2002), soybean (*Glycine max*) (Kirigwi et al., 2004), and chickpea (*Cicer aerietum*) (Khanna-Chopra and Khanna-Chopra, 2004). Various United States Department of Agriculture (USDA) reports have identified drought as the most frequent yield-reducing factor in arid and semiarid regions, although water deficit may occur even in high rainfall areas (Vamerali et al., 2003). Indian production of cereals and pulses dropped by about 30% in 1971 due to drought (Swindale and Bidinger, 1981). In the Sahel in Mauritania and Ethiopia, cereal production decreased by the same magnitude during the same period. The USDA reported that droughts in 1980, 1983, and 1988 significantly reduced US maize and soybean yields (Taiz and Zieger, 1998). A heat and drought wave of 2003 caused significant reductions in gross primary productivity and reduction in maize yield in both Eastern and Western Europe (Ciais et al., 2005). This information presents drought as a potential cause of disaster, especially since it affects almost all areas of the world; and crops, such as rice, that feed much of the world's population are easily affected by drought.

Water stress due to drought can lead to major physiological and biochemical changes, such as reduced photosynthesis (Lawlor and Cornic, 2002; Tezara et al., 1999) and reprogramming of gene expression (Neill and Burnett, 1999: Pattanagul and Madore, 1999: Romo et al., 2001). Physiological changes under drought stress are often reflected at the transcription level, where the levels of mRNA related to key processes such as photosynthesis are down-regulated (Bartels and Salamani, 2003). When plants experience water deficits, stomatal pores progressively close (Lawlor and Cornic, 2002; Saccardy et al., 1996; Tezara et al.,

1999). This process is regulated largely by leaf water potential but can be mediated by abscisic acid (ABA). Stomatal closure leads to decreases in photosynthetic CO_2 assimilation due to restricted diffusion of CO_2 into the leaf and altered CO_2 metabolism. Pelleschi et al. (1997) found that reduced CO_2 diffusion during stomatal closure is mainly responsible for the decline in photosynthesis in C_3 plants subjected to dehydration. However, Tezara et al. (1999) reported that, in sunflower (*Heliathus annuus*) (a C_3 plant) under water stress, the photosynthetic rate is limited more by altered CO_2 metabolism than by reduced diffusion. The lower CO_2 availability inhibits carbon assimilation, and ultimately photosynthetic capacity is lost as a consequence of the reduced stomatal conductance and/or direct damage to carbon metabolism (Bartels and Salamini, 2000; Colom and Vazzana, 2003).

Closure of stomata, as a result of water deficit and consequent decrease in CO_2 concentration in the leaf mesophyll, results in the accumulation of reducing power (NADPH) in the chloroplasts. This is because electrons produced by photolysis of water are in excess compared to NADP. Under such conditions, where NADP is limiting, O_2 acts as an alternative electron acceptor, resulting in the formation of superoxide radical (O_2^-) (Baisak et al., 1994; Gamble and Burke 1984; Sairam et al., 1998). The superoxide radical, through a series of univalent reduction reactions, produces hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Smirnoff, 1993). In addition to these species, superoxide in a reaction with $2H^+$ can also generate singlet oxygen ($^1O_2$) (Thomson et al., 1987). These molecules (O_2^-, H_2O_2, OH^-, and $^1O_2$), are called reactive oxygen species (ROS). They are highly toxic and can damage important cellular biomolecules such as lipids, proteins, nucleic acids, and chlorophyll (Baisak et al., 1994; Fu and Huang, 2001; Li et al., 1998; Cruz de Carvalho, 2008; Thomson et al., 1987). According to Zhang and Kirkham (1996), the harmful effects of the ROS are due primarily to their ability to

initiate autooxidative chain reactions on unsaturated fatty acids leading to lipid peroxidation and membrane destruction. Tissue damage due to ROS under drought has been reported in pea (*Pisum sativum*) (Moran et al., 1994) and *Phillyrea angustifolia* (Munne-Bosch and Penuelas, 2003).

On the other hand, as ROS levels increase under drought stress, they might function as an "alarm" signal that triggers defense responses. A specific transduction pathway that involves H_2O_2 as a secondary messenger has been reported by Cruz de Carvalho (2008). As stated by Dat et al. (2000), ROS seem to have a dual effect under abiotic stress conditions that depend on their overall cellular amount. If present at relatively low levels, they are likely to function as components of a stress signaling pathway, triggering stress defense/acclimation responses. However, when they reach a certain level, ROS can become phytotoxic, damaging cellular co mponents and eventually causing tissue death. Thus, ROS can be viewed both as toxins indicating cellular stress and as secondary messengers involved in stress-signal transduction.

1.2.2. Mechanisms to Cope with Drought in Plants

Mechanisms that plants use to cope with drought can, more or less intuitively, be grouped into drought avoidance and drought tolerance. A combination of these defines drought resistance in its physiological context according to Levitt (1972). Each of these general strategies is described below.

1.2.2.1. Drought Avoidance Mechanisms

Drought-avoiding plants have the ability to complete their life cycle without severe water deficits developing. Some ephemerals have a very short life cycle that can be completed during a brief rainy season. Other plants exhibit adaptations to increase water uptake and reduce water loss and thereby avoid the debilitating consequences of drought that other plants might feel

(Bartels and Salamini, 2001; Laporte et al., 2002; Verslues et al., 2006). Accordingly, developing a more extensive root system is a drought-avoiding strategy. It is an almost universal observation that the root:shoot ratio increases with water stress. Increases in root weight may be due to a greater density or depth of roots (Turner, 1979). Under water stress, new root growth extends into moist soil zones. As water deficits progress, the upper soil layers usually dry first. Thus shallower roots are common in wetter soils as opposed to deeper roots systems in dryer soils (Taiz and Zeiger, 1998). The possession of a deep and thick root system, which allows access to water deeper in the soil is considered important in determining drought resistance in rice (Price et al., 2002), *Arabidopsis thaliana* (Xiong et al., 2006), pea (Chiantante et al., 1999), tall fescue (*Festuca arundinacea*) (Ervin and Koski, 1998), and *Wilwitchia mirabilis* (Fitter and Hay, 2002). Therefore, greater root growth and improved morphological development can result in drought avoidance.

Development in shoots also plays an important role in water stress responses. At the onset of the dry season, a desert plant *Zygophyllum qatarense* responds to water stress with a leaf polymorphism in which it replaces the wet-season foliage with unifoliate, xeromorphic leaves. As the dry season progresses, the plant reduces its leaf area substantially (Sayed, 1996).

Leaves of many plants frequently wilt and droop (or roll, in the case of many grasses) under water stress, and this response reduces the interception of radiation, thereby counteracting the increase in leaf temperature arising from stomatal closure and preventing further development of leaf water deficit (Turner, 1979).

Reduced leaf area can be a mechanism for moderating water use and injury under drought stress (Blum, 2005). Severe drought stress may result in increased levels of ABA and subsequent leaf abscission, thereby reducing transpirational demand. Such developmental changes within a

plant during water stress are important morphological drought-avoiding adaptations that help maintain the plant's water potential at some functional level in the midst of potential water limitation.

1.2.2.2. Drought Tolerance Mechanisms

As drought stress becomes more severe, it becomes increasingly more difficult to avoid dehydration; and mechanisms that allow plants to withstand reduced water content (more negative water potentials) become increasingly important. Drought tolerance is defined as the ability of plants to continue to function at lowered tissue water potentials. Drought-tolerating mechanisms often involve the maintenance of turgor (by accumulation of solutes) and/or desiccation tolerance (by protoplasmic resistance) (Jones et al., 1981). "Desiccation tolerant" plants can recover from a fully air-dried state (Vicre et al., 2004). When dehydrated, these plants are in a metabolically dormant, or cryptobiotic, state. Mesophytic plants, including all crop plants, lack the ability to enter the cryptobiotic state. In fact, mesophytes typically cannot recover from severe (approximately 50% or greater) decreases in water content (Verslues et al., 2006). However, many plants have some ability to tolerate significant water loss, while maintaining metabolic activity.

Biochemical and physiological studies have shown that sugars (e/g., raffinose family oligosaccharides (RFO), sucrose, sorbitol, and mannitol), amino acids (e.g., proline), and amines (e.g., glycine betaine) accumulate under drought stress in different plant species (reviewed by Seki et al., 2007). As soil dries, its water potential becomes more negative. Accumulation of solutes (osmolytes) by plant tissues makes their water potentials more negative, allowing them to take up water and avoid reductions in turgor. This drought-tolerating mechanism is called osmotic adjustment (OA). By contributing towards OA, osmolytes act as protectants for plants

subjected to low water potential (Pandey et al., 2004). OA helps maintain cell turgor, which can allow cell enlargement and plant growth during water stress; and it can allow stomata to remain at least partially open and CO_2 assimilation to continue at water potentials that would be otherwise inhibitory (Alves and Setter, 2004). OA by osmolytes has been reported to be a cause of improved productivity in wheat (Flower and Ludlow, 1987), sorghum (*Sorghum bicolor*), barley (*Hordeum vulgare*), chickpea (*Cicer arietnum*), pigeon pea (*Cajanus cajan*) (Khanna-Chopra and Khanna-Chopra, 2004), and rice (Lanceras et al., 2004). In addition to acting as osmolytes to maintain turgor for a longer period of time, some of the accumulated solutes are thought to participate in stress-protective functions as free radical scavengers and stabilization of macromolecules during drought (Abebe et al., 2003; Seki et al., 2007).

1.2.2.3. Biochemical/Metabolic/Genomic Responses Associated with Drought Resistance

In many plants that adapt to water stress, a set of genes are transcriptionally activated, leading to accumulation of new proteins in seeds and vegetative organs and greater tolerance to drought. Proteins termed LEA (Late Embryonic Abundant), which were first characterized in cotton (*Gossypium hirsutum*), are a set of proteins that accumulate in embryos late in seed development (Xu et al., 1996) where they are associated with acquisition of desiccation tolerance in maturing seeds. These proteins are also found in vegetative tissues in response to exogenous ABA, as well as osmotic and dehydration stress (Liang et al., 2006), at any stage of plant development (Baker et al., 1988). At least six groups of LEA proteins have been categorized by virtue of the similarity in their deduced amino acid sequences; and group 2, also referred to as the dehydrins, contain proteins that are induced by dehydration-related stresses such as osmotic stress and drought (Wang, et al., 2006). Their hydrophilic nature and high solubility indicate that the proteins are maintained in the cytosol, where they are assumed to function as chaperone-like

protective molecules to combat cellular damage (Umezewa et al., 2006) and to act as hydrophilins, retaining water (Reys et al., 2008) during dehydration. An association between tolerance to drought stress and these groups of proteins has been observed in some crop plants. In blueberry (*Vacinium* spp.), the dehydrins were found to accumulate in response to changes in ABA levels during drought stress (Panta et al., 2001). *LEA* genes, when over-expressed in rice (Xiao et al., 2007), tobacco (*Nicotiana tabacum*) (Wang et al., 2006), and *Arabidopsis thaliana* (Figueras et al., 2004) led to drought tolerance in transgenic plants. Although the specific roles of the LEA proteins are not known, it is clear that they are regulated by ABA and cellular water loss.

Plant cells, including photosynthetic cells, are protected against the detrimental effects of reactive oxygen species (ROS) by an antioxidant system that has been associated with stress tolerance in plants. The system is composed of enzymatic and nonenzymatic detoxification mechanisms, which mitigate and repair damage initiated by the ROS (Fu and Huang, 2001; Zhang and Kirkham, 1996). The enzymatic system is made of the enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) (Li et al., 1998; Mittler, 2002). Primarily, SOD reacts with the superoxide radicals to generate H_2O_2, H_2O, and molecular oxygen (O_2). The H_2O_2 so produced is disposed off by APX, POD, and CAT. APX and GR take part in the ascorbate-glutathione cycle, where H_2O_2 is removed and ascorbic acid is regenerated (Li et al., 1998; Smirnoff, 1993; Thomson, 1987; Zhang and Kirkham, 1996). In addition, GR maintains a high ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), which can further protect chloroplasts against oxidative damage (Gamble and Burke, 1984). Other enzymes related to glutathione metabolism, glutathione S-transferase (GST) (acting as peroxidase) and glutathione peroxidase (GPX), have

been reported to be induced under drought stress in moss (*Tortula ruralis*) and leafy spurge (*Euphorbia esula*) and are efficient scavengers of H_2O_2 and lipid peroxide using reduced glutathione and preferably thioredoxin as a reductant (Cruz de Carvalho, 2008).

Recently, increasing evidence for the participation of thioredoxins (TRX) and glutaredoxins (GRX) in plant antioxidant defense has emerged (Meyer et al., 2009; Rey et al., 2005). In higher plants, these proteins are distributed in the cytoplasm, plasma membrane, endoplasmic reticulum, nucleus, apoplast, mitochondrion, and chloroplast (Iqbal et al., 2006). Drought-induced ROS can oxidize –SH-containing enzymes of the chloroplast. TRX act to reduce the oxidized -S-S- groups in these enzymes, reforming them into the active -SH groups (Buchanan and Balmer 2005). These proteins are induced under drought stress and play a role in drought resistance by supplying reducing power to enzymes, repairing oxidized proteins, or regulating scavenging mechanisms (Dos Santos and Rey, 2006). For example, in some cases, glutathione peroxidase (GPX) appears to prefer TRX to glutathione as a reductant during scavenging of H_2O_2 and lipid peroxides (Cruz de Carvalho, 2008). A potato (*Solanum tuberosum*) mutant in TRX CDSP32 exhibited high sensitivity to oxidative stress generated by methyl viologen compared to the wild type and transgenic plants oveexpressing the gene (Rey et al., 2005). Methyl viologen generates ROS from photosystem I, which oxidize the –SH-containing enzymes (Kotabova et al., 2008). TRX is required to reverse this damage.

In wheat, proteomic studies identified differential expression of a TRX between drought-tolerant and -susceptible genotypes (Hajheidari et al., 2007). Expression of the protein was higher in drought-tolerant than in -susceptible genotypes. Therefore, TRX can be seen to play a role in drought tolerance by taking part in antioxidant defense systems in plants.

Wheat leaves exposed to mild water stress (-0.5 MPa) exhibited increased activities of SOD, CAT, APX, and GR, which correlated with reduced lipid peroxidation; but the protective system was not effective at a more severe water stress (-1.5 MPa) (Baisak et al., 1994). These results suggest that the degree of water stress is also important in the systems' protection and that it may represent a drought-tolerance response. Nevertheless, drought tolerance was associated with scavenging systems represented by APX and CAT in wheat (Sairam et al., 1998) and cowpea (*Vigna anguiculata*) (D'Arcy-Lameta et al., 2006). Transgenic plants overexpressing SOD and APX were resistant to oxidative-stress-inducing chemicals methyl viologen (Tang et al., 2006) and paraquat (Lee et al., 2007) in potato and tobacco, respectively; and overexpression of APX in tobacco (Badawi et al., 2004) and GPX in Arabidposis (Miao et al., 2006) enhanced tolerance to water deficit. In *Phaseolus* species, the drought-tolerant *P. acutifolius* has higher constitutive expression of SOD, CAT, and APX than the sensitive *P. vulgaris* (Turkan et al., 2005). Studies with *P. vulgaris* cultivars differing in drought tolerance showed that tolerant cultivars maintain stable GR expression during drought stress compared to susceptible cultivars (Torres-Franklin et al., 2008). Although effectiveness against stress may be determined by the level of stress, these comparative and transgenic approaches show that the enzymatic antioxidant system can be an indicator of drought tolerance in different plants.

The nonenzymatic detoxification mechanisms involve antioxidants such as lipophilic vitamin E (α-tocopherol), hydrophilic vitamin C (ascorbic acid), and carotenoids. Vitamin E reacts with O_2^- as well as $^1O_2$. Vitamin C can react directly with H_2O_2 in a reaction mediated by APX (Smirnoff, 1993). Glutathione is the substrate for the formation of vitamin C, which has the ability to react directly with free radicals such as OH. (Pastori and Trippi, 1992). Vitamin E, Vitamin C, and glutathione have been reported to increase in plants under water stress (Shao et

al., 2008). Transgenic plants with greater levels of transcripts encoding enzymes associated with the glutathione-ascorbate cycle accounted for lower levels of H_2O_2 during drought (Rivero et al., 2007). The transgenic plants had higher photosynthesis and water use efficiency and higher survival rate under severe drought. In addition to their role in energy dissipation in plant tissues, the carotenoids, particularly β-carotene, remove singlet oxygen ($^1O_2$). According to Stuhlfauth et al. (1990), total carotenoids increased after one day of stress in leaf disks of two oak (*Quercus*) species. At low water potential (-2.5 MPa), there was a 25% increase in β-carotene and a correlated decrease in $^1O_2$ levels, suggesting protection against this ROS (Stuhlfauth et al., 1990). Drought tolerance in the tree species *Cistus clusii* was found to be associated with higher levels of α-tocopherol and β-carotene than in *Cistus albidus* (Munne-Bosch et al., 2003). Furthermore, the tolerant species had negligible chlorophyll degradation and de-epoxidation of violaxanthin in the xanthophyll cycle, and this was explained by better protection against oxidative stress by α-tocopherol and β-carotene during drought stress.

In summary, the ROS generated under water deficit can be counteracted in drought-tolerating plants by the activation of antioxidant systems to remove the ROS from the affected cells. Comparative and transgenic studies have identified association between drought tolerance and activities of the antioxidant systems in different plants. However, efficiency of the system might depend on the degree of water deficit, as high levels of stress could also cause damage to the antioxidant system itself.

Another group of proteins that are induced by abiotic stress is the heat shock proteins (HSP). Five major families of HSP are recognized: the HSP70 (DnaK) family, the chaperonins (HSP60), the HSP90 family, the HSP100, and the small heat shock protein (sHSP) family (Wang et al., 2004). Of these families, the sHSP have been most associated with abiotic stress. Initially

detected upon heat treatment, sHSP production has now been observed under drought stress. Although mechanisms by which sHSP are involved in cell protection are not fully understood, there is strong evidence supporting the view that they function as molecular chaperones that bind to misfolded substrate proteins and thereby prevent irreversible aggregation during stress (Sun et al., 2002). Synergistic cooperation between sHSP, p26, and the sugar trehalose in the protection of citrate synthase during thermal stress has been observed in vitro (Viner et al., 2001). Hamilton and Heckathorn (2001) demonstrated that electron transport of mitochondrial complex I was protected by sHSP upon salt stress, with similar effects to that of ascorbate, glutathione, and α-tocopherol as well as SOD and CAT. The results provide evidence that sHSP may protect plants from stress by cooperation with other stress-protective molecules such as sugars, amino acids, or amines and/or by mechanisms involving ROS scavenging.

The involvement of HSP in drought has been studied through comparative and transgenic approaches**.** Qualitative differences in the pattern of synthesis of HSP were observed in maize lines differing in drought and heat tolerance (Ristic et al., 1991). A unique HSP was observed in a drought- and heat-resistant line compared to a sensitive line. Differential HSP expression was also observed in potato genotypes differing in drought tolerance (Vasquez-Robinet et al., 2008). While HSP70, HSP40, and an sHSP whose products localize to the chloroplast were induced in a drought-tolerant genotype, HSP genes targeted to the cytosol were induced in the sensitive genotype. This differential expression in different genotypes and organelles implies roles for HSP in drought tolerance. Overexpression of sHSP enhanced drought tolerance in rice (Sato and Yokoya, 2008) and Arabidopsis (Sun et al., 2001).

1.2.2.4. Drought Avoidance and Tolerance Strategies May Be Integrated

Since drought-avoidance and -tolerance mechanisms were first proposed by Levitt (1972) and others, our understanding of molecular and cellular events during drought stress has increased. It has become clear that many of the molecular events initiated by drought do not fit exclusively into avoidance or tolerance categories. For example, accumulated solutes may play a role as osmolytes, allowing plants to take up more water and as such constitute drought avoidance. At the same time, solutes such as amino acids and sugars may in addition play a protective role against protein and membrane damage. Likewise, dehydrins can function as chaperone-like protective molecules (Close, 1997) (tolerance mechanism), while their hydrophilin activity (Reyes et al., 2008) helps retain water (an avoidance mechanism). The involvement of ABA in ABA-regulated stomatal conductance and root growth (Liu et al., 2005; Schroeder et al., 2001) is important in avoidance; but, at the same time, ABA accumulation has been reported to cause synthesis of dehydrins (Xiao and Nassuth, 2006), which are important for drought tolerance.

1.2.3. Recent Advances in Identifying Drought-Resistance Genes and Pathways

Many of the genes that are known to respond to drought stress have been identified, and the products of these genes can be classified into two groups (Bray, 1997; Yamaguch-Shinozaki and Shinozaki, 2006). The first group includes proteins that probably directly protect against stress such as enzymes for osmolyte biosynthesis, LEA proteins, and detoxification enzymes. The second group consists of proteins involved in the regulation of gene expression and signal transduction of stress responses, such as transcription factors (TF), protein kinases, protein phosphatases, and enzymes involved in biosynthesis of signaling molecules.

The phytohormone ABA accumulates under water deficit. Increasing ABA concentrations lead to many changes in plant development, physiology, and growth. Some of these changes bring about avoidance and tolerance mechanisms (discussed above). At the molecular level, ABA induces expression of several genes mediated through ABA-responsive *cis*- and *trans*- acting factors and protein kinases or phosphatases in a Ca^{2+}-dependent or -independent signaling cascade (Bray, 2002; Nakashima et al., 2009). ABA-dependent pathways regulate stress-responsive gene expression through CBF4, MYB/MYC, and bZIP-type TF, which bind CRT/DRE, MYBR/MYCR-recognition sequences, and ABA-responsive elements (ABRE), respectively. These pathways are induced by drought as well as salinity stress.

A number of genes are known to be induced by drought, salinity, and cold in *aba* (ABA-deficient) and *abi* (ABA-insensitive) Arabidopsis mutants. This suggests the existence of alternative, ABA-independent regulatory systems of gene expression during stress responses. Indeed there is an ABA-independent pathway whose genes have one or several copies of the CRT/DRE *cis* elements in their promoters (Figure 1.1). A family of TF known as CBF or DREB1 binds to this element and activates transcription of the downstream dehydration-responsive genes (Stockinger et al., 1997). DREB1 TF are believed to interact with CRT/DRE and induce expression of stress-tolerance genes in response to cold, whereas DREB2 TF are involved in drought responses (Liu et al., 1998; Seki et al., 2001). Overexpression of the Arabidopsis DREB1A TF under the control of a stress-inducible promoter (RD29A) increased drought tolerance in wheat (Pellegrineschi et al., 2004). In a study with DREB1-expressing tobacco, transgenic plants had improved drought- and low-temperature-stress tolerance (Kasuga et al., 2004). This information indicates that, even in ABA-independent pathways, plants have common mechanisms in their physiological responses and tolerance to drought.

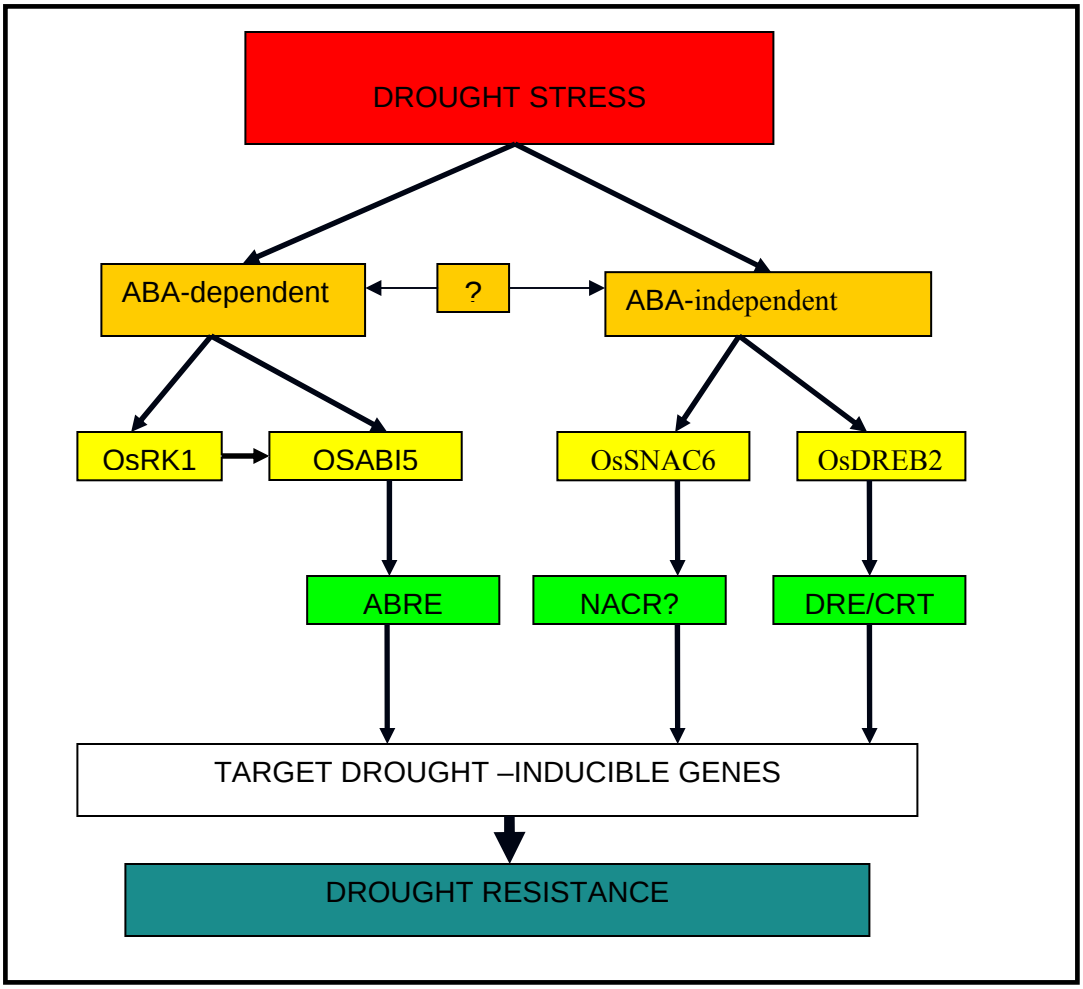

Figure 1.1. Drought-response genes in the model species rice. (adapted from Nakashima et al., 2009) Red (drought effect); darker yellow (signaling pathways); lighter yellow (regulatory genes); green (*cis* elements of drought inducible genes, or transcription factors); white (downstream target genes); blue (plant responses).

While it is important to identify genes that protect and maintain cellular structure and function, the major focus should be to improve crop yield by increasing carbon gain under drought stress. It is therefore desirable to target genes that increase water use efficiency (WUE) without incurring yield penalties. This may be difficult, because improving WUE is often accompanied by decreased photosynthesis (Flexas et al., 2004) and yield (Condon et al., 2004). However, genes that can modify transpiration efficiency by acting on epidermal and mesophyll

development or stomatal density and porosity may circumvent this problem. This implies that traits that affect phenology and development that can be easily engineered for drought stress avoidance and tolerance should be considered.

A gene that directly regulates plant transpiration has been identified in Arabidopsis (Masle et al., 2005). This is the regulatory gene *ERECTA*, encoding a leucine-rich repeat receptor-like kinase. Its expression led to high photosynthesis and low stomatal conductance, which resulted in high WUE (Masle et al., 2005). Other regulatory genes that have been found to control stomatal conductance are *OST,* encoding a protein kinase (Mustilli et al., 2002) and ABF3 and ABF4 TF (Kang et al., 2002). Overexpresion of *OST1*, *ABF3*, and *ABF4* in transgenic plants resulted in reduced transpiration (Kang et al., 2002; Mustilli et al., 2002).

A dominant gain-of-function Arabidopsis mutant termed *hrd-D* has been identified (Karaba et al., 2007). The corresponding *HARDY (HRD)* gene belongs to the ERF/AP2 family of TF. Expression of the Arabidopsis *HRD* gene in rice improves WUE by enhancing photosynthesis and reducing transpiration. In addition, plants exhibited drought tolerance, lowered water consumption, increased shoot biomass under well-watered conditions, and an adaptive increase in root biomass under drought stress. While *HRD* overexpression in Arabidopsis produces smaller plants with thicker leaves and more chloroplast-bearing mesophyll, in rice there is an increase in leaf biomass and bundle sheath cells that probably contribute to enhanced photosynthetic assimilation and efficiency.

Another ERF/AP2 TF gene SHINE (SHN) displays drought resistance in Arabidopsis (Aharoni et al., 2004). The expression of this gene in rice and tomato confers drought resistance, increases WUE and biomass production, and reduces whole-plant transpiration (Karaba, 2007).

1.2.4. Techniques for Plant Gene Expression Profiling and Identification Under Water Deficit

Plant species whose genomes have been sequenced are good models to study the function of genes in important biological pathways. These plants include Arabidopsis (The Arabidopsis Genome Initiative, 2000), rice (International Rice Genome Sequencing Program, Yu Jun et al., 2002; Goff et al., 2003), poplar (*Populus* spp.) (Tuskan et al., 2006), and soybean (DOE Joint Genome Institute, 2008).

Sequencing projects have been producing not only genomic sequences of model plants of interest but also a large number of expressed sequence tags (EST) for many other crop plant species. Plant science has therefore entered a new era, with sequencing of the genomes representing model systems as well as crop plants (rice and soybean). The field now faces the challenge to provide applications for crop improvement. Many tools now enable a large-scale, parallel, quantitative assessment of molecular states. The new high-throughput gene characterization and function analysis technologies can identify candidate genes involved in growth and development and responses to the environment, to include genes involved in drought responses. Determination of the biological functions of these genes is among the greatest challenge for the post-genomic era.

Since its emergence more than a decade ago, DNA microarray technology has been applied to determine transcript abundance for many or all transcripts in a genome by comparing control and experimental treatments. The microarray data have been analyzed for a number of plant processes, such as seed development, wounding responses, pathogen signaling, and environmental stress responses (Rabanni et al., 2003). Expression microarrays also provide new insights into physiological and biochemical pathways of drought tolerance and thus can lead to

identification of novel candidate genes that can rapidly advance breeding for drought tolerance. On the basis of microarray and RNA gel blot analyses, drought-inducible genes were identified in Arabidopsis (Seki et al., 2001). Similar approaches also led to identification of dehydration-inducible genes in sorghum (Buchanan et al., 2005; Pratt et al., 2005) and rice (Zhou et al., 2007). The major limitation of microarrays is that they rely on current genome annotations, which precludes the identification of novel transcripts.

Recent breakthroughs in sequencing technologies have led to innovations such as pyrosequencing (Margulies et al., 2005). Similar to the microarray, gene expression patterns can be monitored with platforms such as GS-FLX 454 pyrosequencing technology. Availability of a large set of EST from plants such as maize and others has triggered the development of large-scale and genome-wide analyses of gene expression patterns. This technology has been applied to study genes involved in olive (*Olea europaea*) fruit development (Alagna et al., 2009), genetic variation among mangroves (*Rhizophora mangle*) and *Heritiera littoralis* (Dessanayake et al., 2009), and responses to drought and salinity in rice (Sunkar et al., 2008). Such methods therefore can advance knowledge gained from model systems to complex crop plants and source germplasm.

Key advantages of the GS-FLX pyrosequencing platform over microarray methods are: 1) the ability to directly determine the identity and the abundance of a transcript rather than inferring it indirectly from measures of hybridization intensity used in microarray experiments; 2) the read length (200 to 400 bases) allows for assembly of the sequences into genes, which is particularly important for species without reference genome information.

1.3. References

Abebe T, Arron AC, Martin B, Cushman JC (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol **131**: 1748-1755.

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004) The shine clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. Plant Cell 16: 2463-2480.

Alagna F, D'Agostino N, Torchia L, Srvili M, Rao R, Pietrella M, Guiliano G, Chiusano ML, Baldoni L, Perrotta G (2009) Comparative 454 pyrosequencing of transcripts from two olive genotypes during fruit development. BMC Genomics **10**: doi:10.1186/147-2164-10-399.

Alves AAG, Setter TL (2004**)** Abscisic acid accumulation and osmotic adjustment in cassava under water deficit. Environ Exp Bot **51**: 259-279.

Badawi GH, Kawano N, Yamaguchi Y, Shimada E, Sasaki R, Kubo A, Tanaka K (2004) Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts. Physiol Plantarum **121**: 231-238.

Baisak R, Rana D, Acharya PBB, Kar M (1994) Alterations in the activities of active oxygen scavenging enzymes of wheat leaves subjected to water stress. Plant Cell Physiol **35**: 489-495.

Baker, JC, Steele C, Dure III L (1988) Sequence and characterization of 6 LEA proteins and their genes from cotton. Plant Mol Biol **11**: 277-291.

Bartels D, Salamini F (2001) Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level. Plant Physiol **127**: 1346-1353.

Blum A (2005) Drought resistance, water-use efficiency, and yield potential: are they comparable or mutually exclusive. Aust J Agr Res **56**: 1159-1168.

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2: 48-54.

Bray EA (2002) Classification of Genes Differentially Expressed during Water-deficit Stress in *Arabidopsis thaliana*: an Analysis using Microarray and Differential Expression Data. Ann Bot 89: 803-811.

Brevedan RE, Egli DB (2003) Short periods of water stress during seed filling, leaf senescence and yield of soybean. Crop Sci **43**: 2083-2088.

Buchanan BB, Balmer Y (2005) Redox regulation: a broadening horizon. Annu Rev Plant Biol **56**: 187-220.

Buchanan CD, Lim S, Salzman RA, Kagiampakis I, Morishige DT, Weers BD, Klein RR, Pratt LH, Cordonier-Pratt M, Klein PE, Mullet JE (2005) *Sorghum bicolor*'s transcriptome response to dehydration, high salinity and ABA. Plant Mol Biol **58**: 699-720.

Cabuslay GS, Ito O, Alejar AA (2002) Physiological evaluation of rice *(Oryza sativa)* to water deficit. Plant Sci **163**: 815-827.

Chiatante D, Di Iorio A, Maiorol L, Scippa SG (1999) Effect of water stress on root meristerms in woody and herbaceous plants during the first stage of development. Plant Soil **217**: 159-172.

Ciasis Ph, Reichstein M, Viovy N et al (2005) Europe-wide reduction in primary productivity caused by heat and drought in 2003. Nature **437**: 529-533.

Colom MR, Vazzana C (2003) Photosynthesis and PS II functionality of drought resistant and drought-sensitive weeping love grass plants. Environ Exp Bot **49**:135-144.

Condon AG, Richards RA, Rebetske GJ, Farquhar GD (2004) Breeding for high water use efficiency. J Exp Bot **55**: 2447-2460.

Cruz de Carvalho MHC (2008) Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signaling and Behavior **3**: 156-165.

D'Arcy-Lamenta T, Ferrari-Iliou R, Contour-Ansel D, Pham-Thi A-T, Zuily-Fodil Y (2006) Isolation and characterization of four ascorbate peroxidase cDNAs responsive to water deficit in cowpea leaves. Ann Bot **97**: 133-140.

Dassanayake M, Haas JS, Bohnert HJ, Cheeseman JM (2009) Shedding light on an extremophile lifestyle through transcriptomics. New Phytol **183**: 764-775.

Dat J, Vandenabeele S, Vranova E, Van Montangu M, Van Breusegem F (2000) Dual action of the active oxygen species during plant stress responses. Cell Molec Life Sci **57**: 779-795.

Dos Santos CV, Rey P (2006) Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci **11**: 1329-1334.

Ervin EH, Koski AJ (1998) Drought avoidance aspects and crop coefficients of Kentucky bluegrass and tall fescue turfs in the semiarid west. Crop Sci **38**: 788-795.

Figueras MJ, Pujal A, Saleh R, Save M, Pages M, Goday A (2004) Maize RAB17 overexpression in *Arabidopsis* plants promotes osmotic stress tolerance. Ann Appl Biol **144**: 251-257.

Fitter AH, Hay RKM (2002) Environmental Physiology of Plants. Academic Press. San Diago.

Flexas J, Bota J, Cifre J, Escalona JM, Galmes J, Gulias J, E.K. Lefi EK, S.F. Martinez-Carnellas SF, Moreno MT, Ribas-Carbo M, Reira D, Sampol B, Medrano H (2004) Understanding down-regulation of photosynthesis under water stress: Future prospects

and searching for physiological tools for irrigation management. Ann Appl Biol **144**: 273-283.

Flower DJ, Ludlow MM (1987) Variation among accessions of pigeon pea (*Cajanus cajan*) in osmotic adjustment and dehydration tolerance of leaves. Field Crop Res **17**: 229-243.

Fu J, Huang B (2001) Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ Exp Bot **45**: 105-114.

Gamble PE, Burke JJ (1984) Effect of water stress on the chloroplast antioxidant system I. Alterations in glutathione reductase activity. Plant Physiol **76**: 615-621.

Goff SA, Ricke D, Lan T-H, et al. (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *Japonica*). Science **296**: 92-100.

Hajheidari M, Eivazi A, Buchanan BB, Wong JH, Majidi I, Salekdeh GH (2007) Proteomics uncovers a role for redox in drought tolerance in wheat. J Proteome Res **6**: 1451-1460.

Hamilton III EW, Heckathorn SA (2001) Mitochondrial adaptation to NaCl. Complex I is protected by antioxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. Plant Physiol **126**: 1266-1274.

Jones MM, Turner NC, Osmond CB (1981) Mechanisms of drought resistance. pp. 15-35. *In* Paleg, L. G., and D. Aspinal. (ed.) The Physiology and Biochemistry of Drought Resistance in Plants. Academic Press. Sydney.

Kang JY, Choi HI, Im MY, Kim SY (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14**: 343-357.

Karaba A, Dixit S, Greco R, Aharoni A, Trijatmiko KR, Marsh-Martinez N, Krishnan A, Nataraja KN, Udayakumar M, Pereira A (2007) Improvement of water use efficiency

in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene. PROC NATL ACAD SCI USA **104**: 15270-15275.

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004) A combination of *Arabidopsis* DREB1A gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stressed tolerance in tobacco by gene transfer. Plant Cell Physiol **45**: 346-350.

Khanna-Chopra M, Khanna-Chopra R (2004) Osmotic adjustment of chickpea in relation to seed yield and yield parameters. Crop Sci **44**: 449-455.

Kirigwi FM, Van Ginkel M, Trethowan R, Sears RG, Rajaram S, Paulsen GM (2004) Evaluation of selection strategies for wheat adaptation across water regimes. Euphytica **135**: 361-371.

Kotabova E, Kana R Helena, Kyselakova K, Lipova L, Novak O, Ilik P (2008) A pronounced light-induced zeaxanthin formation accompanied by an unusually slight increase in non-photochemical quenching: A study with barley leaves treated with methyl viologen at moderate light. J Plant Physiol **165**: 1563-1571.

Lanceras JC, Pantuwan G, Jongdee B, Toojinda T (2004) Quantitative trait loci associated with drought tolerance at reproductive stage in rice. Plant Physiol **135**: 384-399.

Laporte MM, Shen B, Tarzynski MC (2002) Senescing for drought avoidance: expression of maize NADP-malic enzyme in tobacco results in altered stomatal function. J Exp Bot **53**: 699-670.

Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ **25**: 275-294.

Lee Y-P, Kim S-H, Bang J-W, Lee H-S, Kwak S-S, Kwon S-Y (2007) Enhanced tolerance to oxidative stress in transgenic plants expressing three antioxidant enzymes in chloroplasts. Plant Cell Rep **26**: 591-598.

Levitt J (1972) Responses of Plants to Environmental Stresses. Academic Press, New York.

Li L, Van Staden J, Jager AK (1998) Effects of plant growth regulators on the antioxidant system in seedlings of two maize cultivars subjected to water stress. Plant Growth Regul **25**: 81-87.

Liu F, Jensen CR, Shahanzari A, Anderson MN, Jacosen S-E (2005) ABA-regulated stomatal control and photosynthetic water use efficiency in potato (*Solanum tuberosum*) during progressive soil drying. Plant Sci **168**: 831-836.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 binding domain separate two cellular signals transduction pathways in drought- and low-temperature responsive genes in Arabidopsis. Plant Cell **10**: 1391-1406.

Margulies M, Egholm M, Altman WE et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature **437**: 376-380.

Masle J, Gilmore SR, Farquhar GD (2005) The *ERECTA* gene regulates plant transpiration efficiency in Arabidopsis. Nature **436**: 866-870.

Meyer Y, Buchanan BB, Vignols F, Reicheld J-P (2009) Theoredoxins and glutaredoxins: unifying elements in redox biology. Annu Rev Genet **43**: 335-367.

Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P (2006) An Arabidosis glutathione peroxidase functions as both a redox transducer and scavenger in abscisic acid and drought stress response. Plant Cell **18**: 2749-2766.

Mittler R (2002) Antoixodants and stress tolerance. Trends Plant Sci **7**: 405-410.

Moran JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV, Aparicio-Tejo P (1994) Drought induces oxidative stress in pea plants. Planta **194**: 346-352.

Munne-Bosch S, Penuelas J (2003) Photo- and antioxidative protection, and a role for salicyclic acid during drought and recovery in field-grown *Phillyrea angustifolia* plants. Planta **217**: 758-766.

Mustilli AN, Merlot S, Vavasseur A, Fenzi F, Giraudat J (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell **14**: 3089-3099.

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. Plant Physiol **149**: 88-95.

Neill SJ,Burnett EC (1999) Regulation of gene expression during water deficit stress. Plant Growth Regul **29**: 23-33.

Nguyen HT, Babu RC, Blum A (1997) Breeding for drought resistance in rice: physiology and molecular consideration. Crop Sci **37**: 1426-1438.

Pandey R, Agarwal RM, Jeevaratnam K, Sharma GL (2004) Osmotic stress-induced alterations in rice *(Oryza sativa* L.) and recovery on stress release. Plant Growth Regul **42**: 79-84.

Panta GR, Rieger MW, Rowland LJ (2001) Effect of cold and drought stress on blueberry dehydrin accumulation. J Hortic Sci Biotech **76**: 549-556.

Pastori MM, Trippi VS (1992) Oxidative stress induces high rate of glutathione synthesis in a drought-resistant maize strain. Plant Cell Physiol **33**: 957-961.

Pattanagul W, Madore MA (1999) Water deficit effects on raffinose family oligosaccharide metabolism of coleus. Plant Physiol **121**: 987-993.

Pellegrineschi A, Reynolds M, Pachew M, Breto RM, Almeraya R, Yamaguchi-Shinozaki K, Hoisington D (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1 gene delay water stress symptoms under greenhouse conditions. Genome **47**: 493-500.

Pelleschi S, Rocher JP, Prioul JL (1997) Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. Plant Cell Environ **20**: 493-503.

Pratt LH., Liang C, Shah M, Sun F, Wang H, Reid HP, W, Gingle WAR, Paterson AH, Win R, Dean R, Kelin R, Nguyen HT, Ma H, Zhao X, Morishige DT, Mullet JE, Cordonnier-Pratt M (2005) Sorghum expressed sequence tags identify signature genes for drought, pathogenesis, and skotomorphogenesis from a milestone set of 16,801 unique transcripts. Plant Physiol **139**: 869–884.

Price AH, Cairas JE, Horton P, Jones H, Griffiths H (2002) Linking drought resistance mechanisms to avoidance in upland rice using QTL approach: progress and new opportunities to integrate stomatal and mesophyll resistance responses. J Exp Bot **53**: 989-1004.

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol **133**: 1755-1767.

Reyes JL, Compos F, Wei H, Arora R, Yang Y, Karlson DT, Cavarrubias AA (2008) Functional dissection of hydrophillins during in vitro freeze protection. Plant Cell Environ **31**: 1781-1790.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc Natl Acad Sci USA **104**: 19631-19636.

Romo S, E. Labrador E, Dopico B (2001) Water stress-regulated gene expression in *Cicer arietinum* seedlings and plants. Plant Physiol Bioch 39: 1017-1026.

Saccardy K, Cornic G, Brulfert J, Reyss A (1996) Effect of drought stress on net CO_2 uptake by *Zea* leaves. Planta **199**: 589-595.

Sairam RK, Deshmukh PS, Saxena DC (1998) Role of antioxidant systems in wheat genotypes tolerance to water stress. Biol Plantarum **41**: 387-394.

Sato Y, Yokoya S (2008) Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHsp17.7. Plant Cell Rep **27**: 329-334.

Sayed OH (1996) Adaptational responses of *Zygophyllum qatarene* Hadidi to stress conditions in a desert environment. J Arid Environ **32**: 445-452.

Schroeder JI, Kwak JM, Allen GJ (2001) Guard cell abscisic acid signaling and engineering drought hardiness in plants. Nature **410:** 327-330.

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hiyashizaki Y, Shinozaki K (2001) Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell **13**: 61-72.

Seki M, Umezewa T, Urano K, Shinizaki K (2007) Regulatory metabolic networks in drought stress response. Curr Opin Plant Biol **10**: 296-302.

Sminorff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol **125**: 27-58.

Stockginger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to C-repeats/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA **94**: 1035-1040.

Stuhlfauth T, Steuer B, Fock HP (1990) Chlorophyll and carotenoids under water stress and their relation to primary metabolism. Photosynthetica **24**: 412-418.

Sun X, Fontaine JM, Rest JS, Shelden EA, Welsh MJ, Benndorf R (2004) Interaction of Human HSP22 (HSPB8) with Other Small Heat Shock Proteins. J. Biol Chem 279: 2394-2402.

Sunkar R, Zhou X, Zheng Y, Zhang W, Zhu J-K (2008) Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biology **8**: doi:10.1186/1471-2229-8-25.

Swindale LD, Bidinger FR (1981) The human consequences of drought and crop research priorities for their alleviation. pp. 2-13. *In* Paleg, L. G., and D. Aspinal. (eds.) The Physiology and Biochemistry of Drought Resistance in Plants. Academic Press. Sydney.

Taiz L, Zeiger E (1998) Plant Physiology. Sinauer Associates, Inc. Publishers. Sunderland, Massachusetts.

Tang L, Kwon S-Y, Kim S-H, Kim J-S, Choi JS, Cho KY, Sung CK, Kwak SS, Lee HS (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide

dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. Plant Cell Rep **25**: 1380-1386.

Tezara W, Mitchel VJ, Driscoli SD, Lawlor DW (1999) Water deficit inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature **401**: 914-917.

Thomson JE, Legge RL, Barber RF (1987) The role of free radicals in senescence and wounding. New Phytol **105**: 317-344.

Torres-Franklin ML, Contour-Ansel D, Zuily-Fodil Y, Pham-Thi A-T (2006) Molecular cloning of glutathione reductase cDNAs and analysis of GR gene expression in cowpea and common bean leaves during recovery from moderate drought stress. J Plant Physiol **165**: 514-521.

Tuberosa R, Salvi S (2006) Genomics-based approaches to improve drought tolerance of crops. Trends Plant Sci 11: 404-411.

Turkan I, Bor M, Ozdemir F, Koca H (2005) Differential responses of lipid peroxidation and antioxidants in the leaves of drought tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethelene glycol mediated water stress. Plant Sci **168**: 223-231.

Turner NC (1979) Drought resistance and adaptation to water deficit in crop plants. pp. 343-372. In H. Mussell and R. Staples (eds). Stress Physiology in Crop Plants. John Wiley and Sons. New York.

Tuskan GA, Difazio S, Jansson S, et al. 2006. The genome of black cottonwood *Populus trichocarpa* (Torr and Gray). Science **313**: 1596-604.

Umezewa T, Fujita M, Fujita Y, Yamaguchi-Shinozaky K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes unlock the truth. Curr Opin Plant Biotech **17**: 113-122.

Vamerali T, Saccomani M, Bons S, Mosca G, Guarise M, Ganis M (2003) A comparison of root characteristics in relation to nutrient and water stress in two maize hybrids. Plant Soil **255**: 157-167.

Vasquez-Robinet C, Mane SP, Ulanov AV, Watkinson JI, Stromber VK, De Koeyer D, Schafleitner R, Willmot DB, Bonierbale M, Bohnert HJ, Grene R (2008) Physiological and molecular adaptation to drought in Andean potato genotypes. J Exp Bot **59**: 2109-2123.

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu J-K (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J **45**: 523-539.

Vicre M, Farrant JM, Driovich A (2004) Insights into the cellular mechanisms of dessication tolerance among angiosperm resurrection plant. Plant Cell Rep **27**: 1329-1340.

Viner RI, Clegg JS (2001) influence of trehalose on the molecular activity of p26, a small heat shock/crystallin protein. Cell Stress Chaperon **6**: 126-135.

Wang W, Vinocur B, Shoseyou O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci **9**: 244-252.

Wang Y, Jiang J, Zhao X, Liu G, Yang C, Zhan L (2006) A novel LEA gene from *Tamarix androssowii* confers drought tolerance in transgenic tobacco. Plant Sci **171**: 655-662.

Xiao H, Nassuth A (2006) Stress- and development induced expression of spliced and unspliced transcripts from two highly similar dehydrin1 genes in *V. riparia* and *V. vinifera*. Plant Cell Rep **25**: 968-977.

Xiao X, Huang Y, Tang N, Xiong L (2007) Overexpression of a *LEA* gene in rice improves drought resistance under field conditions. Theor Appl Genet **115**: 35-46.

Xiong L, Wang RG, Mao G, Koczan J (2006) Identification of drought tolerance determinants by genetic analysis of root responses to drought stress and abscisic acid. Plant Physiol **142**: 1065-1074.

Xu D, Duan X, Wang B, Hong B, Ho TD, Wu R (1996) Expression of a late abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol **110**: 249-257.

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stress. Annu Rev Plant Biol **57**: 781-803.

Yu J, Hu S, Wang J et al. (2002) A draft sequence of the rice genome (Oryza sativa L. ssp. indica). Science **296**: 79-92.

Yue B, Xue W, Xiong L, Yue X, Luo X, Cui K, Jin D, Xing X, Zhang Q (2005) Genetic basis of drought resistance at reproductive stage in rice: separation of drought tolerance from drought avoidance. Genetics **10**: 1213-1228.

Zhang J, Kirkham MB (1996) Enzymatic responses of the ascorbate-glutathione cycle to drought in sorghum and sunflower. Plant Sci **113**: 139-147.

Zhou J, Wang X, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong X, Zhang Q, Fan L, Deng XW (2007) Global genome expression of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol **63**: 591-608.

Chapter 2. Physiological and Genomic Responses to Progressive Drought Stress in Rice at a Reproductive Stage of Development

Abstract

Drought stress reduces yield in rice (*Oryza sativa*), especially when it coincides with reproductive development. A most sensitive stage is when the panicle is "in the boot" and up to 10 d after heading. In this work, moderate (1 d with leaves rolled) or severe (2 d with leaves rolled) drought stress was applied at panicle emergence, and yield components and changes in gene expression were studied in the cultivar 'Nipponbare'. Depending on the severity of drought, grain yield was reduced by as much as 50%. The reduction was associated with decreases in two yield components: number of spikelets reduced by 20 to 33%, and spikelet fertility ratio reduced by 33 to 50%. Drought also affected vegetative development (leaf "firing" and shoot biomass) and the harvest index. Microarray was used to analyze global gene expression in vegetative (leaf) and reproductive (panicle) tissues and revealed that more than 20% of the genome responded to drought. Comparative analysis of the expressed genes indicated that the vegetative and reproductive tissues responded differentially to drought stress. This indicates that there is organ-specific regulation in response to drought, mediated by organ-specific transcription responses. Alternatively, drought stress was not sensed equally between the two tissues. Novel genes identified in this study can be further assessed for their potential contribution to drought resistance by genetic transformation approaches.

2.1. Introduction

Drought stress can seriously limit rice production and yield stability in rain-fed lowland areas (Dey and Upadyaya, 1996). Yield in rice is ultimately a product of several components: plants per unit area, panicles per plant, fertile spikelets per panicle, and average caryopsis grain weight. Drought most dramatically affects yield – primarily at the spikelet level – when the drought stress takes place during the particularly sensitive stage between booting and 10 d after heading (Selote and Chana-Chopra, 2004). Grain yield reductions due to water deficits at a flowering stage are mainly caused by a reduction in the number of filled, i.e., fertile, spikelets per panicle, without a substantial decrease in spikelet number per panicle (Ekanayake et al., 1989; O'Toole and Namuco, 1983). This observation implies that water deficit affects grain number as determined by fertilization and grain initiation and development. However, the number of spikelets (and consequently grains) can also be reduced if stress induces abscission of spikelets (Ekanayake et al., 1989; O'Toole and Namuco, 1983).

In cereals, drought stress during the most sensitive developmental stage can interfere with reproductive success (and yield) by arresting the development of the male and female gametophytes, preventing fertilization and fruit set, and/or inducing premature abortion of fertilized ovules. In the female tissue, this most sensitive period coincides with meiosis in the megaspore mother cell and subsequent degeneration of the three redundant megaspores in the tetrad (Saini, 1997). Male reproductive development in cereals, particularly between the time of meiosis and fertilization, is likewise highly sensitive to drought, which can cause a serious reduction in grain number and therefore yield. In rice, which is likely the most sensitive cereal in this regard (O'Toole and Moya, 1981), water deficits during anthesis can inhibit anther dehiscence and pollen germination and reduce pollen viability (O'Toole and Namuco, 1983;

Ekanayake et al., 1990) – again reducing grain number. Similar observations have been made in oats (*Avena sativa*) (Sandhu and Horton, 1977) and wheat (*Triticum aestivum*) (Sheoran and Saini, 1996).

Spikelet opening (necessary for successful cross-pollination and development of fertile spikelets) can also be inhibited by untimely drought stress. Drought stress between boot stage and anthesis can affect the degree of expansion of the panicle, leading to formation of sterile spikelets. Under drought stress, the elongation of the panicle and supporting tissues can be delayed such that anthesis (pollen release usually coinciding with stigma exertion) occurs while part of the panicle is still within the leaf sheath. These unexposed spikelets will be sterile.

Plants respond to drought stress at physiological, biochemical, and molecular levels. The response depends on species, genotype, length and severity of water deprivation, stage of development (as already mentioned), and the tissues involved. Under drought stress, these responses may be triggered by the production of abscisic acid (ABA) and reactive oxygen species (ROS), or intracellular messengers such as phospholipids. Recent studies (Hao et al., 2008; Zhang et al., 2007; Xing et al., 2008) suggested that ABA-induced H_2O_2 production mediates nitric oxide (NO) generation, which, in turn activates mitogen-activated protein kinase (MAPK) cascades and eventually results in the up-regulation of antioxidant enzymes and downstream processes. The ABA downstream messengers, or signals, generated by tissues exposed to drought act in the coordination and execution of plant stress responses that include metabolic and developmental adjustments.

In wheat, and likely in all cereals, the ABA produced upon turgor loss in vegetative tissues can be translocated to the inflorescence (panicle), where it accumulates in meiotic spikelets and anthers (Morgan 1980). High ABA levels in developing reproductive structures

may inhibit cell division and impair floret and then seed development with obvious consequences on yield potential; and ABA can also act more directly as an abscission/abortion agent.

Drought can play other, more indirect roles on yield components via its impact on photosynthesis and subsequent levels of sugars. In general, under moderate drought stress, soluble sugars tend to increase (Chaves, 1991; Porcel and Ruiz-Lozano, 2004), while starch content decreases (Chaves, 1991). Under severe dehydration, soluble sugars in transport decrease (Pinheiro et al., 2001). An insufficient supply of sugars can, in essence, "starve" the plant and arrest development of reproductive structures, causing abortion of spikelets, florets, or grains (Makela et al., 2005). In maize (*Zea mays*), reduction in sugar content of ovary wall cells is caused by the curtailing of sucrose delivery, accompanied by depletion of glucose, as starch is consumed in the ovaries (Makela et al., 2005; Mclaughlin and Boyer, 2004).

Drought can also induce oxidative stress through generation of ROS such as superoxide radical ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) (Cruz de Carvalho, 2008; Smirnoff, 1993). The presence of ROS can induce free radical scavengers and other protective mechanisms; but, simultaneously, the ROS can produce injury. Because ABA was shown to induce H_2O_2 production (Hao et al., 2008; Guan et al., 2000; Pei et al., 2000; Xing et al., 2008; Zhang et al., 2007), that particular ROS may be an intermediate signal for ABA in mediating expression of genes involved in ABA signaling and downstream processes. It is clear that ROS contribute to stress damage, as evidenced by observations that transgenic plants over-expressing ROS scavengers, or mutants with higher ROS scavenging ability, show increased tolerance to stress (Bohnert and Sheveleva, 1998; Kocsy et al., 2001). This agrees with the observation that ROS in drought-stressed rice caused spikelet sterility more in a drought-sensitive than in a

drought-resistant cultivar, where the drought-tolerant plants exhibited higher ROS scavenging ability than the sensitive ones (Selote and Khana-Chopra 2004). Drought-induced ABA accumulation can also induce ROS production, and this can bring about leaf senescence (sometimes described as "firing") when antioxidants cannot deal with rapid and uncontrolled accumulation of the ROS (Munne-Bosch and Alegre, 2004). Abscision of organs such as leaves (Roberts et al., 2002) and probably flowers marks the end of senescence. The loss of leaves, which provide photoassimilates to the rest of the plant and flowers, can cause reproductive failure.

Experiments using microarray technology have identified several genes that are induced by abiotic stresses, including drought. These genes can be classified into those that encode products to: 1) directly protect plant cells against stresses or 2) regulate gene expression and signal transduction (Shinozaki et al., 2003; Zhou et al., 2007). Genome-wide identification of genes regulated by drought stress can provide a more comprehensive understanding of the transcriptional responses to drought. Such responses are putatively involved in protecting the plant from continuing drought, through acclimation or other induced mechanisms. This can be a starting point to identify the individual genes, which is essential for future breeding and genetic engineering applications.

Previous studies have identified various transcriptional responses to drought and related stresses in rice (Rabbani et al., 2003; Yazaki et al., 2003; Yazaki et al., 2004; Zhou et al., 2007). However, these studies were on callus or seedlings treated with ABA (Rabbani et al., 2003; Yazaki et al., 2003; Yazaki et al., 2004) or on plants grown on Hoagland's solution with their roots on filter paper (Zhou et al., 2007). In such cases, the drought stress has been rather "artificially" or "instantly" imposed, without a gradual decrease in water potential for the soil

and plant system. Genomic data from experiments that induce water deficit by gradual soil drying in mature rice plants are limited. It is thus unknown to what degree responses to "shock treatments" may be indicative of a plant's capacity to adapt to gradual change in the water potential of the soil. Therefore, a comparative analysis of rice plants subjected to a slow decrease in soil moisture (progressive drought), presumably leading to gradual changes in sensing and signaling of a drought episode, is important to appropriately measure transcriptional responses. The literature and our results here show that physiologically imposed drought stresses at the panicle-emergence stage can greatly reduce yield. Differential gene expression data developed here show that vegetative and reproductive tissues respond differently to drought at this key stage of development. This indicates that there is organ-specific regulation in response to drought, which is mediated by organ-specific transcription responses. Alternatively, drought stress was not sensed equally between the two tissues.

2.2. Materials and Methods

2.2.1. Physiology and Yield Experiment

2.2.1.1. Plant Material and Drought Stress Treatments

Rice (*O. sativa* subspecies *japonica* cv. 'Nipponbare') was grown in the greenhouse in twelve 500-ml pots (one plant per pot) filled with a 1:1 mixture of topsoil and compost (mixture of sphagnum peat moss, vermiculite, and processed bark ash) (Scott-Sierra Horticultural Product Co, Marysville, OH). Pots were placed in a tray filled with water to simulate paddy/flooded conditions. Plants were supplied with a general-purpose fertilizer, 20-20-20, (Scott-Sierra Horticultural Product Co, Marysville, OH) dissolved in water to provide 50 kg each of N, P_2O_5, and K_2O ha^{-1}. Fertilizer application was done twice a month throughout the growing period. Plants were grown under well-watered/flooded conditions until they reached the R4 "boot" stage (when the panicle began to emerge on the main, or primary, culm) (Counce et al., 2000).

When plants reached the R4 stage, eight pots were randomly selected (but blocked according to the day they arrived at R4), removed from the flooded trays, and irrigation was withheld. Four of these were subjected to "moderate drought stress" by withholding irrigation for 7 d, which included 1 d with leaves rolled. The other four were exposed to "severe drought stress", withholding irrigation for 8 d, which included 2 d with leaves rolled. (Preliminary experiments showed that 3 d with leaves rolled during anthesis essentially inhibited all seed set. Therefore 2 d of leaf rolling is being operationally defined as "severe" drought stress, and 1 d leaf rolling as "moderate" drought stress.) Pots for the severe drought stress treatment had irrigation withdrawn 1 d before the moderate drought stress pots, such that subsequent sampling for all treatments was carried out on the same day. The rest of the plants (the other four pots) were the well-watered controls and were irrigated/flooded throughout the experimental period.

This experiment was conducted between April and August (approx. 14-h photoperiod), and the greenhouse temperatures were moderated near 28/22^{o}C day/night.

2.2.1.2. Measurements of Soil Moisture Content, Chlorophyll Fluorescence, and Plant Water Status and Post-Drought Irrigation

A Delta-T theta soil moisture probe (ML2X, Dynamax, Houston, TX) was calibrated by comparison with gravimetric water content measurements from saturation to permanent wilting point for the potting mixture. Gravimetric water contents were obtained from conventional oven-dry weights and multiplied by bulk density (Qiu et al., 2001). This allowed the Delta-T theta soil moisture probe to provide an indirect determination of soil moisture content (SMC) and soil water potential using water release curves following procedures of Olsen (1974).

At the end of the drought period, volumetric soil moisture content was determined with the Delta-T theta probe. Chlorophyll fluorescence was measured using a pulse amplitude modulated fluorometer (OS1-FL, Optiscience, Hudson, NH). Photochemical efficiency of photosystem II (Fv/Fm) was measured on 15-min-dark-adapted youngest fully expanded leaves, while effective quantum yield of photo-system II (ØPS II) was measured on the same but light-adapted leaf (Moradi and Ismail, 2007). Saturating light pulses of >2600 μmol m^{-2} s^{-1} and 600 ms duration were used to determine fluorescence for the dark- and light-adapted leaves. Plant water status was monitored by determination of relative water content (RWC%) according to Smart (1974). Briefly, the same leaf used for Fv/Fm and ØPS II was excised, and an approximately 6-cm section had its fresh weight (FW) determined immediately. The leaf sections were then floated in deionized water at room temperature for 6 h, and their rehydrated weight (RW) was determined. Finally, they were dried in an oven at 70^{o}C overnight and weighed to obtain the dry weight (DW). The RWC% was calculated as: RWC% = (FW - DW)/(RW - DW) x 100.

Drought-stressed plants were re-watered after the 7 or 8 d without irrigation; and, after 3 d, the number of leaves "fired" (leaves with at least 50% of their lamina exhibiting yellowing or necrosis) was recorded. Percent of leaves fired (LF%) was calculated as the ratio of leaves fired to total number of leaves on the plant. The plants were further maintained under well-watered/flooded conditions until they reached grain ripeness, and additional measurements were made at that time (see below).

The experiment was a randomized complete block (RCB) design, with four replications and with single plants (single pots) as the experimental unit. Blocks were created sequentially to account for the variation in staging to R4. Block 1 (with its three treatments) consisted of three plants (pots) that arrived at R4 together, and so forth. Data were subjected to analysis of variance (ANOVA) using the general linear model (Proc GLM) of Statistical Analysis System (SAS). Differences between means were tested by the Least Significance Difference with a 0.05 threshold ($LSD_{0.05}$).

2.2.1.3. Measurements of Spikelet Fertility and Yield Traits

Plants reached maturity (grain was ripe) by 20 weeks after planting. The panicles on the main culm were excised at the point of peduncle bifurcation, and the number of filled (contained a grain) and empty spikelets was recorded. The grains were removed by hand, dried at 37^{o}C for 7 d, weighed, and counted. The peduncle (the upper internode) length (PL) was measured to the point of panicle attachment. The main culms (less their panicles) were harvested at ground level and dried at 70^{o}C for 72 h and then weighed to determine the shoot biomass (SB).

The yield components assessed were total spikelets (both fertile and sterile) per panicle (SP), fertile spikelet ratio (FS) (fertile spikelets divided by total spikelets), grain yield (GY) (weight of dried grain), and single grain weight (SGW) (GY divided by number of grains). The

harvest index (HI) was calculated as the ratio of total grain weight (GY) to total above ground dry weight. A grain in this case means whole grain (or caryopsis) with hulls (palea and lemma).

2.2.2. Rice Drought Transcriptome Experiment

This experiment was, in general, a repeat of the previous one, but with transcriptomic data also collected. The plant material and drought stress treatments and key physiological measurements were as in 2.2.1., except that there was no moderate drought stress treatment and only three replications. Half of the plants were exposed to severe drought stress (withholding irrigation for 8 d, to include 2 d of leaf rolling), and the others were well watered throughout the experimental period. Plants were not taken to maturity, and grain production traits were not examined in this experiment.

On the second day of leaf rolling, the leaf immediately below the flag leaf (hereafter described as "vegetative tissue") and the panicle (hereafter described as "reproductive tissue") were excised from the main/primary culms and immediately put in liquid N_2 and stored at -80^{o}C before RNA isolation. Comparable tissues were taken at the same time from the blocked well-watered controls. RNA was isolated from the tissues with RNeasy plant minikit (Qiagen, Valencia, CA) following manufacturer's instructions. Total RNA was sent to Virginia Bioinformatics Institute (VBI) core laboratory facility, which carried out quality control analysis, cDNA synthesis, and hybridization to the GeneChip Rice Genome Array (Affymetrix) containing probes to query approximately 34000 transcripts. The probe arrays were scanned according to the Affymetrix Microarray Suite (Choe et al., 2005).

Raw numeric values representing the signal of each feature were imported into AffylmGUI (Wettenhall et al., 2006); and the data were background corrected, normalized, and summarized using Robust Multiarray Averaging (RMA) (Irizarry et al., 2003). A linear model

was then used to average data between replicate arrays and to detect differential expression (Smyth and Speed, 2004).The *P* values from the test were converted to *Q* values to correct for multiple hypothesis testing (Storey and Tibshirani, 2003) using the Q-value software (http://faculty.washington.edu/jstorey; accessed 01/10/2010). Genes with $Q<0.05$ were declared as differentially expressed. Gene ontology (GO) analysis was performed with the web based EASYGO software (http://bioinformatics.cau.edu.cn/easygo/; accessed 01/10/2010) in the set of differentially expressed genes in each treatment (Zhou and Su, 2007). The analysis was performed on the aspect of biological processes using the hypergeometric test with false discovery rate (FDR) cut-off = 0.05, and mapping count cut-off = 5. Terms with $P<0.05$ were declared significantly enriched.

2.3. Results

2.3.1. Effect of Drought on Soil Moisture and Plant Physiological Responses

The mild and severe drought treatments decreased soil moisture from 0.44 cm^3/cm^3 (control) to 0.13 and 0.11 cm^3/cm^3, 28% and 23% of the control, respectively (Table 2.1). These three levels of moisture correspond to soil water potentials of -0.03 MPa (control), -0.66 MPa (moderate drought), and -1.03 MPa (severe drought). The plant physiological responses were also significantly affected by the two levels of drought stress (Table 2.1). Relative water content (RWC%) was reduced by 20% under moderate drought and 34% under severe drought treatment, while the percentage of leaf firing (LF) was significantly increased to 25% and 50% of the control under mild and severe drought, respectively. Drought stress also affected the quantum yield of photosystem II (ØPS II), but photochemical efficiency (Fv/Fm) was not significantly altered.

2.3.2. Effect of Drought on Shoot Biomass and Grain Yield Components

As expected, when plants were exposed to drought at panicle emergence, the shoot biomass (SB) was significantly reduced (Table 2.2). Several allometric parameters (spikelets/panicle (SP), fertile spikelet ratio (FS), grain yield (GY), and harvest index (HI)) were reduced by drought stress; but single-grain weight (SGW) was not affected. Reductions in key yield components besides SGW ranged from 15% to 50% following moderate drought and from 20% to 70% following severe drought (Figure 2.1). While yield components were severely affected by drought during anthesis, the vegetative parts of the plants (shoot and peduncle) were less affected. The reductions in shoot biomass and peduncle length (PL) were as low as 11% and 13% respectively under severe drought stress. In short, drought stress applied at the heading stage reduced grain production much more than vegetative growth.

2.3.3. The Rice Drought Transcriptome

A second experiment partially repeated the previous study but focused more on transcriptome analysis. Table 2.3 shows that a severe drought stress (2 d with leaf rolling) invoked physiological responses (SMC, Fv/Fm, QPS II, and RWC%) in the same range as the earlier study. This suggests that the plants in the transcriptome study can also be characterized as experiencing severe drought stress.

To identify drought-responsive genes, RNA from vegetative (leaves) and reproductive (panicles) tissues was analyzed via Affymetrix gene chips. Venn diagrams illustrate the general relationships between the drought responses in the vegetative and reproductive parts of rice (Figure 2.2). Numbers of genes significantly up- or down-regulated by drought in each tissue are shown. Over 11500 genes were differentially expressed in the vegetative (leaf) tissues, with 5812 up-regulated and 5750 down-regulated by drought stress. In the reproductive (panicle) tissues, almost 6000 genes were differentially expressed, with 3548 up-regulated and 2442 down-regulated. Of the up-regulated genes, 1486 were up-regulated in common in both reproductive and vegetative parts.

Over 3600 responsive (up- and down-regulated) genes (about 30% of the total responsive genes) coded for proteins of unknown function, but many genes known to respond to drought in other species were recovered by this microarray analysis. The annotations for some of the genes suggest that they produce regulatory proteins, enzymes involved in diverse metabolic pathways, transporters, and structural proteins. The up-regulated genes include a protein kinase (Os01g50400), a phosphatase (Os06g37660), a dehydrin (Os11g26780), a late embryogenesis abundant (LEA) protein (Os05g46480), a heat shock protein (Os03g16920), an ABA responsive (Os12g29400) gene, an ethylene responsive protein (Os02g47840), and an aquaporin

(Os10g35050) – all of which have been shown to function in drought responses in various species. Among the down-regulated genes, many are also known to be associated with drought or dehydration response. Some of the same functional annotations noted above are also represented in the down-regulated genes. Examples of these genes include a protein kinase (Os10g04720) and a phosphatase (Os01g51890). The down-regulated list also includes genes coding for enzymes involved in photosynthesis and related processes, such as a chlorophyll a-b binding protein (Os05g50340), a photosystem II protein (OS03g21560), and a chloroplast precursor (Os01g55570). Also down-regulated are genes involved in gene expression and related processes to include a ribosome binding factor (Os06g04610) and a ribosome biogenesis protein (Os02g18830).

To look more closely at the biological processes involved in drought stress responses in rice, gene ontology (GO) analyses were performed using the differentially regulated genes, including both differentially up- and down-regulated genes. The results of these analyses are presented Venn diagram style in Figure 2.2. The differentially expressed genes are involved in diverse processes such as metabolism, gene expression, and signal transduction. Over 160 drought-affected processes were detected: 99 in the vegetative tissues only, 17 in reproductive tissues only, and 23 in both tissues. Responses to water stress (GO:0009415), transport (GO:0006810), amino acid metabolism (GO:0006520), and carbohydrate metabolism (GO:0005975) among others were induced in common. On the other hand, almost and equal number of biological processes were down-regulated by drought. Of those, 28 were down-regulated in common, while 65 and 36 processes were down-regulated only in the vegetative or reproductive tissue, respectively. The biological processes down-regulated in common included among others, photosynthesis (GO:0015979, GO:0009765), transport (GO:0006810), gene

expression (GO:0010467), and diverse metabolic processes involved in carbohydrate (GO: 0005975, GO: 0044262, GO: 00116052) and amino acid (GO:0006519 and GO:0006520) metabolism. The two tissues clearly responded differently to drought stress, as there were processes associated with drought that were induced in one tissue and not the other. These included responses to oxidative stress (GO:0006979) induced in only reproductive tissue and polyol metabolism (GO:0019751) induced only in vegetative tissue.

2.4. Discussion

2.4.1. Drought Stress at Panicle Emergence Reduces Yield

In controlled experiments, withholding irrigation resulted in a reduction of soil moisture and soil water potential. This caused the plants to experience drought stress as shown by reductions in relative water content (RWC%) and quantum yield of photosystem II (ØPS II). A reduction in RWC% would correlate with much lowered water potentials in the vegetative and reproductive tissues, resulting in drought stress. By design, the leaves of drought-stressed plants were rolled for 1 or 2 d, very visible evidence of stress. The subsequent firing of leaves and very dramatic reductions in yield were further evidence, or residuals, of the drought stress.

The effects of drought stress on physiology are well documented. When plants experience water deficits, stomatal pores progressively close (Lawlor and Cornic, 2002; Saccardy et al., 1996; Tezara et al., 1999). The closing is regulated largely by leaf water potential but can be mediated by ABA. Stomatal closure leads to decreases in photosynthetic CO_2 assimilation due to restricted diffusion of CO_2 into the leaf and altered CO_2 metabolism. Pelleschi et al. (1997) found that reduced CO_2 diffusion during stomatal closure is mainly responsible for the decline in photosynthesis in C_3 plants subjected to dehydration. However Tezara et al. (1999) reported that, in sunflower (*Helianthus annuus*) (a C_3 plant) under water stress, the photosynthetic rate is limited more by altered CO_2 metabolism than by reduced diffusion. The lower CO_2 availability inhibits carbon assimilation, and ultimately photosynthetic capacity is lost as a consequence of the reduced stomatal conductance and/or direct damage to carbon metabolism (Bartels and Salamini, 2001; Colom and Vazzana, 2003). In addition, closure of stomata as a result of water deficit and consequent decrease in CO_2 concentration in the leaf mesophyll results in the accumulation of NADPH in the chloroplasts. Under such conditions,

where NADP is limiting, ROS can be generated (Baisak et al., 1994; Gamble and Burke, 1984; Hao et al., 2008). The ROS are highly toxic and can damage important cellular molecules such as lipids, proteins, nucleic acids, and chlorophyll (Cruz de Carvalho, 2008; Fu and Huang, 2001; Li et al., 1998; Zhang and Kirkham, 1996; Thomson et al., 1987).

Previous studies have shown that low ØPS II of drought-stressed rice plants was associated with a lower efficiency of photosystem II (Fv/Fm) (Garg et al., 2002). However, in the present study, the Fv/Fm was not reduced, while ØPS II was. Pieters and El Souki (2005) reported similar results in rice. They suggested that, compared to a more drought-sensitive variety, in a more drought-tolerant variety, Fv/Fm is less important in determining the decrease in ØPS II. Rather a decreased photochemical quenching of chlorophyll a fluorescence (qP) is linked to degradation of reaction center protein D1 (part of the photosystem II complex). D1 protein turnover plays an important role in the regulation of PS II electron transport and represents a major target for photo-induced damage under water stress.

The increase in leaf "firing" (LF) and reduction in shoot biomass (SB) due to water stress could be a result of processes responding to ABA accumulation, reduction in photosynthesis (as indicated by low ØPS II), and/or tissue death due to ROS (Munne-Bosch and Alegre, 2004).

Grain production traits were significantly reduced by drought at both levels of stress. Drought reduced spikelet number (SP), fertility of spikelets (FS), grain yield (GY), and harvest index (HI) but not single-grain weight (SGW). Rice grain yield can be assessed at the culm or tiller level using SP, FS, and SGW (Kim et al., 2009). The number of spikelets is determined soon after formation of the panicle and spikelet meristems (Sakamoto and Matsuoka, 2008). Drought stress during the earlier stages affects number of panicles and SP, while drought exposure at later reproductive stages affects FS (Kim et al., 2009). However, in this study, it was

observed that SP was reduced when drought was applied at panicle emergence (Table 2.2), and at this stage all the spikelets are formed. This implies that spikelets were aborted in response to drought. The same processes that affect the vegetative tissues (ROS generation and damage to tissues) could cause spikelet abortion. In addition, dehydration may occur to exposed lemma, palea, and anthers as they are exserted from the glumes. Furthermore, the spikelets are reported to be sensitive to high temperatures, due to drying of the floral parts that can cause sterility (Ekanayake et al., 1989). The reduced spikelet number and spikelet fertility ratio under drought may have been caused by loss of spikelets (due to abscission) and sterility of the remaining floral parts.

Tissue water deficits can directly reduce turgor, which can affect cell expansion (Hsiao, 1973). The hydrostatic pressure exerted by the lodicules and elongating anthers on the glumes cause floret opening in rice. Turgor potential is positively correlated with anthesis in rice (Ekanayake et al., 1989). Therefore, low turgor in a drought situation could inhibit flower opening, and this could affect pollination and fertilization, leading to low FS.

It appears that rice has a pre-determined period when peduncle elongation occurs, and anthesis follows about a day thereafter (O'Toole and Moya, 1981). Drought can slow peduncle elongation due to reduced turgor, and this may occur while part of the panicle is still within the leaf sheath. Unexserted spikelets will be sterile (Liu et al., 2006; Ji et al., 2005). In fact, final peduncle elongation was affected in this study, and the observed reduction in GR (Figure 2.1; Table 2.2) may be a result of spikelet sterility due to incomplete exsertion from the leaf sheath.

Apart from the above mentioned factors, drought stress might have affected reproductive development through abortion of the fertilized ovules and ovaries. In maize, water deficit inhibits ovary growth and decreases kernel number, while decreasing levels of reducing sugars (glucose

and fructose) and starch and increasing sucrose (Zinselmeier et al., 1995). The sink strength of developing reproductive tissues might also be limited by availability of water.

2.4.2. Drought Stress Induces Differential Gene Expression in Vegetative and Reproductive Tissues

The number of differentially expressed genes following drought stress in this study indicated that about 17% of the genes were up-regulated in the vegetative tissue examined, while about 10% were in the reproductive tissues. For down-regulated genes, the numbers were 17% and 7% in vegetative and reproductive tissues, respectively. According to Ji et al. (2005) and Seki et al. (2002), depending on the organ and growth stages, drought stress transcriptional responses are normally observed on up to 10% of the genome. In this study more than 20% of the genes probed responded to drought. The variation in responses at genome level may be due to species differences, severity of drought conditions, length of exposure, or (perhaps most likely) sensitivity of the methodologies used to detect transcriptomic responses.

Among the genes regulated by drought stress, some have known functions, while others were previously predicted to have functions related to drought stress or ABA (Zhou et al., 2007). This study shows that induced and repressed genes in both vegetative and reproductive tissues respond with alterations in water stress processes, cell metabolism, transport, cell communication and signal transduction, and protein biosynthesis. Generally the results agree with previous drought stress global gene expression studies in rice (Zhou et al., 2007; Reddy et al., 2001), Arabidopsis (Seki et al., 2003), and maize (Andjelkovic and Thomson, 2006). The diversity of biological processes affected suggests a high level in regulation of cell processes in mitigating effects of drought. Further delving into specific processes and their likely connections is needed and will occur as this work is developed for peer-reviewed publications.

2.4.3. There Is Limited Overlap of Drought Responsive Genes and Processes between Vegetative and Reproductive Tissues

The analysis of differentially expressed genes as displayed in the Venn diagrams (Figure 2.2) shows overlaps between vegetative and reproductive tissues for expressed genes. Despite these overlaps, there are clear indications that the two tissues are responding differently to drought. Of the 7874 detected induced genes, only 18% (1486) occurred in both vegetative and reproductive tissues; and of the 7270 detected repressed genes, only 12% were found in both tissue types.

Gene ontology (GO) analysis likewise revealed that many of processes affected by drought are induced in either vegetative or reproductive tissue, but not both; and some of these are known drought-related processes. In vegetative tissue, the tissue-specific processes included polyol metabolism (GO:0019751), hexose catabolism (GO:0019320), and GTPase-mediated signal transduction (GO:0051056), as well as regulation of GTPase activity (GO:0007264, GO:0051056, GO:0043087), ion transport (GO:0006811), and cation transport (GO:0006812). According to Bohnert et al. (1995), accumulation of polyols (mannitol, sorbitol, *myo*-inositol) is correlated with tolerance to drought. Polyols of various types have been reported to accumulate in response to drought stress in redbud (*Cercis canadensis*) (Griffin et al., 2004) and ash (*Fraxinus excelsior*) (Guicherd et al., 1997). These metabolites function in osmotic adjustment, osmoprotection, and scavenging ROS.

The GO-detected processes in vegetative (leaf) tissues included some that are associated with stomatal closure that may be regulated exclusively in the shoot. Closure of the stomata has been ascribed to the activation of Ca^{2+}, K^{+} and Cl^{-} ion channels (Fan et al., 2004; Ward and Schroeder, 1994). It is conceivable that transport proteins produced in response to drought and

ABA will be highly expressed at solute and water exchange sites to determine hydraulic conductivity of the xylem parenchyma cells and guard cells. Recently Lemichez et al. (2001) reported ABA inactivation in Arabidopsis of a small guanosine triphosphatase (GTPase) protein called Rac1, which is thought to be essential for stomatal closure. In another study (Zheng et al., 2002), the association of ABA and an Arabidopsis GTPase in ABA signaling was demonstrated.

On the other hand, some GO-detected processes were drought-responsive only in the reproductive and not in the vegetative tissues. Examples of these included glucan biosynthesis (GO:0009250), response to oxidative stress (GO:0006979), and galactose metabolism (GO:0006012). In members of the Poaceae, to which rice belongs, (1-3),(1-4)-ß-glucan is a characteristic component of cell walls, especially those of the endosperm (Fincher and Stone, 1987; Carpita, 1996). Glucans are important because: 1) they form the major part of callose, which during normal plant growth and development is found as a transitory component of the cell plate in dividing cells; 2) they are a major component of pollen mother cell walls and pollen tubes; and 3) they are a structural component of plasmodesmatal canals. Glucan-rich callose is also synthesized in response to wounding, physiological stress, pathogen infection (Delmer, 1987), and accumulation of ROS (Benitez-Alfonso et al., 2009; Schraudner et al., 1992). The formation of glucan callose can be a response to drought-induced accumulation of ROS, and this could be a reproductive-organ-specific response in rice.

Gene ontology analysis of biological processes also indicated that hexose metabolism was altered by drought in vegetative tissue, while galactose metabolism was specifically enriched in the reproductive tissues. Hexoses (glucose, fructose, and galactose) increase in response to water stress in rice (Morsey et al., 2007), potato (*Solanum tuberosum*) (Vasquez-Robinet et al., 2008), and *Populus euphratica* (Guignard et al., 2005). However, various studies

have reported drought-induced increases in glucose and fructose in leaves of rice (Pieters and el Souki, 2005), oak (*Quercus petraea*) (Epron and Dryer, 1996), and pigeon pea (*Cajanus cajan*) (Keller and Ludlow, 1993). While data on the relationships between drought stress and hexose responses in rice reproductive tissues are limited, it is possible that glucose, fructose, and galactose are more drought regulated in the vegetative parts, while only galactose metabolism may be affected in the reproductive parts. The hexose response to drought could be explained by increased activities of disaccharide-hydrolyzing enzymes, e.g., invertase, or by inhibition of hexose-utilizating pathways, e..g., glycolysis, in the cases of glucose and fructose. On the other hand, drought can also reduce these simple sugars, which will indicate that disaccharide hydrolyzing enzymes have been inhibited or that photosynthesis, which generates the disaccharide was affected. Organ specific differences in terms of which sugar is affected may be due to factors that affect gene expression in each organ, or the extent to which stress is sensed by specific organs.

In conclusion, drought at panicle emergence and anthesis reduced yield by affecting processes in vegetative and reproductive organs. The reduction in yield is reflected in reduced yield components, especially fewer spikelets and reduced numbers of fertile spikelets. Drought stress at this stage of development invokes differential gene expression. Many drought-responsive genes have been annotated as proteins of unknown function. These may be novel genes associated with drought responses in rice. The changes in expression at both gene and biological process levels differ between the vegetative and reproductive parts. This indicates that there is organ-specific regulation in response to drought, which is mediated by organ specific transcription responses. Alternatively, progressive drought may not have been sensed equally

between the two organs. Processes that affect photosynthesis, carbohydrate metabolism, and responses to ROS seem particularly sensitive to drought – and fertile for further study.

2.5. References

Andjelkovic V, Thompson R (2006) Changes in gene expression in maize kernel in response to water and salt stress. Plant Cell Rep **25**: 71-79.

Baisak R, Rana D, Acharya PBB, Kar M (1994) Alterations in the activities of active oxygen scavenging enzymes of wheat leaves subjected to water stress. Plant Cell Physiol **35**: 489-495.

Bartels D, Salamini F (2001) Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level. Plant Physiol **127**: 1346-1353.

Benitez-Alfonso Y, Cilia M, San Roman A, Thomas C, Maule A, Hearn S, Jackson D (2009) Control of *Arabidopsis* meristem development by thioredoxin-dependent regulation of intercellular transport. Proc Natl Acad Sci USA **106**: 3615-3620.

Bohnert HJ, Sheveleva E (1998) Plant stress adaptation: Making metabolism move. Curr Opin Plant Biol **1**: 267-274.

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell **7**: 1099-1111.

Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol **47**: 445–476.

Chaves MM (1991) Effects of water deficit on carbon assimilation. J Exp Bot **42**: 1-6.

Choe SE, Boutros M, Michelson AM, Church GM, Halfon MS (2005) Preferred analysis methods for Affymetrix GeneChips revealed by wholly defined control data set. Genome Biol **6:** 1-16.

Colom MR, Vazzana C (2003) Photosynthesis and PS II functionality of drought resistant and drought-sensitive weeping love grass plants. Environ Exp Bot **49**: 135-144.

Counce PA, Keisling CT, Mitchell (2000) A uniform, objective and adaptive system for expressing rice development. Crop Sci **40**: 437-443.

Cruz de Carvalho MHC (2008) Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signaling Behavior **3**: 156-165.

Delmer DP (1987) Cellulose biosynthesis. Annu Rev Plant Physiol **38**: 259-290

Dey MM, Upadhyaya HK (1996) Yield loss due to drought, cold and submergence in rice: In Everson, RE, Herdt RW, Hossain M (Eds.), Rice in Asia: Progress and priorities. CAB International, Wallington, UK, pp. 291-303.

Ekanayake IJ, De Datta SK, Steponkus PL (1989) Spikelet sterility and flowering response to rice water stress at anthesis. Ann Bot **63**: 257-264.

Ekanayake IJ, De Datta SK, Steponkus PL (1990) Sensitivity of pollination to water deficit at anthesis to upland rice. Crop Sci **30**: 310-315.

Epron D, Dryer E (1996) Starch and soluble carbohydrates in leaves of water stressed oak saplings. Ann For Sci **53**:263-268.

Fan L-M, Zhao Z, Assman SM (2004) Guard cells: a dynamic signaling model. Curr Opin Plant Biol **7**: 537-546.

Fincher GB, Stone BA (1981) Metabolism of non-cellulosic polysaccharides. *In* W Tanner, FW Loewus, (eds.) Encylopedia of Plant Physiology, New Series 13B. Springer-Verlag, Berlin, pp 68-132.

Fu J, Huang B (2001) Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ Exp Bot **45**: 105-114.

Gamble PE, Burke JJ (1984) Effect of water stress on the chloroplast antioxidant system I. Alterations in glutathione reductase activity. Plant Physiol **76**: 615-621.

Garg AK, Kim J, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA **99**: 15898-15903.

Griffin JJ, Ranney TG, Pharr DM (2004) Heat and drought influence photosynthesis and carbohydrates in two ecotypes of redbud (*Cercis canadensis*). J Amer Soc Hort Sci **129**: 497-502.

Guan LM, Zhao J, Scadalios JG (2000) Cis-element and trans-factors that regulate expression of the maize *cat1* antioxidant gene in response to ABA and osmotic stress: H_2O_2 is the likely intermediary signaling molecule for the response. Plant J **22**: 87-95.

Guicherd P, Peltier JP, Gout E, Marigo RGB (1997) Osmotic adjustment in *Fraxinus excelsior* L.: malate and mannitol accumulation in leaves under drought conditions. Trees **11**: 155-161.

Guignard C, Jouve L, Bogeat-Triboulot BM, Dreyer E, Hausman J-F, Hoffmann L (2005) Analysis of carbohydrates in plants by high-performance anion-exchange chromatography coupled with electrospray mass spectrometry. J Chromatogr **1085**: 137-142.

Hao G-P, Xing Y, Zhang J-H (2008) Role of nitric oxide dependence on nitric oxide synthase-like activity in the water stress signaling of maize seedlings. J Integr Plant Biol **50**: 435-442.

Hsiao TC (1973) Plant responses to water stress. Ann Rev Plant Physiol **24**: 519-570.

Irrizarry RA, Hobbs B, Collin F, Beazr-Barclay YD, Antonellis KJ, Scherf U, Speed T (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4**: 249-264.

Ji XM, Raveendran M, Oane R, Ismail A, Laffite HR, Bruskiewich R, Cheng SH, Bennet J (2005) Tissue-specific expression and drought responsiveness of cell-wall invertase genes of rice at flowering. Plant Mol Biol **59**: 945-964.

Keller F, Ludlow MM (1993) Carbohydrate metabolism in drought-stressed leaves of pigeon pea (*Cajanus cajan*)**.** J Exp Bot **44**: 1351-1359.

Kim EH, Kim YS, Park SH, Koo YJ, Choi YD, Chung YY, Kim J (2009). Methyl jasmonate reduces grain yield by mediating stress signals to alter spikelet development in rice. Plant Physiol **149**: 1751-1760.

Kocsy G, Galiba G, Brunold C (2001) Role of glutathione in adaptation and signaling during chilling and cold acclimation in plants. Physiol Plant **113**: 158-168.

Lamichez E, Wu Y, Sanchez J, Mettouchi J, Chua N (2001) Inactivation of AtRac1 by abscisic acid is essential for stomatal closure. Genes Dev **15**: 1808-1816.

Lawlor W, Cornic G 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ **25**: 275-294.

Li L, Van Staden J, Jager AK (1998) Effects of plant growth regulators on the antioxidant system in seedlings of two maize cultivars subjected to water stress. Plant Cell Environ **20**: 493-503.

Liu JK, Liao DQ, Oane R, Estenor L, Yang XE, Li ZC, Bennet J (2006) Genetic variation in the sensitivity of anther dehiscence to drought stress in rice. Field Crops Res. **97**: 87-100.

Makela P, Mclaughlin JE, Boyer JS (2005) Imaging and quantifying carbohydrate transport to the developing ovaries of maize. Ann Bot **96**: 939-949.

Mclaughlin JE, Boyer JS (2004) Glucose localization in maize ovaries when kernel number decreases at low water potential and sucrose is fed to the stems. Ann Bot **94**: 75-86.

Moradi F, Ismail AM S (2007) Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. Ann Bot **102**: 1-13.

Morgan JM (1980) Possible role of abscisic acid in reducing seed set in water stressed wheat plants. Nature **289**; 655-657.

Morsy MR, Jouve L, Hausman JF, Hoffmann L, Stewart JM (2007) Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. J Plant Physiol **164**: 157-167.

Munne-Bosch S, Alegre L (2004) Die and let live: leaf senescence contribution to plant survival under drought stress. Funct Plant Biol **31**: 203-216.

O'Toole JC, Moya TB (1981) Water deficit and yield in upland rice. Field Crop Res **4**: 247-259.

O'Toole JC, Namuco OS (1983) Role of panicle exsertion in water stress induced sterility. Crop Sci **23**: 1093-1097.

Olson KR (1979) Saran coated method for determining bulk densities, soil moisture values, and linear extensibility. Agron. Mimeogr. 79-85. Soil Characterization Laboratory. Cornell University, Ithaca, N.Y.

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. Nature **406**: 731-734.

Pelleschi S, Rocher JP, Prioul JL (1997) Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. Plant Cell Environ **20**: 493-503.

Peters AJ, El Souki S (2005) Effects of drought during grain filling on PS II activity in rice. J Plant Physiol **162**: 903-911.

Pinheiro C, Chaves MM, Ricardo CP (2001) alterations in carbon and nitrogen metabolism induced by water deficit in the stems and leaves of *Lupinus albus* L. J Exp Bot **52**: 1063-1073.

Porcel R, Ruiz-Lozano J (2004) Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. J Exp Bot **55**: 1743-1750.

Qiu, Y., Fu, B., Wang, J., Chen, L. (2001) Soil moisture variation in relation to topography and land use in a hillslope catchment of the Loess Plateau, China. Journal Hydrol **240**: 243–263.

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol **133**: 1755-1767

Reddy AR, Ramakrishna W, Chandra Sekhar A, Ithal N, Ravindra Babu P, Bonaldo MF, Soares MB, Bennetzen JL (2001) Novel genes are enriched in normalized cDNA

libraries from drought-stressed seedlings of rice (*Oryza sativa* L. subsp. *indica* cv. Nagina 22). Genome **45**: 204-211.

Robberts JA, Elliot KA, Gonzalez-Carranza ZA (2002) Abscision, dehiscence, and other cell separation processes. Annu Rev Plant Biol **53**: 131-158.

Saccardy K, Cornic G, Brulfert J, Reyss A (1996) Effect of drought stress on net CO_2 uptake by *Zea* leaves. Planta **199**: 589-595.

Saini HS (1997). Effects of water stress in gametophyte development in plants. Sex Plant Reprod **10**: 67-73.

Sakamoto T, Matsuoka M (2008) Identifying and exploiting grain yield genes in rice. Curr Opin Plant Biol **2:** 209-214.

Sandhu BS, Horton ML (1977) Response of oat to water deficits I: Physiological characteristics. Agron J **69**: 357-360.

Schraudner M, Ernst D, Langebartels C, and Sandermann Jr H (1992) Biochemical plant responses to ozone III. Activation of the defense-related proteins β-1,3-glucanase and chitinase in tobacco leaves. Plant Physiol **99**: 1321-1328.

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enjo A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi_Shinozaki K, Carninci P, Kawai J, Hayashikazi Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought stress, cold and high-salinity stresses using full-length cDNA microarray. Plant J **31:** 279-292.

Seki M, Satou M, Sakurai T, Akiyama K, Lida K, Ishida J, Nakajima M, Enjo A, Narusaka M, Fujita M, Oono Y, Kamei A, Yamaguchi-Shinozaki K, Shinozaki K (2003)

RIKEN Arabidopsis full-length (RAFL) cDNA and its applications for expression profiling under abiotic stress conditions. J Exp Bot **55**: 213-223.

Selote DS, Khanna-Chopra R (2004) Drought induced spikelet sterility is associated with an inefficient antioxidant defense in rice panicles. Physiol Plant **121**: 462-471.

Sheoran IS, Saini HS (1996) Drought-induced male sterility in rice: Changes in carbohydrate levels and enzyme activities associated with inhibition of starch accumulation in pollen. Sex Plant Reprod **9**: 161-169.

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold responses. Curr Opin Plant Biol **6**: 410-417.

Smart RE, Bingham GE (1974) Rapid estimation of relative water content. Plant Physiol **53**: 258-260.

Smirnoff N (1993) The role of active oxygen in the response of plant to water deficit and desiccation. New Phytol **125**: 27-58.

Smyth GK, Speed TP (2003) Normalization of cDNA microarray data. Methods **31**: 265-273.

Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. Proc Natl Acad Sci USA **100**: 9440-9445.

Tezara W, Mitchel VJ, Driscoli SD, Lawlor DW (1999) Water deficit inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature **401**: 914-917.

Thomson JE, Legge RL, Barber RF (1987) The role of free radicals in senescence and wounding. New Phytol **105**: 317-344.

Vasquez-Robinet C, Mane SP, Ulanov AV, Watkinson JI, Stromberg VK, De Koeyer R, Willmott DB, Bonierbale M, Bohnert HJ, Grene R (2008) Physiological and molecular adaptations to drought in Andean potato genotypes. J Exp Bot 59: 2109-2123.

Ward JM, Schroeder JI (1994) Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. Plant Cell **6**: 669-683.

Wettwnhall Jm, Simpson KM, Satterley K, Smyth GK (2006). AffylmGUI: a graphical user interface for linear modeling of single channel microarray data. Bioinformatics **22**: 897-899.

Xing Y, Jiang W, Zhang J (2008) atMKK1 mediates ABA-induced CAT1 expression and H2O2 production via atMPk6-coupled signaling in Arabidopsis. Plant J **54**: 440-451.

Yazaki J, Kishimoto N, Nagata Y, Ishikawa M, Fujii F, Hashimoto A, Shimbo K, Shimatani Z, Kojima K, Suzuki K, Yamamoto M, Honda S, Endo A, Yoshida Y, Sato Y, Takeuchi K, Toyoshima K, Miyamoto C, Wu J, Sasaki T, Sakata K, Yamamoto K, Iba K, Oda T, Otomo Y, Murakami K, Matsubara K, Kawai J, Carninci P, Hayashikazi Y, Kikuchi S (2003) Genomics approach to abscisic acid and gibberellin-responsive genes in rice. DNA Res **10**: 249-261.

Yazaki J, Shimatani Z, Hashimoto A, Nagata Y, Fujii F, Kojima K, Suzuki K, Taya T, Tonouchi M, Nelson C, Nakagawa A, Otomo Y, Murakami K, Matsubara K, Kawai J, Carninci P, Hayshikazi Y, Kikuchi S (2004) Transcriptional profiling of genes responsive to abscisic acid and gibberellin in rice: phenotyping and comparative analysis between rice and Arabidopsis. Physiol Genomics **17**: 87-100.

Zhang AY, Jiang MY, Zhang JH, et al., (2007) Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of mitogen-activated protein kinase cascade involved in antioxidant defense in maize leaves. New Phytol 175: 36-50.

Zhang J, Kirkham MB (1996) Enzymatic responses of the ascorbate glutathione cycle to drought in sorghum and sunflower. Plant Sci **113**: 139-147.

Zheng Z-L, Nafisi M, Tam A, Li H, Crowell DN, Chary N, Shroeder JI, Shen J, Yang Z (2002) Plasma membrane-associated ROP10 small GTPase is a specific negative regulator of abscisic acid responses in Arabidopsis. Plant Cell **14**: 1-11.

Zhou J, Xiangfeng W, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong L, Zhang Q, Fan L, Deng XW (2007) Global genome expression analysis of rice in response to drought and high salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol **63**: 591-608.

Zhou X, Zhen Su Z (2007) EasyGO: Gene Ontology-based annotation and functional enrichment analysis tool for agronomical species. BMC Genomics 8: doi/ 10.1186/1471-2164-8-246.

Zinselmeier C, Westgate ME, Schussler Jr, R (1995) Low water potential disrupts carbohydrate metabolism in maize *(Zea mays* L.) ovaries. Plant Physiol **107**: 385-391.

Table 2.1. Physiological parameters in rice affected by moderate and severe drought conditions at the panicle-emergence stage

Treatment	Soil Moisture Content (SMC) (cm^3/cm^3)	Chlorophyll Fluorescence		Relative Water Content (RWC) (%)	Fired Leaves (FL) (%)
		Fv/Fm	ØPS II		
Control (WW)	0.44[a]	0.794[a]	0.740[a]	96.9[a]	23.1[a]
Moderate Drought	0.13[b]	0.790[a]	0.605[ab]	75.9[b]	28.1[b]
Severe Drought	0.10[c]	0.732[a]	0.520[b]	65.8[c]	49.4[c]
LSD(0.05)	0.001	0.090	0.158	11.9	3.2
P value	<0.0001	0.244	0.041	0.003	<0.0001

WW = well-watered (throughout) controls. Moderate drought = irrigation withheld for 7 d, including 1 d of leaf rolling. Severe drought = irrigation withheld for 8 d, including 2 d of leaf rolling. SMC = volumetric soil moisture content. Fv/Fm = photochemical efficiency of photosystem II. ØPS II = effective quantum yield of photosystem II. FL = percent of leaves with >50% of lamina showing chlorosis or necrosis. Means within columns followed by the same letter are not significantly different at p=0.05. For each data point, n = 4.

Table 2.2. Yield components and allometric parameters in rice plants affected by moderate and severe drought conditions applied at panicle emergence

Treatment	Spikelet/ Panicle (SP)	Fertile Spikelet Ratio (FS)	Grain Yield (GY) (g)	Harvest Index (HI)	Single Grain Weight (SGW) (g)	Shoot Biomass (SB) (g)	Peduncle Length (PL) (cm)
Control (WW)	186[a]	0.35[a]	1.63[a]	0.41[a]	0.023[a]	2.33[a]	29.9[a]
Moderate Drought	149[b]	0.23[b]	0.81[b]	0.31[ab]	0.024[a]	2.04[b]	27.2[b]
Severe Drought	123[c]	0.17[b]	0.48[c]	0.19[b]	0.025[a]	1.72[c]	25.5[c]
LSD(0.05)	25.56	0.06	0.52	0.13	0.935	0.25	1.49
P value	0.0024	0.0006	0.0043	0.018	0.122	0.0031	<0.0011

WW = well-watered (throughout) controls. Moderate drought =irrigation withheld for 7 d, including 1 d of leaf rolling. Severe drought = irrigation withheld for 8 d, including 2 d of leaf rolling. Means within columns followed by the same letter are not significantly different at p=0.05. For each data point, n = 4.

Table 2.3. Comparative analysis of the physiological parameters measured in the physiological and the transcriptome experiments

Treatment	Soil Moisture Content (SMC) (cm^3/cm^3)	Chlorophyll Fluorescence		Relative Water Content (RWC) (%)
		Fv/Fm	ØPS II	
Physiological Experiment				
Control (WW)	0.44[a]	0.794[a]	0.740[a]	96.9[a]
Moderate Drought	0.13[b]	0.790[a]	0.605[ab]	75.9[b]
Severe Drought	0.11[c]	0.732[a]	0.520[b]	65.8[c]
Transcriptome Experiment				
Control (WW)	0.43[a]	0.791[a]	0.742[a]	96.1[a]
Severe Drought	0.13[c]	0.702[c]	0.487[bc]	63.2[c]
LSD (0.05)	0.002	0.080	0.114	8.36
P value	<0.0001	0.0109	0.0012	<0.0001

WW = well-watered controls. Moderate drought = irrigation withheld for 7 d, including 1 d of leaf rolling. Severe drought = irrigation withheld for 8 d, including 2 d of leaf rolling. Fv/Fm = photochemical efficiency of photo-system II. ØPS II = effective quantum yield of photo-system II. Means within columns followed by the same letter are not significantly different at p=0.05. For each data point in the physiological experiment, n = 4. In the transcriptome experiment, n = 3.

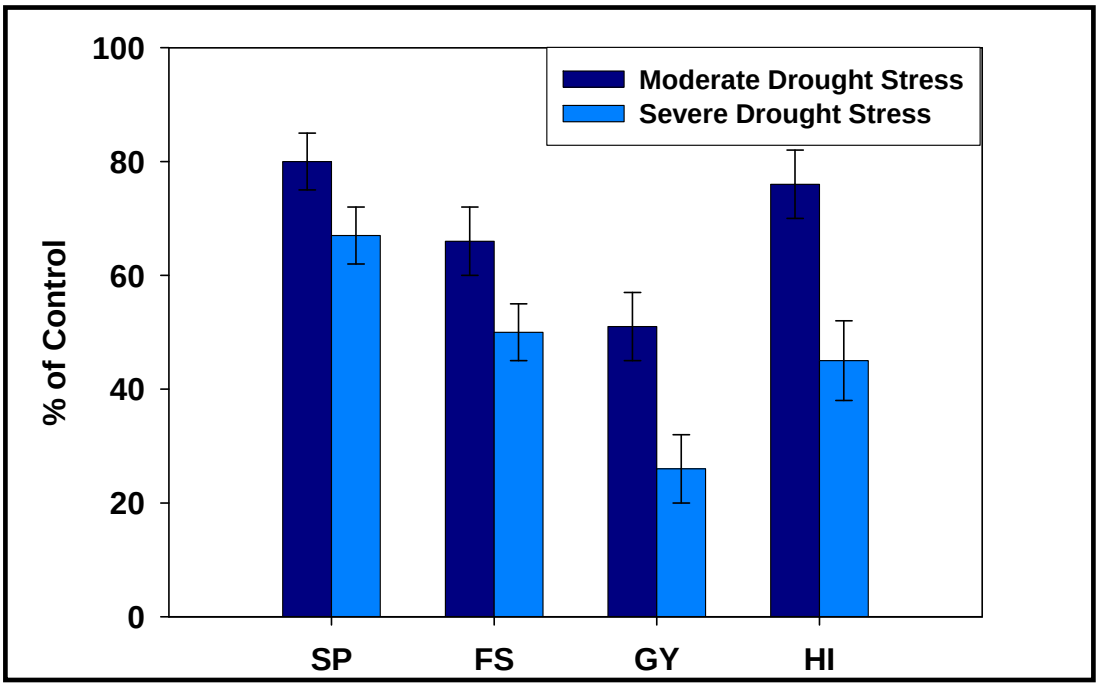

Figure 2.1. Seed production traits in rice plants affected by moderate and severe drought stress. Moderate drought = irrigation withheld for 7 d, including 1 d of leaf rolling. Severe drought = irrigation withheld for 8 d, including 2 d of leaf rolling. Controls were well watered throughout the experiment. SP = spikelets/panicle, FS = fertile spikelet ratio, GY = grain yield, HI = harvest index. Error bars = standard error of means. For each data point, n = 4.

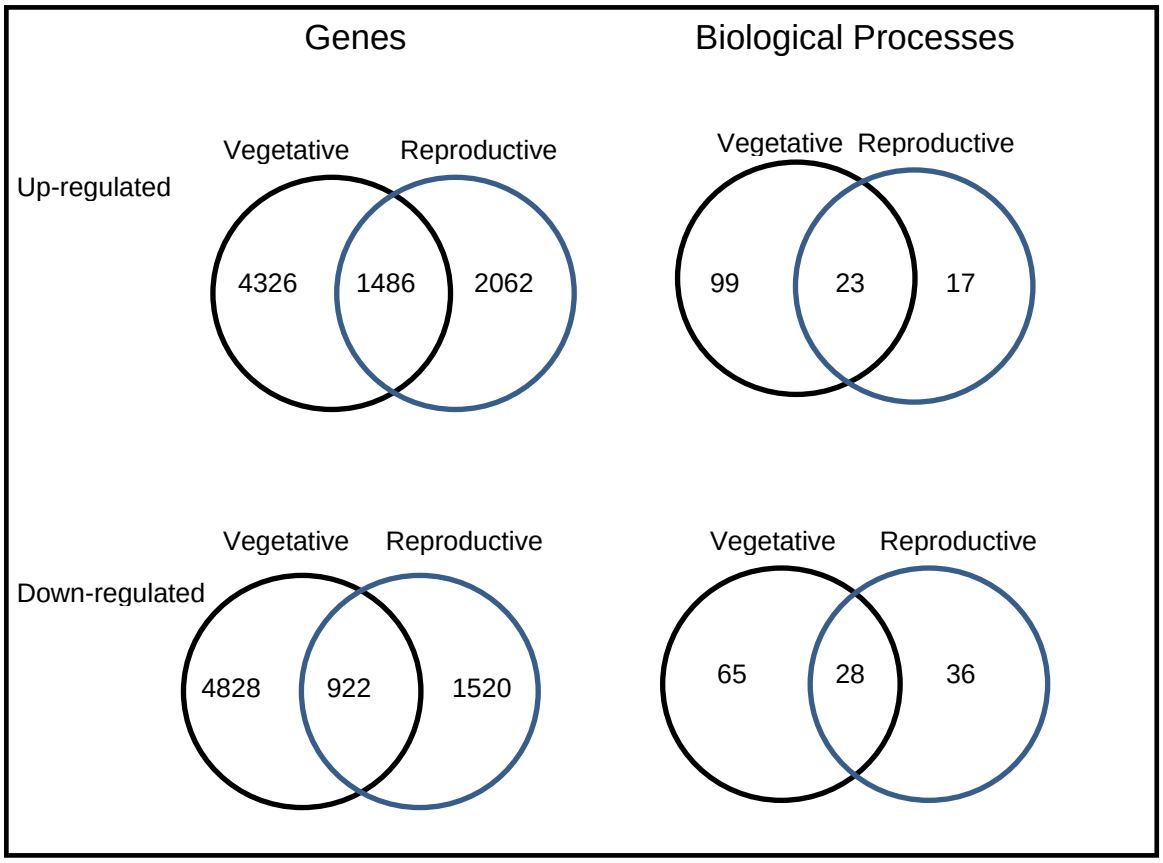

Figure 2.2. Venn diagrams showing the overlap between transcript expression and biological processes in the vegetative and reproductive tissues of rice subjected to drought stress. Drought was imposed by withholding irrigation for 8 d, including 2 d of leaf rolling (severe drought). Transcript profiling and data analysis were performed as described in Materials and Methods.

Chapter 3. Comparative Genomic Analysis of Maize and Rice Responses to Drought

Abstract

Drought stress is a major challenge for the production of rice (*Oryza sativa*) and maize (*Zea mays*), especially when water deficits coincide with the crops' reproductive stages of development. This study was undertaken with the notion that the two species might share common physiological and genomic responses to drought stress. Microarray (rice) and GS-FLX pyrosequencing (maize) data were used to identify drought-regulated gene orthologs in these two major cereal species. The identified genes were used for gene ontology enrichment analysis to look more closely at biological processes. Gene ontology analysis indicated that, in both species, drought stress causes a transition from protein synthesis to degradation and that the light reactions of photosynthesis are dramatically repressed. In addition, a Calvin cycle gene was also down-regulated, which implies that both the light and carbon fixation reactions of photosynthesis were repressed by drought. The in-common drought-responsive genes identified within the two species' genomes are potential candidates to study for their broader importance in drought stress by using transformation and mutagenesis.

3.1. Introduction

Drought stress limits production and yield stability in rice (*Oryza sativa*). The effect is most dramatic when the plants are exposed to drought during reproductive development (Selote and Chana-Chopra, 2004). Likewise, in maize (*Zea mays*) reductions in grain yield are greatest when drought occurs during reproductive stages (Zinselmeier et al., 1995). This work was undertaken with the notion that drought might be affecting similar processes to cause yield reductions in these two cereals; and, if there are such shared processes, they might well involve the expression of similar or shared genes. This notion is supported by comparative mapping showing that individual gene sequences and chromosomes of many grasses exhibit extensive synteny (Devos, 2005). The recently released maize draft genome sequence identifies about 8000 orthologous genes between rice, maize, sorghum (*Sorghum bicolor*), and even Arabidopsis (Schnable et al., 2009). Functional orthologs would be expected to produce similar phenotypic expressions across species. For example, mutants of the orthologous *GAI, Rht-1,* and *D8* dwarfing genes reduce plant height in Arabidopsis, wheat (*Triticum aestivum*), and maize, respectively (Peng et al., 1999). Functional orthologs have also been shown to be involved in responses to drought (Nelson et al., 2007), cold (Tondelli et al., 2006), and salinity (Hamada et al., 2001). These genes presumably code for functional and regulatory proteins that play important roles in stress responses.

In the past, identifying key stress response genes has relied on physiological and biochemical studies, whereby pathways that are rate limiting under stress can be identified (Bartels and Nelson, 1994). Today, high-throughput methods and functional genomics can be used in combination to select from tens of thousands of candidate genes, i.e., the entire genome, a few target genes.

Many of the advances in genomics research for gene identification have been founded around Arabidopsis, but resources and information in rice and maize have gained importance recently. Rice is a model species for cereal crops and has perhaps the richest resources available for plant genomic studies (RCSC, 2003).

Drought responses in rice have been analyzed at a transcriptome level (Rabanni et al., 2003; Yazaki et al., 2004; Zhou et al., 2007). Such studies have identified many drought-responsive genes. However, the studies were done on ABA-treated callus (Rabanni et al., 2003; Yazaki et al., 2004) or with hydroponic plants that were air-dried to simulate drought (Zhou et al., 2007), i.e., not under typical physiological conditions. In some of these studies, the microarrays used represented only parts of the rice genome; and many drought-responsive genes are likely not included in those analyses.

In the maize studies reported here and in the rice studies of Chapter 2 of this dissertation, a more physiologically relevant soil dry-down has been used to impose drought. The subsequent application of microarrays (in rice) and GS-FLX pyrosequencing (in maize) has identified a number of drought-responsive genes. The rice genes were used to extract their maize orthologs. The in-common responsive genes were further analyzed for biological processes that might be important for drought responses in both species. Such genes can be useful resources for transgenic analysis, such as overexpression, antisense suppression, and double stranded RNAi silencing.

Commonly regulated genes in rice and maize present evidence that drought stress causes a transition of metabolism from protein synthesis (by repressing amino acid biosynthesis and translation) to protein degradation (by inducing the ubiquitin-proteasome pathway). In addition, photosynthesis is severely inhibited by repression of genes involved in both light and dark

reactions. The results further indicate that carbohydrate metabolism may be one of the targets of drought stress in reproductive tissues.

3.2. Materials and Methods

3.2.1. Experiment to Elicit Drought Stress and Obtain Genomic Data from Maize

3.2.1.1. Plant Materials, Drought Stress Treatments, and Tissue Sampling

Maize inbred line B73 was grown in a greenhouse in 12, 10-l pots (one plant per pot) with 1:1:1 mix of peat:vermiculite:perlite with 6 g pulverized limestone, 35 g $CaSO_4$, 42 g powdered $FeSO_4$, and 1 g of fritted trace element (Setter et al., 2001). The pots were hand-irrigated daily; and, to replace the leached nutrients, plants were supplied on a weekly basis with a general purpose fertilizer, 15-16-17, (Scott-Sierra Horticultural Product Co, Marysville, OH) dissolved in water to provide 50 kg each N, P_2O_5, and K_2O ha^{-1}.

Plants were grown under well-watered conditions until they reached the silking stage (onset of silk emergence). At this stage/size, the plants were able to deplete their pots' water supply very quickly. Their leaves would wilt (roll) by the second day of withholding water. Preliminary studies showed that, when irrigation was withheld at this stage for 4 d, including 3 d of leaf rolling, seed set was totally inhibited. Accordingly, for this study, 2 d of leaf rolling was operationally defined as "severe drought stress", and 1 d of leaf rolling as "moderate drought stress".

Eight of the pots were randomly selected, and irrigation was withheld from them. Four of these were subjected to "moderate drought" by withholding irrigation for 2 d, which included 1 d with leaves rolled. The other four were exposed to "severe drought", withholding irrigation for 3 d, which included 2 d with leaves rolled. Pots for the severe drought stress treatment had irrigation withdrawn 1 d before the moderate drought stress pots, such that sampling for all treatments was carried out on the same day. The rest of the plants (the other four pots) were the

well-watered controls and were irrigated throughout the experimental period. Twenty-four hours before sampling, plants were hand pollinated.

At the end of the drought periods (2 or 3 d after withholding irrigation), soil moisture content (SMC), chlorophyll fluorescence (Fv/Fm and PS II), relative water content (RWC), and soil water potential were measured following procedures in Chapter 2, section 2.2.2. After these measurements, vegetative tissue (a leaf lamina segment taken 2 cm above the ligule) containing leaf meristem (Tardieu and Granier, 2000), and reproductive tissue (ovaries taken 1 d after pollination) (Andersen et al., 2002) were sampled on the plants from well-watered and each level of drought-stressed pots. Tissue samples, which were immediately extracted for RNA (see below), were identified as control (well-watered) leaves (LC), control (well-watered) ovaries (OC), drought-stressed leaves (LD1 and LD2), and drought-stressed ovaries (OD1 and OD2). Means and their standard error were calculated for SMC and chlorophyll fluorescence using Excel.

3.2.1.2. Identification of Drought-Responsive Genes in Maize by GS-FLX Pyrosequencing

The leaf and ovary samples collected in 3.2.1.1. were used for GS-FLX pyrosequencing. RNA was extracted from each replicate sample using the RNAeasy plant minikit (Qiagen), following manufacturer's instructions. The RNA isolates from tissues of moderately (LD1 and OD1) and severely stressed plants (LD2 and OD2) were then pooled into single samples representing drought-stressed leaves (LD) and drought-stressed ovaries (OD). Similarly the RNA extracts from well-watered plants were pooled to produce samples representing the controls from leaf (LC) and ovaries (OC). The four pooled RNA samples were submitted to the Virginia Bioinformatics Institute core laboratory facility, where quality controls, cDNA synthesis, normalization, library preparation, and sequencing were carried out with the Roche/454 GS FLX

pyrosequencing platform (Roche Applied Science, Indianapolis, IN, USA). This generated sequence libraries corresponding to each tissue type: LD, LC, OD and OC.

The sequence reads from the four libraries, which were already trimmed for low quality and primer sequences, were masked for plant repeat sequences. Reads with unmasked (non-repeat) sequence lengths of at least 100 bp were used for further analyses. These reads were mapped to the *Zea mays* var: B73 genomic cDNA squences (www.maizesequence.org; accessed 01/08/210) by BLASTn. Reads mapping with a BLASTn e-value $<10^{-5}$ and a bit score >100 were used for further analyses. The number of reads mapping to the total length of the maize cDNAs, and separately to the last 1000 bp of the sequence, were recorded. In a given library, assuming that a single read matching a gene might be present just at the background level of expression, genes with two or more matching reads were considered present in that library. Comparisons were made between the libraries, LC against LD and OC against OD; and genes present in at least one of them were used for the calculation of the level of differential expression in terms of log (base 2) of the ratio of the number of hits within the last 1000 bp of a gene. This constant length at the 3-prime end of the gene was chosen to normalize for varying gene length and, whenever possible, differentiate between members of a gene family. Maize-rice orthologs were identified using INPARANOID4 (http://inparanoid.cgi.ki.se (accessed 01/08/2010); O'Brien et al., 2005). The genes were arrayed in Venn diagram analysis against the rice drought transcriptome described in Chapter 2.2.4. to identify genes regulated in common.

3.2.2. Experiments to Examine Chlorophyll Fluorescence, Seed Set, and Quantitative RT-PCR under Drought Stress

3.2.2.1. Seed Number Analysis

This experiment was, in fact, two concurrent or nested experiments, set up following the same general procedures and drought-stress treatments outlined in 3.2.1. It began with 18 pots (one plant per pot) with all plants well watered until they reached silking stage. At that time, 11 pots were randomly assigned to drought-stress treatments – seven to severe stress (2 d with leaves rolled) and four to moderate stress (1 d with leaves rolled). At the end of the drought-stress period, soil moisture content (SMC) and chlorophyll fluorescence (Fv/Fm and PS II) were measured, following procedures described in section 2.2.2. At that time, three of the severe-drought-stressed plants and three of the controls were sacrificed for qRT-PCR analysis (see below) using the same tissue sampling procedures as in 3.2.1.1.; and the remaining four plants in each level of drought stress were re-watered and grown to maturity under continuously well-watered conditions for another 10 weeks. (The "controls" were well watered throughout the experiment.) At maturity, the ears were harvested and the number of seeds, or caryopses, on each ear was counted.

The experiment that measured SWC, fluorescence, and ultimately yield was arranged as a completely randomized design (CRD) with four replications. (One plant or pot equals an experimental unit.) Data were subjected to analysis of variance (ANOVA) using the general linear model (Proc GLM) of Statistical Analysis System (SAS). Differences between means were tested by Least Significance Difference, with a 0.05 threshold ($LSD_{0.05}$).

3.2.2.2. Quantitative RT-PCR Analysis

To validate the GS-FLX data, gene expression was examined with qRT-PCR in tissues collected from three severely drought-stressed and three well-watered plants. The differential expression in the rice microarray (Chapter 2 of this dissertation) and the GS-FLX experiments with vegetative and reproductive tissue of maize (3.2.1) and rice (Chapter 2) drew attention to a few key genes – those associated with drought stress. The genes, their annotations and expression levels in terms of Log (base 2) are shown in Table 3.5.

Total RNA was isolated from the maize ovary tissues of both control and drought treated plants using the RNeasy plant kit (Qiagen, USA) according to the manufacturer's protocol. The GS-FLX expression results were validated by performing qRT-PCR on a set of selected transcripts. The PCR primers were designed using Molecular Beacon software, primer length 20 to 25 nucleotides, and an expected amplicon size of 100 to 125 bp (Primer Biosoft International, Palo Alto, CA). The expression of tubulin was determined in both well-watered and drought-stressed samples, and it was determined that the gene did not respond to drought stress. The comparative threshold cycle (Ct) method of quantitation was used with the tubulin gene as a reference. The relative fold-change for each of the selected genes was detected from both the control and drought-stressed plants. For each sample, 500 ng total RNA from one of the biological replicates was converted into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Validation experiments were performed in a Bio-Rad iQ5™ thermocycler using iQ™SYBR® Green Supermix (Bio-Rad, Hercules, CA). The temperature regime used was 95°C for 2 min followed by 40 cycles each of 10 s at 95°C, 30 s at 53°C, and 45 s at 72°C, with the melting curve analysis by increasing temperature from 53°C to 95°C (0.05°C/s). Relative

expression level for each gene in each sample was calculated according to Livak and Schmittgen (2001) and Udvardi et al. (2008).

3.3. Results

3.3.1. Maize Physiological and Genomic Responses to Drought Stress

Maize B73 inbred plants were exposed to drought stress at flowering, as described in Materials and Methods, and tissue samples were collected for GS-FLX transcriptome studies. Physiological measurements were made on the same plants to be able to make correlations to gene expression studies. The soil moisture content was 0.43 (±0.03), 0.17 (±0.03), and 0.11 (±0.04) cm^3/cm^3 for control, moderate (1 d of leaf rolling) drought, and severe (2 d of leaf rolling) drought, respectively. The average PSII fluorescence values were 0.712 (±0.037), 0.523 (±0.142), and 0.403 (±0.121) for the control, moderate, and severe drought stress, respectively.

As a nested/contemporaneous experiment, plant samples were collected for quantitative real time polymerase chain reaction (qRT-PCR) analyses as well as for yield studies. Analysis of the physiological results indicated that the experiments were repeatable. The 2-d and 3-d drought were designated as “moderate” and “severe” drought, respectively. During the drought stress period, the soil moisture content decreased from 0.46 cm^3/cm^3 (controls) to 0.15 (moderate drought), and to 0.12 (severe drought). These translated into soil water potentials of -0.03, -0.77, and -1.34 MPa, respectively. Relative water content (RWC) also dropped from 96.7% (control) to 86.6% (mild drought) and to 77.7% (severe drought). These levels of drought also reduced the PS II, but not the Fv/Fm. The number of seed set was also significantly reduced by severe but not moderate drought stress (Table 3.1).

3.3.2. The Rice-Maize Drought-Responsive Orthologs

The GS-FLX sequencing and maize-rice INPARANOID ortholog analyses identified 2199 genes in the four libraries, out of which 1284 were in the leaf libraries and 915 were in the ovary libraries (Table 3.2). Over 600 genes were differentially regulated in the maize leaf tissue,

with 349 up-regulated and 329 down-regulated (Figure 3.1). On the other hand, 431 genes were differentially regulated in the ovary tissue; and, of these, 386 were up-regulated and 45 were down-regulated.

The Venn diagram arrays show that 219 orthologs were responsive to drought in vegetative tissue, while 55 were found in reproductive tissue (Figure 3.1). The in-common maize-rice orthologous up-regulated genes were 101 in vegetative tissue and 43 in reproductive tissue. On the other hand, 118 maize-rice common orthologs were down-regulated in the vegetative tissues, while only 12 were found in the reproductive tissues

The similarly regulated orthologs were used for gene ontology (GO) analysis. The results show that, in total, 15 biological processes were regulated similarly in the four different tissues (maize vegetative, rice vegetative, maize reproductive, and rice reproductive). The shared up-regulated processes in the vegetative (leaf) tissues included: catabolic processes (GO:0030163 and GO:0043282) and processes involved in homeostasis such as (GO:0045454, GO:0042592 and 0019725). Carbohydrate metabolism was the only biological process that was up-regulated in common in the reproductive tissues (maize ovaries or rice panicles). In the vegetative tissues, photosynthetic processes (GO:0015979 and GO:0019684), translation (GO:0006412), and generation of metabolites and energy (GO:0006091) were notably among the down-regulated processes. In addition to photosynthesis (GO:0015979), generation of metabolites and energy (GO:0006091) were the only down regulated processes in the reproductive tissue. These processes were also significantly regulated in the rice microarray experiment in Chapter 2.

The distribution of genes across the four categories of tissues was analyzed and is represented in a Venn diagram in Figure 3.2. The analysis shows that, in the vegetative tissue, 39 and 70 genes were respectively up- and down- regulated. In the reproductive tissues, 14 and five

genes were up- and down- regulated, respectively. Between the four tissues, six genes were commonly up-regulated and three were down-regulated. Some of these genes were selected and further analyzed by qRT-PCR. Out of the 10 genes selected for qRT-PCR only one gene, lipid binding protein (LBP), was regulated in the opposite direction. However, the results accurately validate the GS-FLX data.

3.4. Discussion

3.4.1. Drought Stress at Silking/Anthesis Stage in Maize Induces Seed Losses

Drought stress of maize during the time of pollination reduced soil moisture content to -0.77 (moderate) or -1.34 (severe) MPa and induced physiological responses. RWC and photosynthesis were reduced, and this was later reflected in a reduction in the number of seed. The reduction of seed number in maize exposed to drought at silking stage may be due to reduced female receptivity that causes failure of pollination (Bassetti and Boyer, 1993; Bassetti and Westgate, 1994). Alternatively newly formed zygotes are sensitive to dehydration and may abort even if stress has been relieved before pollination (Westgate and Boyer, 1986). Although mechanisms underlying abortion are largely unclear, these might be explained by effects of drought on generation of reactive oxygen species (ROS) and subsequent damage to biomolecules by ROS (Baisak et al., 1994; Cruz de Carvalho, 2008; Thomson et al., 1987) and reduction of photosynthesis. Reduction in photosynthetic rates and disruption of carbon metabolism in leaves can lead to reduced assimilate availability to export to sink tissues such as developing kernels, and thereby cause reproductive abortion. Loss of kernel set in maize due to drought is correlated with the extent of loss of photosynthesis and photosynthate influx into the kernels (Schussler and Westgate, 1995).

Previous studies in maize have indicated that ovary/seed abortion can involve inadequate hexose supply to the growing tissue. Drought decreases activities of both vacuolar and cell-wall-bound acid invertases (Zinselmeier et al., 1995), induces invertases in vegetative tissues, and causes their repression in ovaries (Kim et al., 2000). This suggests that sucrose export from source tissues may be restricted, and/or the disaccharide could accumulate in sink tissues such as the ovaries. It has been suggested that drought-induced sucrose accumulation in reproductive

organs of maize and rice may be partially due to low activity of invertases, which fail to cleave incoming sucrose into hexoses (Anderson et al., 2002). In addition, invertase-derived hexoses were found to be critical morphogenic factors for normal development of carrot embryos (Tang et al., 1999); and hexoses have been closely associated with cell division and expansion, with sucrose favoring storage and maturation in legume seed development (Gibson 2005; Wobus and Weber, 1999). Therefore, drought stress in reproductive tissues may limit – through consequences on invertase – formation of hexoses important for development; and the consequent increase in sucrose may inhibit normal development, resulting is low reproductive success (low seed yield).

3.4.2. Transcriptome Studies Using Microarrays Coupled with GS-FLX Pyrosequencing Can Be Used for Drought-Related Gene Discovery in Related Species

Global gene expression, including microarray analysis, has already provided valuable insight into genes and processes related to drought stress in Arabidopsis (Seki et al., 2002); barley (*Hordeum vulgare*) (Ozturk et al., 2002), rice (Zhou et al., 2007), and maize (Zinselmeier et al., 2002). By surveying expression patterns, classes of drought-inducible genes were identified. These genes take part in diverse processes believed to be important for drought resistance.

The application of the two technologies (microarray and GS-FLX pyrosequencing) in this study has enabled identification of drought-inducible rice-maize orthologs. About 80% of the genes in Arabidopsis are also found in rice (Yu et al., 2002), and this reinforces the likelihood that many genes and physiological processes controlling drought tolerance are shared among rice and maize. In this study, not all genes responsive to drought in maize were also responsive in rice. This could be due to inherent drought responsive differences between the two genomes or

the fact that stresses were not equally imposed or sensed between the two species. RWC was lower in rice (63.2%) than in maize (77.7%) under "severe drought" as defined here, even though the soil water potential in maize was lower at -1.34 MPa. This demonstrates one of the difficulties encountered in comparative genomics of species with differences in size, growth habits, morphological and physiological characters in making correlation to different plant characteristics.

Despite this, a number of commonly responsive genes were identified in the vegetative and reproductive tissues. To understand the functional significance of these genes, the Web-based tool EasyGO (Zhou and Su, 2007; http://bioinformatics.cau.edu.cn/easygo/, accessed 01/12/2010) was used to find statistically over-repressed functional terms. Gene ontology (GO) analysis for biological processes of the commonly up-regulated genes revealed that catabolism of protein and biopolymers and establishment of homeostasis were significantly enriched in the drought-stressed vegetative tissues. Genes associated with two processes (protein and biopolymer catabolism) were cullin1 (Os11g26910), SKP1-like protein 1B (Os02g11050), and 26S-protease regulatory subunit (Os01g27160) (Table 3.4a). These genes are part of the ubiquitin- and proteasome proteolytic pathway involved in the turnover of misfolded proteins and hormone-mediated signal transduction (Ingavardsen and Veirskov, 2001; Zhang et al., 2008). Protein degradation is a normal cellular activity, but an increase is degradation in response to drought can be interpreted as the result of excessive protein damage. The protein turnover is necessary for the removal of abnormal or damaged proteins and for altering the balance of proteins and, in a worst-case scenario, degradation of damaged cells (apoptosis). Protein degradation can be an adaptive response to drought, as Lee et al. (2009) demonstrated that removal of a drought sensitivity protein by the protease was important for drought tolerance

in *Arabidopsis thaliana*. Drought tolerance was also enhanced in tobacco (*Nicotiana tabacum*) when genes involved in the ubiquitin-proteasome were overexpressed (Guo et al., 2008). The up-regulation of protein/biopolymer catabolic processes in the vegetative tissue can be viewed as a response to severity of stress and an adaptation to it.

Other processes that were enriched in the vegetative tissues are those generally involved in maintenance of cellular homeostasis. Examples are GO:0045454 (cell redox homeostasis) and GO:0065008 (regulation of biological quality). The genes involved in these processes belong to the theoredoxin superfamily (Houston et al., 2005). These genes are thioredoxin_H type (Os07g08840), theoredoxin_M type (Os02g42700), PDIL2-2 protein disulphide isomerase (Os01g23740), and OsGrx_C2.2 glutaredoxin subgroup I (Os04g42930) (Table 3.4a). Drought stress can result in changes in the chloroplast, mitochondria, and cytosol redox state, leading to oxidative damage to biological membranes and proteins. Lines of evidence indicate that theoredoxins can relieve oxidative stress by modulating both the activity of enzymes scavenging ROS and other functions related to control of cell redox homeostasis (Broin and Rey, 2003; Dos Santos and Rey, 2006) by re-reducing the oxidized -S-S- groups in enzymes involved in metabolism (Buchanan and Balmer, 2005). Induction of these genes by drought likely results from the changes in cellular redox state, and the proteins participate in response to oxidative stress within organelles (chloroplasts and mitochondria) and in the cytosol upon oxidative stress.

In the reproductive tissue, carbohydrate catabolism (GO:0030163) was the only in-common enriched biological process. Genes involved in these process are periplasmic beta-glucosidase precursor (Os03g53800), Os4bglu13-beta-glusosidase homolog (Os04g39900), Os8bglu28-beta-glucosidase homolog (Os08g39870), glycosyl hydrolase fam17 (Os01g64170), and beta 1,3:1,4 glucan synthase (Os08g06380) (Table 3.4a). Beta-glucosidases and glycosyl

hydrolases are involved in the selective cleavage of glucose from polysaccharides; and the glucose may then re-enter sugar-nucleotide interconversion pathways (Leah et al., 1995), contributing to the synthesis of new polysaccharides and other types of polymers. Most b-glucosidases also possess glucotransferase activity, which may enable them to act on glucose units to form a diversity of oligosaccharides (Leah et al., 1995; Amiard et al., 2003), and other carbohydrates that may be important for drought tolerance. Previous studies in maize show that drought stress inhibits invertases and that glucose from sucrose is rendered unavailable under such conditions (Zinselmeier et al., 1995). This could represent an attempt by plant cells to derive sucrose from other sources for primary metabolism to continue during stress. Perhaps the cleaved glucose may be used in the synthesis of 1,3:1,4-glucan (callose), which could be an indication that the chemical composition of the cell wall is altered in these tissues in response to drought as reported by Ingram and Bartels (1996). The results suggest that carbohydrate metabolism may be one of the targets of drought stress or an adaptive response in vegetative tissues.

Analysis of drought-stress down-regulated as well as up-regulated genes is important in understanding general biological responses to stress. In this study, 118 genes were down-regulated in common the vegetative tissues of rice and maize. Those genes were further analyzed to identify the biological processes affected significantly under drought. Photosynthesis (GO:0015979 and GO:0019684) processes and energy metabolism (GO:0006091) were among the down-regulated processes (Table 3.4b). The same processes (from 13 genes) are also down-regulated in the reproductive tissues. Genes taking part in these processes are: five photosystem I reaction center subunits, three chlorophyll a-b binding proteins, a photosystem II reaction center subunit, and two photosystem II oxygen-evolving enhancer proteins (Table 3.4b). Generation of

precursor metabolites and energy (GO:0042592) in which two ATP synthase genes take part was also repressed. These genes constitute part of the processes involved in the light reactions of photosynthesis from oxygen evolution to ATP synthesis by the ATP synthase. In addition to the light reaction genes, an enzyme (fructose-bisphosphate aldolase: Os11g07020) that takes part in the Calvin cycle was among the genes in the generation of precursor metabolites and energy (GO:0042592). Fructose-bisphosphate aldolase catalyzes the conversion of dihydroxyacetone phosphate into fructose-bisphosphate (Xue et al., 2008). Expression of this gene and its homologs has been previously reported to be repressed by drought in Arabidopsis (Chaves et al., 2009) and wheat (Xue et al., 2008). This therefore shows that drought stress in the vegetative tissues of rice and maize down-regulate the photosynthetic genes involved in both light and dark reactions.

These results echo the reduced photosynthesis (ØPS II) observations (Table 3.1). This is consistent with many previous studies that drought stress inhibits photosynthesis (e.g., Tezara et al., 1999). Down-regulation of photosynthetic genes under drought stress has been observed in rice and barley under long-term drought in the field (Hazen et al., 2005; Ozturk et al., 2002) and under more controlled conditions (Talame et al., 2007). Inhibition of photosynthesis can be direct, as CO_2 availability is limited by diffusion through stomata (Flexas et al., 2007) or the alteration of photosynthetic metabolism (Lawlor and Comic, 2002). Or reduced photosynthesis can arise due to oxidative stress, which cause damage to the photosynthetic machinery. The down-regulation of photosynthesis can also be an adaptive mechanism to reduce further damage to the machinery.

Processes involved in amino acid biosynthesis, such as cellular amino acid and derivative metabolic process (GO:0006519) and organic acid metabolism (GO:0006082) and translation

(GO:0006412), were also down-regulated as a result of drought stress in vegetative tissue. This shows that biosynthesis of amino acids and translation of gene transcripts into proteins were repressed. Because the synthesis of stress-induced proteins occur under drought stress, differential translation of mRNA may be a component of the stress response. However, when drought reaches severe levels, as in this case, synthesis of any new proteins may not be achievable.

In conclusion, the evidence from an examination of genes regulated in common in rice and maize suggests that drought stress causes a transition of metabolism from protein synthesis by repressing amino acid biosynthesis and translation to degradation by inducing the ubiquitin-proteasome pathway. In addition, photosynthesis is severely inhibited by repression of genes involved in both the light and dark reactions. The in-common drought-responsive genes identified within the two genomes are potential candidates to study for their broader importance in drought stress by using transformation and mutagenesis.

3.5. References

Amiard V, Morvan-Bertrand A, Billard J-P, Huault C, **Keller F, Prud'homme M-P** (2003) Fructans, but not the sucrosyl-galactosides, raffinose and loliose, are affected by drought stress in perennial ryegrass. Plant Physiol **132**: 2218-2229.

Anderson MN, Asch F, Wu Y, Jesnsen CR, Naested H, Mogensen VO, Koch KE (2002) Soluble invertase expression is an early target of drought stress during the critical, abortion-sensitive phase of young ovary development in maize. Plant Physiol **130**: 591-604.

Baisak R, Rana D, Acharya PBB, Kar M (1994) Alterations in the activities of active oxygen scavenging enzymes of wheat leaves subjected to water stress. Plant Cell Physiol **35**: 489-495.

Bartels D, Nelson D (1994) Approaches to improve stress tolerance using molecular genetics. Plant Cell Environ **17**: 1659-1667.

Bassetti P, Boyer JS (1993) Water deficit affects receptivity of maize silks. Crop Sci **33**: 279-282.

Bassetti P, Westgate ME (1994) Floral synchrony and kernel set in maize quantified by image analysis. Agron J **86**: 699-703.

Broin M, Rey P (2003) Potato plants lacking the CDSP32 plastidic thioredoxin exhibit overoxidation of the BAS1 2-cysteine peroxiredoxin and increased lipid peroxidation in thylakoids under photooxidative stress. Plant Physiol **132**: 1335-1343.

Buchanan B, Balmer Y (2005) Redox regulation: a broadening horizon. Annu Rev Plant Biol **56**: 187-220.

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot **103**: 551-560.

Cruz de Carvalho MH (2008) Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signaling and Behavior **3**: 156-165.

Devos KM (2005) Updating the 'Crop Circle'. Curr Opin Plant Biol **8**: 155-162.

Dos Santos CV, Rey P (2006) Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci **11**: 1329-1334.

Flexas J, Diaz-Espejo A, Galmes J, Kaldenhoff R, Medrano H, Ribas-Carbo M (2007) Rapid variations in mesophyll conductance in response to changes in CO_2 concentration around leaves. Plant Cell Environ **30**: 1284-1298.

Gibson SI (2005) Control of plant development and gene expression by sugar signaling. Curr Opin Plant Biol **8**: 93-102.

Guo Q, Zhang J, Gao Q, Xing S, Li F, Wang W (2008) Drought tolerance through overexpression of monoubiquitin in transgenic tobacco. J Plant Physiol **165**: 1745-1755.

Hamada A, Shono M, Xia T, Ohta M, Hayashi Y, Tanaka A, Hayakawa T (2001) Isolation and characterization of a Na1/H1 antiporter gene from the halophyte *Atriplex gmelini*. Plant Mol Biol **46**: 35–42.

Hazen SP, Pathan MS, Sanchez A, Baxter I, Dunn M, Estes B, Chang HS, Zhu T, Keps JA, Nguyen HT (2005) Expression profiling of rice segregating for drought tolerance QTLs using a rice genome array. Funct Integr Genomics **5**: 104-116.

Houston NL, Fan C, Xiang QY, Schulze JM, Jung R, Boston RS (2005) Phylogenetic analyses identify 10 classes of the protein disulfide isomerase family in plants, including single-domain protein disulfide isomerase-related proteins. Plant Physiol **137**: 762-778.

Ingram I, D. Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol **47**:377–403.

Invardsen C, Veirskov B (2001) Ubiquitin- and proteasome-dependent proteolysis in plants. Physiol Plant **112**: 451-459.

Kim JY, Mahe A, Brangeon J, Prioul JL (2000) A maize vacuolar invertase, IVR2, is induced by water stress: organ/tissue specificity and diurnal modulation of expression. Plant Physiol **124**: 71-84.

Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ **25**: 275-294.

Leah R, Kigel J, Svendsen IB, Mundy J (1995) Biochemical and molecular characterization of barley seed b-glucosidases. J Biol Chem **270**: 15789-15797.

Lee KL, Cho SK, Son O, Xu Z, Hwang I, Kim WT (2009) Drought stress-induced Rma1H1, a ring membrane-anchor E3 ubiquitin ligase homolog, regulates aquaporin levels via ubiquitin in transgenic arabidopsis plants. Plant Cell: doi/10.1105/tpc.109.071548.

Livak, KJ, Schmittgen, TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$CT method. Methods **25**: 402-408.

Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstrom DC, Bensen RJ, Castiglioni PP, Donnarummo MG, Hinchey BS, Kumimoto RW, Maszle DR, Canales RD, Krolikowski KA, Dotson SB, Gutterson N, Ratcliffe OJ, Heard JE (2007) Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci USA **104**: 16450-16455.

O'Brien KPO, Maido Remm M, Sonnhammer ELL (2005) Inparanoid: a comprehensive database of eukaryotic orthologs. Nucleic Acids Res **33**: 476-480.

Ozturk ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ (2002) Monitoring large scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol **48**: 551-573.

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD, Harberd NP (1999) Green revolution genes encode mutant gibberellin response modulators. Nature **400**: 256–261.

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol **133**: 1755-1767.

Rice Chromosome 10 Sequencing Consortium (RCSC) (2003) In-depth view of structure, activity, and evolution of rice chromosome 10. Science **300**: 1566-1569.

Selote DS, Khanna-Chopra R (2004) Drought induced spikelet sterility is associated with an inefficient antioxidant defense in rice panicles. Physiol Plant **121**: 462-471.

Schussler JR, Westgate ME (1995) Assimilate flux determines kernel set at low water potential in maize. Crop Sci **35**: 1074-1080

Schnable PS, Ware D, Fulton RS, (et al.) (2009) The B73 maize genome: complexity, diversity, and dynamics. Science **326**: 112-115.

Setter TL, Flannigan BA, Melkonian J (2001) Loss of kernel set due to water deficit and shade in maize: carbohydrate supplies, abscisic acid, and cytokinins. Crop Sci **41**: 1530-1540.

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Kazuko Yamaguchi-Shinozaki K,

Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J **31**: 279-292.

Talame V, Ozturk NZ, Bohnert HJ, Tuberosa R (2007) Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. J Exp Bot **58**: 229-240.

Tang GQ, Luscher M, Sturm A (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. Plant Cell **11**: 177-189.

Tardieu, F., and C. Granier (2000) Quantitative analysis of cell division in leaves: methods, developmental patterns and effects of environmental conditions. Plant Mol Biol **43**:555-567.

Tezara W, Mitchel VJ, Driscoli SD, Lawlor DW (1999) Water deficit inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature **401**: 914-917.

Thomson JE, Legge RL, Barber RF (1987) The role of free radicals in senescence and wounding. New Phytol **105**: 317-344.

Tondelli A, Francia E, Barabaschi D, Aprile A, Skinner JS, Stockinger J, Stanca AM, Pecchioni N (2006) Mapping regulatory genes as candidates for cold and drought stress tolerance in barley. Theor Appl Genet **112**: 445–454.

Udvardi MK, Czechowski T, Scheible W-R (2008) Eleven golden rules of quantitative RT-PCR. Plant Cell **20**: 1736-17-37.

Westgate ME, Boyer JS (1986) Reproduction at low water silk and pollen water potential in maize. Crop Sci **26:** 951-956.

Wobus U, Weber H (1999) Sugars as signal molecules in plant seed development. Biol Chem **380**: 937-944.

Xue G-P, McIntyre CL, Glassop D, Shorter R (2008) Use of expression analysis to dissect alterations in carbohydrate metabolism in wheat leaves during drought stress. Plant Mol Biol **67**: 197-214.

Yazaki J, Shimatani Z, Hashimoto A, Nagata Y, Fujii F, Kojima K, Suzuki K, Taya T, Tonouchi M, Nelson C, Nakagawa A, Otomo Y, Murakami K, Matsubara K, Kawai J, Carninci P, Hayashizaki Y, Kikuchi S (2004) Transcriptional profiling of genes responsive to abscisic acid and gibberellin in rice: phenotyping and comparative analysis between rice and *Arabidopsis*. Physiol Genomics: **10**. 1152/physiolgenomics.00201.2003.

Yu, J, Hu S, Wang J, et al. (2002). A draft sequence of the rice genome. Science **296**: 79-91

Zhang Y, Xu W, Li Z, Deng XW, Wu W, Xue Y (2008) F-Box protein DOR functions as a novel inhibitory factor for abscisic acid-Induced stomatal closure under drought stress in Arabidopsis. Plant Physiol **148**: 2121-2133.

Zhou J, Wang X, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong X, Zhang Q, Fan L, Deng XW (2007) Global genome expression of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol **63**: 591-608.

Zhou X, Zhen Su Z (2007) EasyGO: Gene Ontology-based annotation and functional enrichment analysis tool for agronomical species. BMC Genomics **8**: doi: 10.1186/1471-2164-8-246.

Zinselmeier C, Sun Y, Helentjaris T, Beatty M, Yang S, Smith H, Habben J (2002) Use of gene expression profiling to dissect the stress sensitivity of reproductive development in maize. Field Crops Res **75:** 111-121.

Zinselmeier C, Westgate ME, Schussler JR, Jones RJ (1995) Low water potential disrupts carbohydrate metabolism in maize (*Zea mays* L.) ovaries. Plant Physiol **107**: 385-391.

Table 3.1. Physiological parameters in maize affected by moderate and severe drought stress at silking stage

Treatment	Soil Moisture Content (SMC) (cm^3 cm^{-3})	Chlorophyll Fluorescence		Relative Water Content (RWC%)	Seed Number (#/plant)
		Fv/Fm	ØPS II		
Control (WW)	0.46[a]	0.757[a]	0.706[a]	96.7[a]	121[a]
Moderate Drought	0.15[b]	0.735[a]	0.421[b]	86.6[b]	100[ab]
Severe Drought	0.12[c]	0.738[a]	0.511[b]	77.7[c]	40[b]
LSD (0.05)	0.018	0.047	0.158	11.87	68.1
P value	<0.0001	0.05146	0.0118	<0.0001	0.0424

WW = well-watered controls, Moderate drought = withheld irrigation for 2 d; severe drought = withheld irrigation for 3 d; SMC = volumetric soil moisture content; Fv/Fm = photochemical efficiency of photosystem II; ØPS II = effective quantum yield of photosystem II. Means within columns followed by the same letter are not significantly different at p = 0.05. For each data point, n = 4.

Table 3.2. Summary of rice-maize gene orthologs identified by the GS-FLX pyrosequencing platform

Library	Read Numbe[1]	Maize Gene Best Hits[2]	Genes Present[3]	Comparable Genes[4]	Os-orthologs Drought Responsive Genes
LC	14 472	3 424	569		
LD	17 718	4 420	863	1 284	219
OC	39 768	390	138		
OD	16 499	4 244	837	915	55

[1]Number of reads with at least 100 bp were used for analysis.
[2]Reads that mapped to maize (B73) genomic cDNA sequences with a BLASTn e-value $<10^{-5}$ and a bit score greater than 100 were used for further analysis.
[3]Genes with at least two read matches are considered present.
[4]Comparisons between libraries LC against LD and OC against OD.
LC = control (well-watered) leaf tissue
LD = drought-stressed leaf tissue
OC = control (well-watered) ovary tissue
OD = drought-stressed ovary tissue

Table 3.3a. Genes involved in the up-regulated processes in the rice and maize vegetative and reproductive tissues

Up-regulated in the vegetative tissues		
ID	**Annotation**	**P/Q-Value[¶]**
GO:0030163	**Protein catabolism (172)[Ω]**	**0.0475**
Os05g05700	Cullin	0.0037
Os11g26910	Skip like protein 1B	0.0049
Os02g11050	26S protease regulatory subunit	0.0006
Os01g27160	Cullin	0.0205
GO: 0043282	**Biopolymer catabolism (188)**	**0.0464**
Os05g05700	Cullin	0.0037
Os11g26910	Skip like protein 1B	0.0049
Os02g11050	26S protease regulatory subunit	0.0006
Os01g27160	Cullin	0.0205
GO:0045454	**Cell redox homeostasis (114)**	**0.0427**
Os07g08840	Theoredoxin_H type	0.0011
Os01g23740	OsPDIL2-2 protein disulphide isomerase	0.0077
Os04g42930	OsGrx_C2.2-glutaredoxin subgroup I	0.0047
Os02g42700	Thioredoxin_M type	0.0135
GO: 0042592	**Homeostatic process (136)**	**0.0427**
Os07g08840	Theoredoxin_H type	0.0011
Os01g23740	OsPDIL2-2 protein disulphide isomerase	0.0077
Os04g42930	OsGrx_C2.2-glutaredoxin subgroup I	0.0047
Os02g42700	Thioredoxin_M type	0.0135
GO:0019725	**Cellular homeostasis (129)**	0.0427
Os07g08840	Theoredoxin_H type	0.0011
Os01g23740	OsPDIL2-2 protein disulphide isomerase	0.0077
Os04g42930	OsGrx_C2.2-glutaredoxin subgroup I	0.0047
Os02g42700	Thioredoxin_M type	0.0135
GO: 0065008	**Regulation of biological quality (161)**	**0.0493**
Os07g08840	Theoredoxin_H type	0.0011
Os01g23740	OsPDIL2-2 protein disulphide isomerase	0.0077
Os04g42930	OsGrx_C2.2-glutaredoxin subgroup I	0.0047
Os02g42700	Thioredoxin_M type	0.0135
Up-regulated in the reproductive tissues		
GO:0005975	**Carbohydrate metabolic process (838)**	**0.0198**
Os03g53800	Periplasmic beta-glucosidase precursor	0.0006
Os04g39900	Os4bgl13-beta-glucosidase homolog	0.0049
Os08g39870	Os8bglu28-beta-glucosidase homolog	0.0074
Os01g64170	Glycosyl hydrolase fam17	0.0078
Os08g06380	Beta1,3;1,4 glucan synthase	0.0005

The commonly up-regulated genes in rice and maize were further used for GO analysis using the EasyGO program. [¶]P-Values were used for processes, and Q-Values obtained from microarray analysis were used for genes. Numbers in parentheses are genes in the rice genome associated with the process.

Table 3.3b. Genes involved in the down-regulated processes in the rice and maize vegetative and reproductive tissues

Down-regulated in vegetative tissues		
ID	**Annotation**	**P/Q-Value[¶]**
GO:0015979 Photosynthesis (100) [Ω]		**0.0002**
Os08g44680	Photosystem I reaction center subunit	0.0005
Os02g42570	Ferrdoxin-thioredoxin reductase	0.0070
Os07g25430	Photosystem I reaction center subunit IVA	0.0002
Os03g56670	Photosystem I reaction center subunit III	0.0013
Os07g37550	Chlorophyll a-b binding protein of LHCII type III	0.0001
Os12g23200	Photosystem I reaction center subunit XI	0.0001
Os07g38960	Chlorophll a-b binding protein chloroplast precursor	0.0002
Os01g71190	Photosystem II reaction center PSBP28	0.0017
Os09g04840	Oxygen evolving enhancer 2	0.0004
Os12g08770	Photosystem I reaction center subunit N	0.0001
Os08g33820	Chlorophyll a-b binding protein 4	0.0004
Os02g36850	Oxygen evolving enhancer protein	0.0005
Os07g36080	Oxygen evolving enhancer 3	0.0010
GO: 0019684 Light harvesting reaction, photosynthesis (18)		**0.0262**
Os07g38960	Chlorophll a-b binding protein chloroplast precursor	0.0001
Os07g37550	Chlorophyll a-b binding protein of LHCII type III	0.0001
Os08g33820	Chlorophyll a-b binding protein 4	0.0004
GO:0006412 Translation (683)		**0.0013**
Os07g08660	Ribosomal protein S15	0.0424
Os03g03020	L11 domain containing protein	0.0003
Os01g58220	Translation initiation factor SUI1	0.0035
Os0238210	Elongation factor Tu	0.0035
Os03g24200	Ribosomal protein L6	0.0001
Os02g15900	50S ribosomal protein L21	0.0086
Os12g03880	60S acidic ribosomal protein Po	0.0009
Os03g54040	Ribosomal protein L6	0.0001
Os02g04460	Ribosomal protein L3	0.0015
Os05g01110	Ribosomal protein L28	0.0021
Os03g49710	30S ribosomal protein S13	0.0002
GO: 0042592 Generation of precursor metabolite and energy(309)		**0.0321**
Os07g37550	Chlorophyll a-b binding protein of LHCII type III	0.0001
Os07g32880	ATP synthase gamma chain	0.0001
Os03g17070	ATP synthase B chain	0.0002
Os07g38960	Chlorophll a-b binding protein chloroplast precursor	0.0002
Os08g33820	Chlorophyll a-b binding protein 4	0.0004
Os11g07020	Fructose-bisphosphate aldolase	0.0001
GO: 0006082 Organic acid metabolism (488)		**0.0009**
Os07g46460	Ferridoxin-dependent glutamate synthase	0.0001
Os03g52170	4-hydroxy-3-mthylbut-2-enyl disphosphate reductase	0.0239

Table 3.3b Continued		
Os02g42570	Ferridoxin-theoredoxin reductase	0.0070
Os03g25940	Cystathionine gamma-synthase	0.0037
Os11g26860	Serine hydroxyl methyltransferase	0.0005
Os10g35960	NAD-dependent malic enzyme	0.0023
Os10g26110	Tyrosine decarboxylase	0.0371
Os01g73450	Amino acid kinase	0.0225
Os01g74650	Cysteine synthase	0.0011
Os11g31900	Acyl carrier protein	0.0006
GO:0006519 Cellular amino acid & and derivative metabolism(388) 0.0071		
Os07g46460	Ferridoxin-dependent glutamate synthase	0.0001
Os03g25940	Cystathionine gamma-synthase	0.0037
Os11g26860	Serine hydroxyl methyltransferase	0.0005
Os10g26110	Tyrosine decarboxylase	0.0371
Os01g73450	Amino acid kinase	0.0225
Os01g74650	Cysteine synthase	0.0011
Down-regulated in the reproductive tissues		
GO:0015979 Photosynthesis (100)	**0.0004**	
Os06g21590	Chlorophyll a-b binding protein 6A	0.0002
Os07g05480	Chlorophyll a-b binding protein of LHC II	0.0001
Os07g05480	Photosystem I reaction center subunit psaK	0.0049
Os08g33820	Chlorophyll a-b binding protein 4	0.0004
GO:0006091 Generation of precursor metabolite and energy (309) 0.0058		
Os06g21590	Chlorophyll a-b binding protein 6A	0.0002
Os07g37550	Chlorophyll a-b binding protein of LHCII type III	0.0001
Os08g33820	Chlorophyll a-b binding protein 4	0.0004

The commonly down-regulated genes in rice and maize were further used for GO analysis using the EasyGO program. ¶P-Values were used for processes, and Q-Values obtained from microarray analysis were used for genes. Numbers in parentheses are genes in the rice genome associated with the process.

Table 3.4. Genes selected for qRT-PCR analysis.

Os-Locus ID	Annotation	LOG_2 ratio	Zm-ID
Os04g33920	Lipid binding protein	7.94326779	GRMZM2G136364
Os02g44870	Dehydrin_COR410	3.68251825	GRMZM2G147014
Os01g40094	ABI-2 (PP2C)	0.54725743	GRMZM2G134628
Os05g10670	Zinc finger protein_CCCH type	2.62365553	GRMZM2G173124
Os09g25090	CIPK-like protein_1	1.06235794	GRMZM2G129018
Os03g16030	Class_1 Heat shock protein	1.50790266	GRMZM2G137839
Os07g49400	Cytosolic Ascorbate peroxidase	1.13215971	GRMZM2G046382
Os03g57220	Hydroacid oxidase_1	-1.1399541	GRMZM2G129246
Os03g44740	Cytochrome_P450_93A3	-0.4514745	GRMZM2G139467
Os04g56320	RUBISCO activase	-1.6590266	GRMZM2G162200

The genes were selected based on expression in the maize reproductive tissue and any other tissue (maize vegetative, rice vegetative and reproductive tissues) in Figure 3.2.

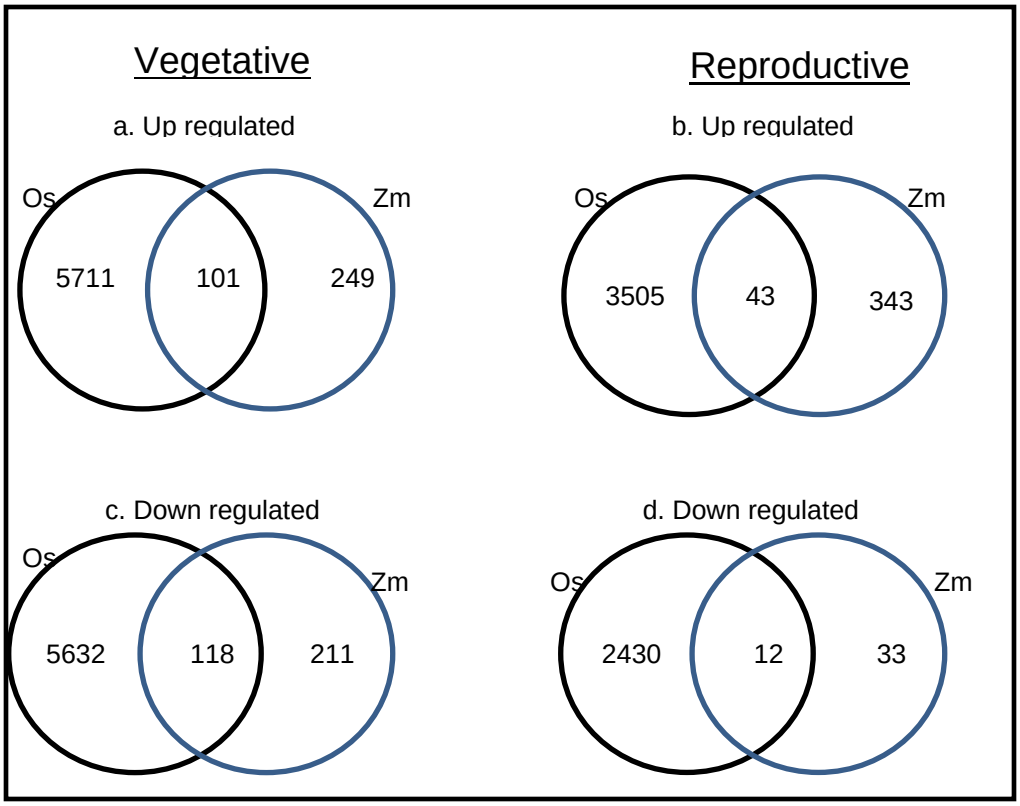

Figure 3.1. Venn diagram of differentially expressed genes in the vegetative and reproductive tissues of rice (Os) and maize (Zm) reproductive tissues under drought stress. The rice genes were identified by microarray analysis, while the maize genes were identified by GS-FLX sequencing. Number of genes identified in each tissues were rice vegetative [5812 (5750)], rice reproductive [3548 (2442)]; maize vegetative [350 (329)], and maize reproductive [386 (45)]. The two numbers in each parenthesis indicate up- and down-regulated genes in each tissue.

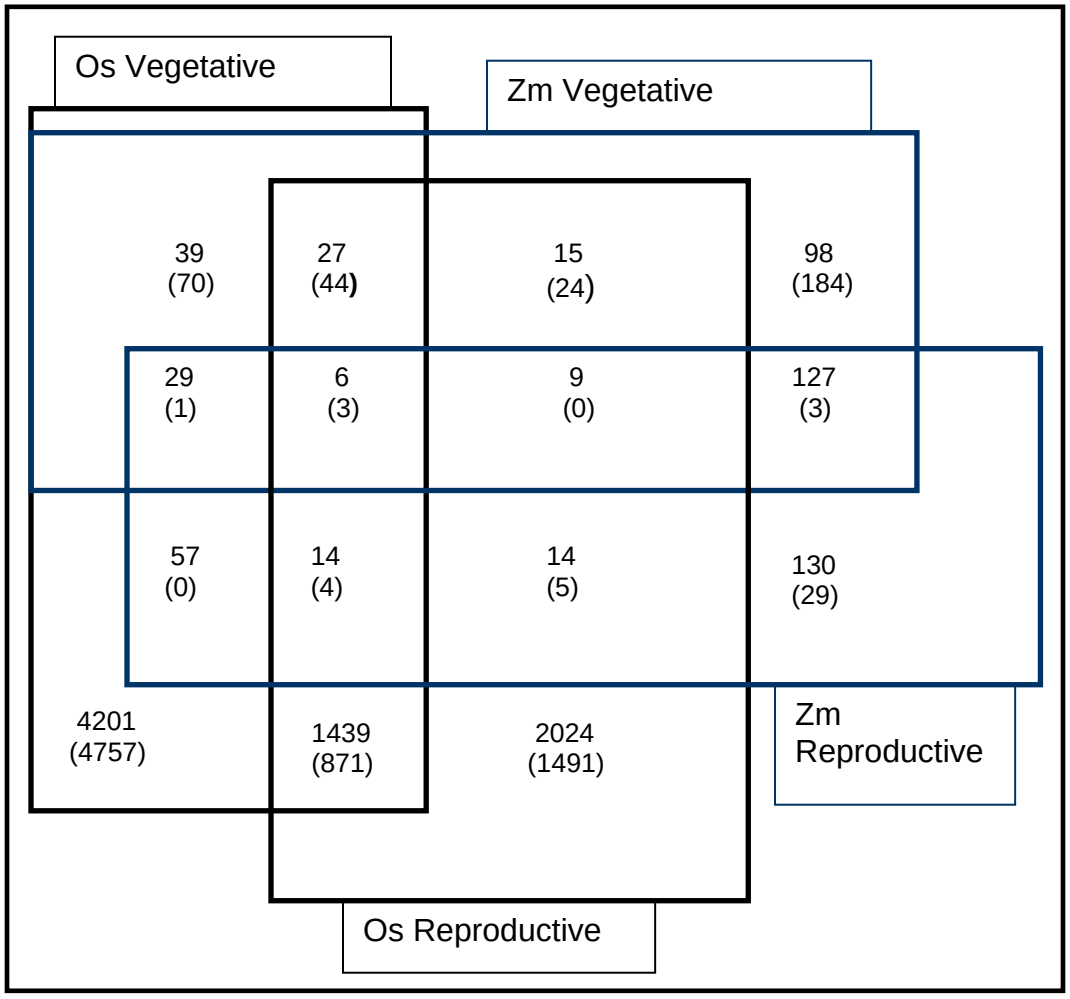

Figure 3.2. Venn diagram multiple comparisons between genes regulated by drought stress in rice (Os) and maize (Zm) vegetative and reproductive tissues. Numbers in parentheses are down regulated genes. Number of genes identified in each tissue were: rice vegetative [5812 (5750)], rice reproductive [3548 (2442)], maize vegetative [350 (329)], maize reproductive [386 (45)].

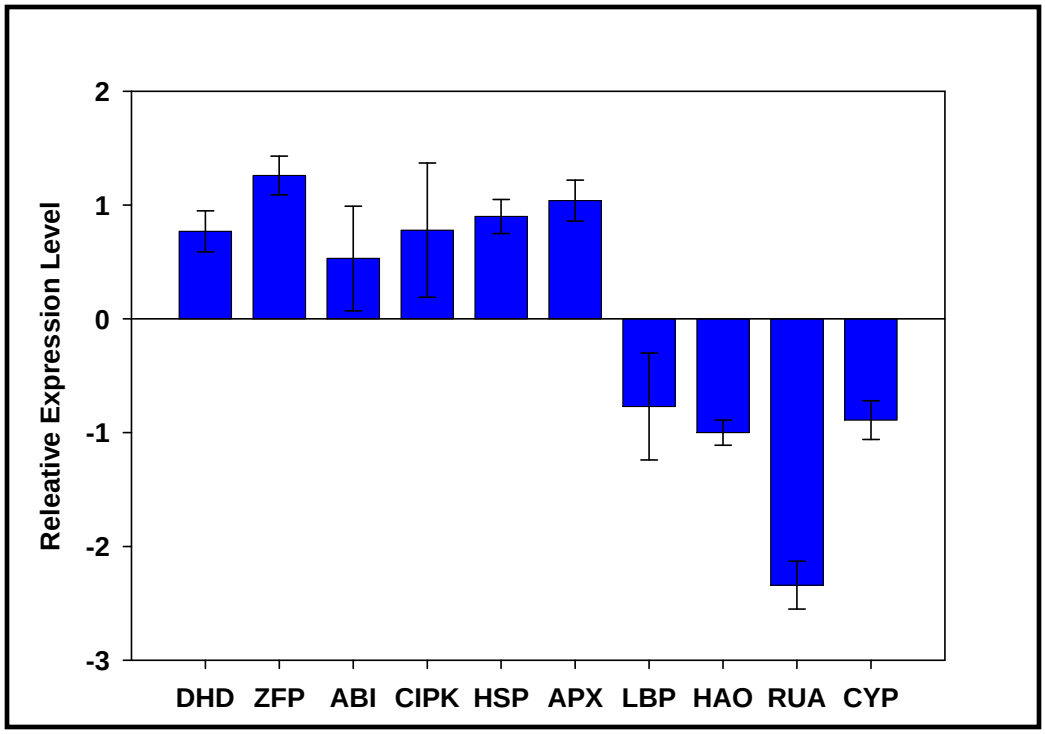

Figure 3.3. qRT-PCR analysis of drought-responsive genes in maize ovary tissue. qRT-PCR procedures are described in Materials and Methods. DHD = Dehydrin, ZFP = Zinc finger protein_CCCH type, ABI = ABI-2 (PP2C), CIPK = CIPK-like protein1, HSP = Class_1_heat shock protein, APX = Cytosolic ascorbate peroxidase, LBP = Lipid binding protein, HAO = Hydroacid oxidase_1, RUA = Rubisco activase, CYP = Cytochrome_P450_93A3. Locus IDs for each is shown in Table 3.4. Bars = +/- 1 standard error. For each data point, n = 3.

Chapter 4. HYR, a Rice AP2/ERF-like Transcription Factor Improves Yield, Water Use Efficiency, and Drought Tolerance in Rice

Abstract

Lack of water is a major environmental factor limiting plant productivity. Plant productivity and efficient water use under water-limited conditions are some of the most important traits for drought resistance, especially in crops such as rice (*Oryza sativa*). The results of overexpression of the HIGHER YIELD RICE (HYR) gene, an AP2/ERF transcription factor identified from rice drought transcriptome analysis, are described here. The HYR-overexpressing plants had increased shoot biomass, grain yield, and water use efficiency (WUE). The enhanced biomass was correlated with increased net photosynthesis but no greater transpiration, resulting in higher WUE. The increase in net photosynthesis was observed under both well-watered and drought-stressed conditions. The HYR plants also accumulated higher levels of soluble sugars (glucose, sucrose, and fructose) and maintained higher relative water content under drought-stress conditions. The increased yields were a function both of more spikelets and of larger grains. The results demonstrate the application of genetic manipulation of a plant transcription factor for the improvement of plant productivity with or without water-limiting conditions.

4.1. Introduction

Drought stress is among the most serious challenges to production worldwide for the major cereals: maize (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) (Pellergrineschi et al., 2004). Major research efforts have been and are being directed at understanding the mechanism of plant responses to drought stress in order to identify gene products that confer adaptation to water deficit. Molecular mechanisms of water stress response have been investigated primarily in the model plant species *Arabidopsis thaliana* (Liu et al., 1998). Upon exposure to drought stress conditions, many stress-related genes are induced, and their products are thought to function as cellular protectors from stress-induced damage (Oh et al., 2009; Shinozaki et al., 2003). The expression of stress-related genes is largely regulated by transcription factors (TF). The rice and *Arabidopsis thaliana* genomes code for more that 1500 TF, and about 45% of them are reported from plant-specific families (Kikuchi et al., 2003). Various drought stress studies have identified TF families with putative functions in drought including MYB, bZIP, Zinc finger, NAM, and APETALA2 (AP2) (Oh et al., 2009; Zhou et al., 2007). The AP2 family is one of the plant-specific TF whose members share a highly conserved DNA-binding domain known as AP2 (Weigel, 1995). Members of this family have been associated with various developmental processes (Chuck et al., 1998) and with stress tolerance (Haake et al., 2002; Liu et al., 1998).

Many approaches might be taken to boost intrinsic yield, including increasing photosynthetic capacity, modifying plant architecture, and enhancing the rate of growth. An algorithm to identify key points of regulation for enhancing plants' rate of photosynthesis has been described (Zhu et al., 2007). The prospects for controlling photosynthetic capacity have been reviewed by Horton et al. (2000) and Long et al. (2006), and some of the specific

opportunities identified are excellent targets for TF-based genetic manipulation. In addition to yield improvement, significant commercial efforts are being focused on stabilizing yield in the face of environmental pressures such as drought. Two trait areas, yield and drought tolerance, offer high-value returns on products and are major targets for TF-based genetic improvement (Century et al., 2008).

The AP2 TF CBF4 (also known as DREB1 [dehydration-responsive element-binding protein]) is probably the most studied in drought. Overexpression of CBF4 was found to lead to drought adaptation in Arabidopsis (Haake et al., 2002) and wheat (Pellegrineschi et al., 2004). Another AP2 Arabidopsis TF called HARDY was recently reported to provide enhanced drought tolerance in Arabidopsis and rice (Karaba et al., 2007). Ectopic expression of these genes confers drought tolerance and/or adaptation by modifying cellular structures of leaves and roots, CO_2 exchange, and parameters such as water use efficiency (WUE), which correlate with the transformed plants' ability to withstand drought. Taken together, these and other findings indicate that AP2 TF offers the potential to engineer plants in a way that makes them more productive under stress conditions.

Although drought stress can alter the growth and development of a plant at any time during its life cycle, water limitations during reproductive growth stages can be especially conducive to yield losses in crops like rice (Selote and Khana-Chopra, 2004) and maize (Zinselmeier et al., 1995). Accordingly, the reproductive phases in these plants should be an important stage to study for identifying stress-responsive genes that might have a protective, or yield altering, function in drought. Advances in plant genomics, including the availability of the complete genome sequence of rice, have provided an opportunity to identify stress-related TF that control drought-related traits. To this end, a genome-wide analysis of drought stress

responses was conducted (Chapter 2 of this dissertation) and led to the identification of a candidate drought-induced AP2/ERF TF in reproductive tissues.

To determine if the TF could play a role in enhancing the tolerance of rice and possibly other crops to drought stress, transgenic plants were generated that contain the candidate gene driven by the CaMV 35S promoter. The transgenic plants here are referred to as HIGHER YIELD RICE (HYR), as they showed higher grain yield even under well-watered conditions. In addition, the HYR lines had higher shoot biomass, photosynthesis, sugar levels, and WUE under well-watered and drought-stressed conditions. The drought-stressed plants maintained higher water status as determined by relative water content. The enhanced productivity and the drought-resistant phenotype of the transgenic plants compared to the wild type are discussed. These studies provide clues to understanding plant productivity in combination with stress tolerance.

4.2. Materials and Methods

4.2.1. Generation and Selection of Rice Transformants with the HYR gene

The rice drought-stress transcriptome experiment (Chapter 2 of this dissertation) showed the gene Os03g02650 (HYR), annotated as an AP2/ERF TF, to be up-regulated (Log_2 ratio = 0.7779) in reproductive tissues. To make an overexpression construct of the gene, the full-length cDNA was cloned behind the 35S Cauliflower Mosaic Virus (CaMV35S) promoter for constitutive expression, generating the 35S:HYR vector. The construct was then introduced into rice (cultivar 'Nipponbare') by *Agrobacterium*-mediated transformation (Hei et al., 1994; Nishimura et al., 2007). Briefly mature seeds were de-husked and sterilized in 70% (vol/vol) ethanol for 1 to 2 min and then transferred to 50% (vol/vol) chlorox solution for 30 min with gentle shaking. The seeds were rinsed five times with sterile water.

The sterilized seeds were then plated for callus induction on Murashige and Skoog (MS) medium supplemented with 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.3 g/L casamino acid, 30 g/L sucrose, 3 g/L proline, 0.1 g/L *myo*-inositol, and 3 g/L gellan gum at pH 5.8 (MSCI) and grown for 21 to 28 d. Three to four weeks after callus induction from the scutellar region of the rice embryo, calli were immersed in *Agrobacterium tumefaciens* suspension for 10 min with gentle shaking to infect them.

Infected calli were co-cultivated with Agrobacterium in MSCI supplemented with 0.5 g/L casamino acid, 100 μM acetosyringone, 68.5 g/L sucrose, 36g g/L glucose, 0.9 g/L L-glutamine, 0.3 g/L L-aspartic acid, and 3 g/L KCl at pH 5.2 (MSCC). After 3 d of co-cultivation, calli were washed five times with sterile water followed by 225 mg/L cefotaxime and further with 250 mg/L carbenicillin. The calli were rinsed three times with sterile water to wash off the antibiotics and blotted on sterile filter paper. The calli were plated on MSCI supplemented with 50 mg/L

hygromycin, 225 mg/L cefotaxime, 250 mg/L carbinicillin, and 3 g/L gellan gum at pH 5.8 (MSSE) and incubated in a tissue culture growth chamber at 28^{o}C and 250 µmol PAR/m^{2}/s. The calli were subcultured on MSSE every two weeks until plant regeneration was observed. The MS tissue culture media, its supplements and antibiotics were sourced from Sigma, St. Louis, MO.

The regenerated plantlets were grown on MS media supplemented with 0.1 g/L *myo*-inositol, 30 g/L sucrose, and 100xMS vitamins at pH 5.8 (MSPG) in a tissue culture growth chamber at 28^{o}C, 250 µmol PAR/m^{2}/s, 16-h light/8-h dark period for two to three weeks. The regenerated plantlets (putative primary transformants, T0) were transplanted into pots and grown in environmental controlled plant growth chamber for another three weeks and transferred to the greenhouse and grown further to maturity.

Seeds from the putative transformants were tested for hygromycin resistance by germinating them on 50 mg/l hygromycin (Sigma, Saint Louis MO), following the procedures of Nishimura et al. (2007). Five hygromycin-resistant lines (HYR-2, HYR-4, HYR-12, HYR-16, and HYR-45) were identified (Figure 4.1.). Seeds from the hygromycin-resistant seed lots were planted, and individual plants were genotyped by PCR using primers to amplify the hygromycin phosphotransferase (hpt) gene marker. DNA was isolated from three-week-old seedlings. About 3 to 5 cm leaf material was ground in liquid nitrogen. 400 µL 1x isolation and 400 µL phenol:chloroform were added, and the material was gently shaken for 5 min. The supernatant was removed into fresh tubes, and 250 µL isopropanol was added and mixed and DNA was precipitated for 5 min at room temperature, followed by centrifuging at 7500 rpm for 20 min. The isopropanol was discarded and the pellet dried at room temperature for 1 h. The pellet was washed by adding 200 µL 70% ethanol and centrifuging for 5 min at 7500 rpm. The ethanol was poured off, and the pellet dried for 1 h at room temperature. The DNA pellet was dissolved in 50

μL of 10 μg/ml RNase A. The DNA was used as template for PCR analysis. The primer sequence used was *hpt* forward, CGATTGCGTCGCATCGACCCTGCGC and *hpt* reverse, CGACCTGATGCAGCTCTCCGAGGGC. PCR was carried out with *Taq* DNA polymerase (New England Biolabs Inc., Ipswich, MA) in 20 μL reaction volume in a thermal cycler (IQ5, Bio-Rad Laboratories, Hercules, CA). The PCR cycle program consisted of initial denaturation at 95^{o}C for 3 min, followed by 30 cycles of denaturation at 95^{o}C for 1 min, annealing at 55^{o}C for 30 s, and extension at 72^{o}C for 1 min, followed by a final extension at 72^{o}C for 5 min. The PCR product was resolved by 0.8% agarose gel electrophoresis in 1x Tris-acetate-EDTA (TAE) buffer along with 1-kbp DNA ladder as marker (New England Biolabs Inc., Ipswich, MA). The *hpt* gene positive plants (Figure 4.1e) were used for experiments.

4.2.2. Experiment to Measure Water Use

250-ml pots were filled with a 1:1 mix of topsoil and compost (Scott-Sierra Horticultural Product Co, Marysville, OH). The compost was made from sphagnum/peat moss, vermiculite, and bark ash. Soil at essentially field capacity was weighed (450 g) and filled into tared pots. One-week old seedlings of HYR and wild type (WT) rice were transplanted into the pots (one plant per pot). The pots were placed in water-filled trays to simulate flooded/paddy conditions and supplied with a general purpose 20-20-20 fertilizer (Scott-Sierra Horticultural Product Co, Marysville, OH) dissolved in water to provide 50 kg N, P_2O_5, and K_2O/ha. Fertilizer was applied once a week throughout the growing period.

Twenty-eight days after planting (DAP) the pots were mulched with a layer of perlite of tared weight to minimize evaporative water loss from the soil surface. The pots were removed from the water-filled trays and placed on tared bases. Sixteen pots of each genotype were divided in two, with eight for drought-stress treatment and eight for well-watered controls. The drought-

stress treatments involved withholding irrigation until an average of 130 g of water were lost (soil + water = 320 g), i.e., until the soil + water weight declined by 30%, which took 3 d. At this time (31 DAP), shoots on half of the pots (four for drought and four for control treatment) for each line were harvested and dried at 72^{o}C for 96 h, and this biomass was designated as BIO_{31}.

For the unharvested plants, pots were maintained such that soil + water was 320 g (drought stressed) or 450 g (field capacity) by replacing water lost through transpiration. These moisture adjustments were made at mid-morning and late afternoon. The amount of water added each day to maintain each pot at drought stress or field capacity was noted for calculation of cumulative water used (WUc). This watering regime was continued for 14 d. At that point (45 DAP), shoots were harvested and dried at 72^{o}C for 96 hr, and this biomass was designated BIO_{45}. WUc was calculated using the formulae: daily WU = (Total weight) – (tared pot + tared base + tared mulch), and WUc = $\sum${daily WU} over 14 d. Gravimetric water use efficiency (WUEg) was calculated as: $WUEg = [(BIO_{45}) – (BIO_{31})]/(WUc)$.

The experiment was a 2 x 6 factorial, with factors being watering regime (drought-exposed or well-watered) and genotype (five HYR genotypes plus WT) arranged in a completely randomized design (CRD) and replicated four times. Data were subjected to analysis of variance (ANOVA) using the general linear model (Proc GLM) of Statistical Analysis System (SAS). Differences between means were tested by the Least Significance Difference with a 0.05 threshold ($LSD_{0.05}$).

4.2.3. Experiment to Measure Gas Exchanges

Three lines (HYR-2, HYR-4, and HYR-16) exhibiting higher biomass production and greater WUE in the previous experiment (4.2.2.) were selected for further physiological analysis. The transgenic and WT plants were grown in tared pots under well-watered/semi-flooded

conditions for eight weeks following procedures as in 4.2.2. At that point, half of the plants from each genotype were allowed to dry down for 7 d until soil + water weight declined 25%. On the morning of day 8, before gas exchange measurements, the soil moisture in the pots with drought stress was adjusted to that same -25% by adding water. Then net photosynthesis (Pn), stomatal conductance (Gs), and transpiration rate (E) were measured on the youngest fully expanded leaves (one per pot) with a portable photosynthesis system LI-COR 6400 (LI-COR Inc. Lincoln, NE, USA). These measurements were taken between 10:30 a.m. and noon. An Arabidopsis leaf chamber (LI-COR) was used for gas exchange measurements. It provided an irradiance of 400 µmol PAR/m^2/s photosynthetically active radiation (PAR), a temperature of 25 to 28^{0}C, a CO_2 concentration of 400 µl/l, an air flow rate of 400 µmol/s, and a relative humidity of 55 to 60%. After the gas exchange measurements, soil moisture content was measured with a Delta-T theta soil moisture probe (ML2X, Dynamax, Houston, TX). (At that time, the average soil moisture content was 0.43 cm^3/cm^3 in the well-watered controls and 0.14 cm^3/cm^3 in the drought-stress treatments.) Instantaneous water use efficiency (WUEi) was calculated using the formula: WUEi = (Pn/E) (Martin and Thorstensen, 1988). Plant water status was monitored by determination of relative water content (RWC%) according to Smart and Bingham (1974). Briefly, the same leaf used for photosynthesis measurement was excised, and an approximately 6-cm section had its fresh weight (FW) determined immediately. The leaf sections were then floated in deionized water at room temperature for 6 h, and their rehydrated weight (RW) was determined. Finally, they were dried in an oven at 70^{0}C overnight and weighed to obtain the dry weight (DW). The RWC% was calculated as: RWC% = (FW - DW)/(RW - DW) x 100.

The experiment was a 2 x 4 factorial with factors being watering regime (drought stressed or well-watered) and genotype (three HYR types and WT) arranged as a completely randomized

design (CRD) with four replications. Two-way analysis of variance (ANOVA) to test for the effects of drought, genotype, and their interactions was conducted using the general linear model (Proc GLM) of Statistical Analysis System (SAS). Differences between means were tested by the Least Significance Difference with a 0.05 threshold ($LSD_{0.05}$).

4.2.4. Experiment to Examine Soluble Carbohydrates

The same three transformants (HYR-2, HYR-4, and HYR-16) plus the WT were used to examine common photosynthetic products. The plants were grown as in 4.2.2. but in an environment-controlled growth chamber with the following environmental settings: 14-h day length, temperature of 28/25^{o}C day/night, 60% relative humidity, and an irradiance at canopy height of 350 µmol PAR/$m^{2/}$s PAR. Plants were grown under these conditions for eight weeks. At that point, half of the plants in each genotype were drought stressed by withholding water for 3 d. A day before sampling for sugars, pot weights (and presumably soil moisture contents) for all drought-stressed plants were adjusted to an equal weight by adding water as necessary to the lighter pots. Soil moisture content (SMC) was determined on the sampling date with a Delta-T theta moisture probe. For sampling of sugars, plants were harvested (all above-ground biomass) and dried at 40^{0}C for 72 h. Glucose, fructose, and sucrose were extracted and analyzed according to the procedures of Hendrix (1993) with modifications (Zhang et al., 2006). Sugars were extracted from 20-mg ground samples in 2 ml of 80% ethanol in an 80^{0}C water bath for 15 min. The crude extract was cooled to room temperature and then centrifuged at 3000 *g* for 10 min. To 1.5 ml of the supernatant, 20 mg charcoal were added. The extract was centrifuged at 2200 *g* for 15 min and 20 µl were transferred to a microtiter plate and dried at 50^{o}C for 1.5 h. A series of standard solutions of glucose, fructose, and sucrose was co-analyzed with the extracts. After drying, 20 µl deionized-distilled water were added to each well, and the plate was covered for 1

h. 100 µl of glucose reagent (Sigma, St. Louis, MO) were added to each well, and the plate was kept at room temperature for 30 min. Glucose was measured on a microplate reader (SpectroMax plus 386, Molecular Devices Corp. Sunnyvale, CA) at 340 nm. 10 µl of 0.25 enzyme unit (EU) phosphoglucose isomerase were added to each well and incubated at room temperature for 30 min, and fructose was measured at 340 nm. 10 µl of 83 EU invertase solution were added and incubated for 30 min before measuring at 340 nm for sucrose.

The experiment was a 2 x 4 factorial, with factors being watering regime and genotype arranged in a completely randomized design (CRD) with four replications. Two-way analysis of variance (ANOVA) to test the effects of drought, genotype, and their interaction was performed using the general linear model (Proc GLM) of Statistical Analysis System (SAS). Differences between means were tested by the Least Significance Difference with a 0.05 threshold ($LSD_{0.05}$).

4.2.5. Experiment to Determine Yield Under Well-Watered Conditions

The same three transgenic lines and WT plants were grown in the greenhouse in 500-ml pots (one plant per pot) filled with a 1:1 mixture of topsoil and compost (Scott-Sierra Horticultural Product Co, Marysville, OH). Pots were placed in a tray filled with water to simulate flooded/paddy conditions. Plants were supplied with a general-purpose (20-20-20) fertilizer (Scott-Sierra Horticultural Product Co, Marysville, OH) dissolved in water to provide 50 kg N, P_2O_5, and K_2O ha^{-1}. Fertilizer was applied once a week throughout the growing period. This experiment was carried out between April and September (approximately 14-h photoperiod), and the greenhouse temperatures were moderated near $28/22^0$C day/night.

Plants were grown to maturity (stage R9, with all filled grains having brown hulls) (Counce et al., 2000). The panicle on the main culm was harvested, and spikelets with grains and unfilled spikelets were counted. The grains (caryopses) with hulls [palea and lemma] attached)

were threshed by hand and dried at 37^0C for 7 d and weighed. The main culm was also harvested and dried at 70^0C for 72 h and weighed. The yield components assessed were number of spikelets (SP), spikelet fertility (SF) (number of spikelets with filled grains divided by the total number of spikelets), grain yield (GY) (weight of grain), and average single-grain weight (GY divided by grain number). The harvest index (HI) was calculated as the ratio of GY to total above ground dry weight.

The experiment was a completely randomized design (CRD) with four replicates. Data were subjected to analysis of variance (ANOVA) using the general linear model (Proc GLM) of Statistical Analysis System (SAS). Differences between means were tested by the Least Significance Difference with a 0.05 threshold ($LSD_{0.05}$)

4.3. Results

4.3.1. Selection of Rice Transformants with the HYR Transcription Factor

The rice drought stress transcriptome studies (Chapter 2 of this dissertation) revealed the gene Os03g02650 (HYR), annotated as an AP2/ERF transcription factor, is up-regulated (Log_2 ratio = 0.7779) in reproductive tissue. An overexpression construct of the gene, under control of the CaMV 35S promoter was transformed into rice cultivar Nipponbare. Seeds (T1 progeny) from the putative transformants were tested for germination in hygromycin, and five hygromycin-resistant lines (HYR-2, HYR-4, HYR-12, HYR-16, and HYR-45) were identified (Figure 4.1.a to d). The segregation pattern of the hygromycin-resistance gene is shown in Table 3.1. The transgenic locus is hemizygous; and, as the transgene provided a gain-of-function phenotype (hygromycin resistance), a 3:1 Mendelian segregation ratio for the transgenic to wild type was expected in the T1 progeny. While some genotypes were below or above this ratio, the average inheritance of the hygromycin resistance gene was 3:1, which is the expected inheritance pattern of the transgene. Seedlings from the hygromycin-resistant seed lots were PCR genotyped for the hygromycin phosphotransferase (*hpt*) gene (Figure 4.1.e). All the plants used for the experiments described in sections 4.3.2 to 4.3.5 tested positive for the *hpt* gene. They will be further tested for the occurrence of Os03g02650 (HYR) prior to publication of this study.

4.3.2. Gravimetric Water Use and Water Use Efficiency Measurements

One-week-old *hpt*-positive seedlings, progeny of five transformants (HYR-2, HYR-4, HYR-12, HYR-16, and HYR-45) expressing the HYR gene and WT seedlings were transplanted into pots and grown under well-watered conditions till 28 DAP, as described in section 4.2.2. Half the plants were then exposed to a chronic drought stress (soils at a defined -130-g/pot water deficit) for 14 d). Plants from each watering regime were harvested at 31 or 45 DAP for biomass.

Their cumulative water use (WUc) and gravimetric water use efficiency (WUEg) for the 14-d interval were calculated as described. Analysis of variance (ANOVA) showed no interaction between drought stress and genotype for shoot biomass, cumulative water use (WUc), or gravimetric water use efficiency (WUEg) – the latter two parameters determined specifically for the period from 31 to 45 DAP. There were genotypic differences for shoot biomass and WUEg, but not for WUc. Drought stress reduced biomass and WUc and increased WUEg. The HYR rice lines developed higher shoot biomass than WT under both well-watered and drought-stressed conditions (Figure 4.2.a). In the longer-running, continuously watered experiment from section 4.2.5., the higher shoot biomass was evident as noticeably larger plants at different growth stages, which in older plants also led to more tillering (Figure 4.3). However, only two lines (HYR-2 and HYR-4) had higher WUEg under both well-watered and drought conditions, while HYR-16 had increased WUEg only under drought stress.

4.3.3. Gas Exchange Measurements

Three transgenic lines (HYR-2, HYR-4, and HYR-16) with higher biomass and WUE based on the gravimetric analyses (4.3.2.) were selected for further physiological studies. In one study, gas exchange measurements were made with a LI-COR 6400 portable photosynthesis system on eight-week-old plants exposed or not to 7 d of withholding irrigation and soils adjusted to 25% below the mass associated with field capacity. At the time of measurement, the average soil moisture content was 0.43 cm^3/cm^3 in the well-watered controls and 0.14 cm^3/cm^3 in the drought-stress treatments.

Using the gas exchange measurements (Pn and E), WUEi was calculated. The ANOVA revealed no interactions between drought and genotype for Pn, Gs (stomatal conductance), E, or WUEi (data not shown). Drought stress reduced Pn, Gs, and E and increased WUEi. There were

genotypic differences for Pn and WUEi, while there were no genotypic differences for Gs and E. The HYR lines had higher Pn and WUEi under both watering regimes (Figure 4.4), while Gs and E were not affected by soil moisture content (at the two levels tested). This suggests that WUEi is increased by an increase in Pn. The drought-stressed HYR plants had higher RWC% relative to the WT, whereas the soil moisture content was not significantly different. At equal soil moisture content, the transgenic plants maintained higher Pn, WUEi, and RWC% compared to WT.

4.3.4. Soluble Carbohydrates

To study more closely the effects of a higher photosynthetic rate on metabolism in two of the HYR lines, an analysis of their soluble carbohydrates was carried out. Plants grown in a controlled environment under well-watered or drought-stressed conditions were harvested, and their dry matter was analyzed for glucose, fructose, and sucrose as described in section 4.2.4. In the well-watered controls, the average SMC was 0.41 cm^3/cm^3, while in the drought stressed plants it was 0.13 cm^3/cm^3. Drought stress increased glucose, fructose, and sucrose; and there were genotypic differences for these sugars (Figure 4.5). However, there were no interactions between genotype and watering regime for any of the sugars (ANOVA data not shown). The two HYR lines produced more free glucose, fructose, sucrose, and total sugars than the WT under well-watered and drought-stressed conditions.

4.3.5. Grain Yield (Main-Culm Panicle) of Well-Watered Plants

The results to this point showed that HYR lines developed higher biomass, had higher rates of photosynthesis, and contained more soluble sugars. Selected lines (HYR-2, HYR-4, HYR-45) were further analyzed for grain yield of the main/primary culm and yield components when grown under continuously well-watered conditions. The HYR lines had increased main

culm biomass, more yield, more grains, and larger single-grain weights relative to the WT (Table 4.2). Only HYR-45 had significantly more total spikelets (SP) than the WT. However, spikelet fertility and harvest index were not significantly different in the HYR lines. This means that grain yield increased due to larger grains and increased SP. In summary, the results indicate that the HYR lines produced larger panicles with more and larger grains as well as more total biomass under well-watered conditions.

4.4. Discussion

4.4.1. The AP2/ERF Transcription Factor Is a Drought-Induced Gene in Rice Reproductive Tissues

The rice genome is predicted to contain 139 AP2/ERF domain containing transcription factor genes (Nakano et al., 2006). To identify stress-inducible *AP2* genes, a rice drought microarray experiment was conducted (Chapter 2 of this dissertation), which identified HYR as one of the novel (unpublished) transcription factors (TF) induced by drought in the reproductive tissue. This work provides a functional characterization of the gene for its effects on plant performance under well-watered and water-limited conditions.

An important part of stress responses is the differential regulation of the plant transcriptome by TF, which regulate the temporal and spatial expression patterns of specific genes. Previous experiments have shown that genes with putative functions in drought include NAM, HLH, G-box, Zinc finger, and AP2 TF (Zhou et al., 2007). Most of the AP2/ERF TF whose transcription properties have been studied are activators of transcription, although some are repressors (Fujimoto et al., 2000). The AP2/ERF family proteins have a DRE cis-element binding motif, believed to be involved in the expression of dehydration-responsive genes. Homologs of the protein under study have been found to respond to dehydration or drought stress (Fujimoto et al., 2000; Haake et al., 2002) or function in drought responses, such as DREB2A (Sakuma et al., 2006). Therefore it is not surprising that the TF identified responded to drought stress.

4.4.2. HYR Transgenic Plants Have Increased Shoot Biomass and Grain Yield

All HYR transgenic lines tested in this study accumulated more shoot biomass (Figure 4.2), exhibited larger phenotypes (Figure 4.3), and had higher grain yields (Table 4.1). To examine possible reasons for the increase in biomass and yield, the HYR lines were further characterized for gas exchange and water-use parameters. The results showed that the transgenic plants had higher net photosynthesis (Pn) compared to the WT. The HYR lines also showed higher water use efficiency (WUEg and WUEi), measured by independent methods (gravimetric and gas exchange). The higher Pn of HYR lines suggests an explanation for the higher biomass produced. Various studies have shown increased biomass production coinciding with improved Pn in Arabidopsis (Kebeish et al., 2007), mulberry (*Morus alba*) (Chaitanya et al., 2002), and rice (Karaba et al., 2007). In rice, biomass accumulation before heading affects final yield performance (Chen et al., 2008). The higher grain yields measured in the HYR lines studied under well-watered conditions here were also associated with an increased number of grains and single-grain weights. Taken together, these data suggest a higher Pn during the vegetative stage produced larger HYR plants, which produced more and then larger grains.

The primary stable product of photosynthesis and the phloem-mobile form of sugar is sucrose. High rates of photosynthesis and/or reduced sink sizes can lead to sucrose accumulation and a feedback inhibition of photosynthesis (Vassey and Sharkey, 1989). Sucrose and its immediate metabolic products (glucose and fructose) were therefore examined in selected HYR lines and WT. The analysis shows that the higher-Pn HYR lines accumulated higher levels of sucrose, glucose, fructose, and total soluble sugars than WT. We cannot determine from the data at hand whether the putative feedback loop (whereby "excess" sucrose inhibits Pn) is less "sensitive" in HYR or if sucrose levels at critical sites (presumably the chloroplasts) are less

because of stronger sink activity in the larger, faster growing plants. In either case, it can be reasoned that the higher sugar levels in these plants are probably a direct result of the higher photosynthesis. Higher photosynthesis presumably leads to increased sucrose synthesis and greater export to various sinks. In the soluble sugar study, the plants were in a vegetative stage, when roots are the primary sinks. In a reproductive stage, the larger, more numerous grains would become the primary sinks. In either case, increased sink size might prevent carbohydrate accumulation in the leaves, which could down-regulate photosynthesis (Murchie et al., 1999). Again, though, we cannot rule out the possibility that HYR mutants have a reduced sensitivity to sucrose's inhibition of photosynthesis.

4.4.3. HYR Rice Plants Exhibit Drought Resistance

The HYR transgenic lines were phenotyped for drought resistance parameters. Compared to the WT, HYR lines had higher photosynthesis (Pn), WUEg, and WUEi under drought stress, with no significant changes in stomatal conductance (Gs) or transpiration rate (E). Stomatal closure typically leads to decreases in photosynthetic CO_2 assimilation due to restricted diffusion of CO_2 into the leaf and altered CO_2 metabolism. Pelleschi et al. (1997) found that reduced CO_2 diffusion during stomatal closure is mainly responsible for the decline in photosynthesis in C_3 plants subjected to dehydration. In C_3 plants, WUE is determined by, among other factors, stomatal control of the ratio of the instantaneous rates of photosynthesis and transpiration (Farquhar and Sharkey 1982). The results for the HYR plants imply that WUE is not determined by stomatal control of photosynthesis. However, Tezara et al. (1999) reported that, in sunflower (*Helianthus annuus*) (a C_3 plant) under water stress, the photosynthetic rate is limited more by altered CO_2 metabolism than by reduced diffusion. The results in this study (Figure 4.4) suggest that inhibition of photosynthesis under drought stress was not limited by stomatal control but by

CO_2 fixation as reported by Tezara et al. (1999). Therefore, CO_2 metabolism in the HYR lines is probably more resistant to dehydration than the WT.

The HYR lines had significantly higher RWC% than the WT under drought stress conditions. Maintenance of plant water status, as expressed by RWC% is an indication of drought resistance (Babu et al., 2003). One of the factors that contributed to high RWC% in the HYR lines could be the accumulation of sugars, especially sucrose, leading to osmotic adjustment. In osmotic adjustment, leaves develop a more negative osmotic potential by accumulating solutes. They can then maintain a higher RWC% during a period of leaf water potential reduction. Solute accumulation and osmotic adjustment have been associated with drought tolerance in ber (*Ziziphus mauritiana*) (Clifford et al., 1998), pea (*Pisum sativum*) (Rodriguez-Maribona et al., 1992), tall fescue (*Festuca arundinacea*) (Elmi and West, 1995), wheat (Morgan, 1995), and sorghum (*Sorghum bicolor*) (Ludlow et al., 1990).

Soluble carbohydrates (glucose, sucrose, fructose, sorbitol, and mannitol) have been reported to accumulate in plants under drought stress (Abebe et al., 2003; Dancer et al., 1990; Gebre et al., 1998). This is due to a shift in C-partitioning from non-soluble carbohydrates (starch) to soluble carbohydrates, which can help maintain turgor for a longer period during drought (Wang et al., 1995) and also participate in stress-protective functions (Abebe et al., 2003).

In conclusion, overexpression of the HYR transcription factor TF in rice has enabled the plants to be more productive due to efficient mechanisms that enabled the plant to carry out higher levels of Pn and use water more efficiently. The plants are also drought resistant, due to adaptation that enable the plant to continue functioning in the presence of soil water deficits.

4.5. References

Abebe T, Arron AC, Martin B, Cushman JC (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol **131**: 1748-1755.

Babu RC, Zhang J, Blum A, Ho T-H, R WU, Nguyen HT (2004) HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa*) via cell membrane protection. Plant Sci **166**: 855-862.

Century K, Reuber TL, Ratcliffe OJ (2008) Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products. Plant Physiol **147**: 20-29.

Chaitanya KV, Masimanani S, Jutur PP, Reddy AR (2002) Variation in photosynthetic rates and biomass productivity among four mulberry cultivars. Photosynthetica **40**: 305-308.

Chen S, Zeng F, Pao Z, Zhang Z, Guo Z (2008**)** Characterizatiion of high-yield performance as affected by genotype and environment in rice. J. Zhejiang Univ Sci B **9**: 363-370.

Chuck G, Meely RB, Hake S (1998) The control of maize spikelet meristem fate by APETALA2-like gene indeterminate spikelet1. Genes and Dev **12**: 1145-1154.

Clifford SC, Arndt SK, Corlet JE, Joshi S, Sankhla N, Popp M, Jones HG (1998) The role of solute accumulation, osmotic adjustment in drought tolerance in *Ziziphus mauritiana* (Lamk.). J Exp Bot **49**: 967-977.

Counce PA, Keisling CT, Mitchell (2000) A uniform, objective and adaptive system for expressing rice development. Crop Sci **40**: 437-443.

Dancer J, David M, Stitt (1990) Water stress leads to a change in partitioning in favor of sucrose in heterotrophic cell suspension culture of *Chenopodium rubrum*. Plant Cell Environ **13**: 957-963.

Elmi AA, West CP (1995) Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of tall fescue. New Phytol **131**: 61-67.

Farquhar GD, Sharkey TD (1982) Stomatal conductance and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **33**: 3117-345.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression. Plant Cell **12**: 393-404.

Gebre GM, Tschaplisnski TJ, Shirshac TL (1998) Water relations of several hadwood species in response to through fall manipulation in upland oak forest during a wet year. Tree Physiol **18**: 299-305.

Haake V, Cook D, Riechmann, Pineda O, Thomashaw MF, Zhang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiol **130**: 639-648.

Hendrix DL (1993) Rapid extraction and analysis of nonstructural carbohydrates in plant tissues. Crop Sci **33**: 1306-1311.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J **6**: 271-282.

Horton P (2000) Prospects for crop improvement through genetic manipulation of photosynthesis: morphological and biochemical aspects of light capture. J Exp Bot **51**: 475-485.

Karaba A, Dixit S, Greco R, Aharoni A, Trijatmiko KR, Marsch- Martinez N, Krishnan A, Nataraja KN, Udayakumar M, Pereira A (2007) Improvement of water use efficiency

in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene. Proc Natl Acad Sci USA **104**: 15270-15275.

Kebeish R, Niessen M, Thiruveedhi K, Bari R, Hirsh J-H, Rosenkranz R, Stabler N, Schonfeld B, Kreuzaler F, Peterhansel C (2007) Chloroplastic photoresperatory bypass incrases photosynthesis and biomass production in *Arabidopsis thaliana*. Nat Biotechnol **25**: 593-599.

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, et al. (2003) Collection, mapping, and annotation of over 28000 cDNA clones from japonica rice. Science **301**: 376-379.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two separate signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10**: 1391-1406.

Long SP, Zhu XG, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yields? Plant Cell Environ **29**: 315-330.

Ludlow MM, Santamaria JM, Fukai S (1990) Contribution of osmotic adjustment to grsin yield in *Sorghum bicolor* (L.) Moench under water-limited conditions. II. Water stress after anthesis. Aust J Agr Res **42**: 67-78.

Martin B, Thorstensen YR (1989) Stable carbon isotope composition, water use efficiency, and biomass productivity of *Lycopersicon esculentum*, *Lycopersicon pennellii*, and their F1 hybrid. Plant Physiol **88**: 213-217.

Morgan JM (1995) Growth and yield of wheat lines with differing osmoregulative capacity at high soil water deficit in seasons of varying evaporative demand. Field Crop Res **40**: 143-152.

Murchie EH, Sarrobert C, Contard P, Betsche T, Foyer CH, Galtier N (1999) Overexpression of sucrose-phosphate synthase in tomato plants grown with CO_2 enrichment leads to foliar carbohydrate accumulation relative to untransformed controls. Plant Physiol Bioch **37**: 251-260.

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol **140**: 411-432.

Nishimura A, Aichi A, Matsuoka M (2007) A protocol for *Agrobacterium*-mediated transformation in rice. Nature Protocols **1**: 2796-2802.

Oh S-J, Kim YS, Kwon C-W, Park HK, Jeong JS, Kim J-K (2009) Overexpression of the transcription factor AP37 in rice improves grain yield under drought conditions. Plant Physiol **150**: 1368-1379.

Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K, Hoisington D (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. Genome **47**: 493-500.

Pelleschi S, Rocher JP, Prioul JL (1997) Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. Plant Cell Environ **20**: 493-503.

Rodriguez-Maribona B, Tenorio JL, Conde JR, Ayerbe L (1992). Correlation between yield and osmotic adjustment of peas (*Pisum sativum*) under drought stress. Field Crop Res **29**: 15-22.

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozakia K (2006) Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell **18**: 1292-1309.

Selote DS, Khanna-Chopra R (2004) Drought induced spikelet sterility is associated with an inefficient antioxidant defense in rice panicles. Physiol Plant **121**: 462-471.

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol **6**: 410-417.

Smart RE, Bingham GE (1974) Rapid estimation of relative water content. Plant Physiol **53**: 258-260.

Tezara W, Mitchel VJ, Driscoli SD, Lawlor DW (1999) Water deficit inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature **401**: 914-917.

Vassey TL, Sharky (1989) Mild water stress of *Phaseolus vulgaris* plants leads to reduced phosphate synthase activity. Plant Physiol **89**: 1066-1070.

Wang Z, Quebedeaux B, Stutte GW (1995) Osmotic adjustment: effect of water stress on carbohydrates in leaves, stems and roots of apple. Australian J Plant Physiol **22**: 747-754.

Weigel D (1995) The APETALA2 domain is related to a novel type of DNA binding domain. Plant Cell **7**: 388-389.

Zhang X, Ervin EH, LaBranche AJ (2006) Metabolic defense responses of seeded bermudagrass during acclimation to freezing stress. Crop Sci **46**: 2598-2605.

Zhou J, Xiangfeng W, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong L, Zhang Q, Fan L, Deng XW (2007) Global genome expression analysis of rice in response to drought and high salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol **63**: 591-608.

Zhu XG, de Sturler E, Long SP (2007) Optimizing the distribution of resources between enzymes of carbon metabolism can drastically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. Plant Physiol. **145**: 513-526.

Zinselmeier C, Westgate ME, Schussler Jr, R (1995) Low water potential disrupts carbohydrate metabolism in maize *(Zea mays* L.) ovaries. Plant Physiol **107**: 385-391.

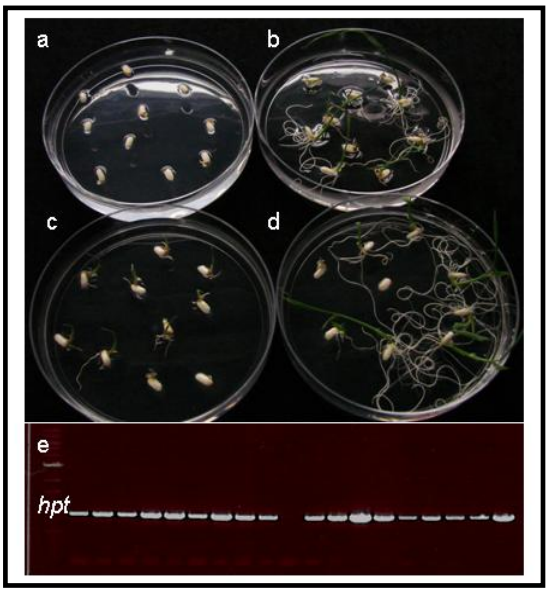

Figure 4.1. Selection of putative transgenic plants for hygromycin resistance. a. wild type; b to d. putative HYR-transgenic types; and e. PCR-genotyping for the hygromycin resistance *(hpt*) gene in the transgenic plants.

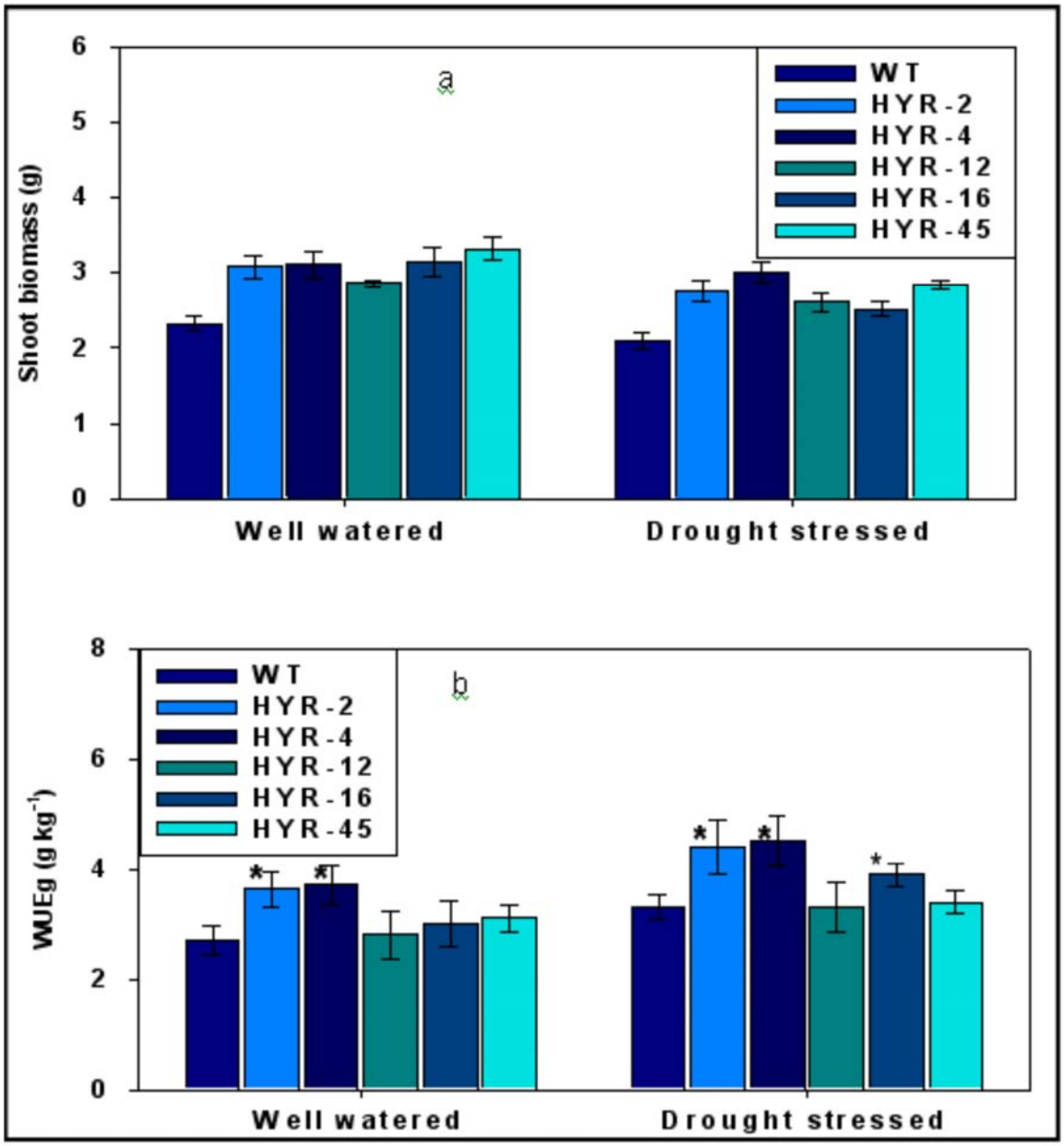

Figure 4.2. Analysis of shoot biomass (a) and gravimetric water use efficiency (WUEg) (b) in wild type (WT) and five HYR-transformed rice lines under well-watered and drought-stressed conditions. WUEg was calculated using the formula in 4.2.2. In (b), *indicates significant difference between HYR and WT within the watering treatments. In (a), each HYR line is different than WT for both well-watered and drought-stressed plants. Drought stress = 14 d with soil + water mass at 30% below mass associated with field capacity. Error bars are standard error of the means, n = 4.

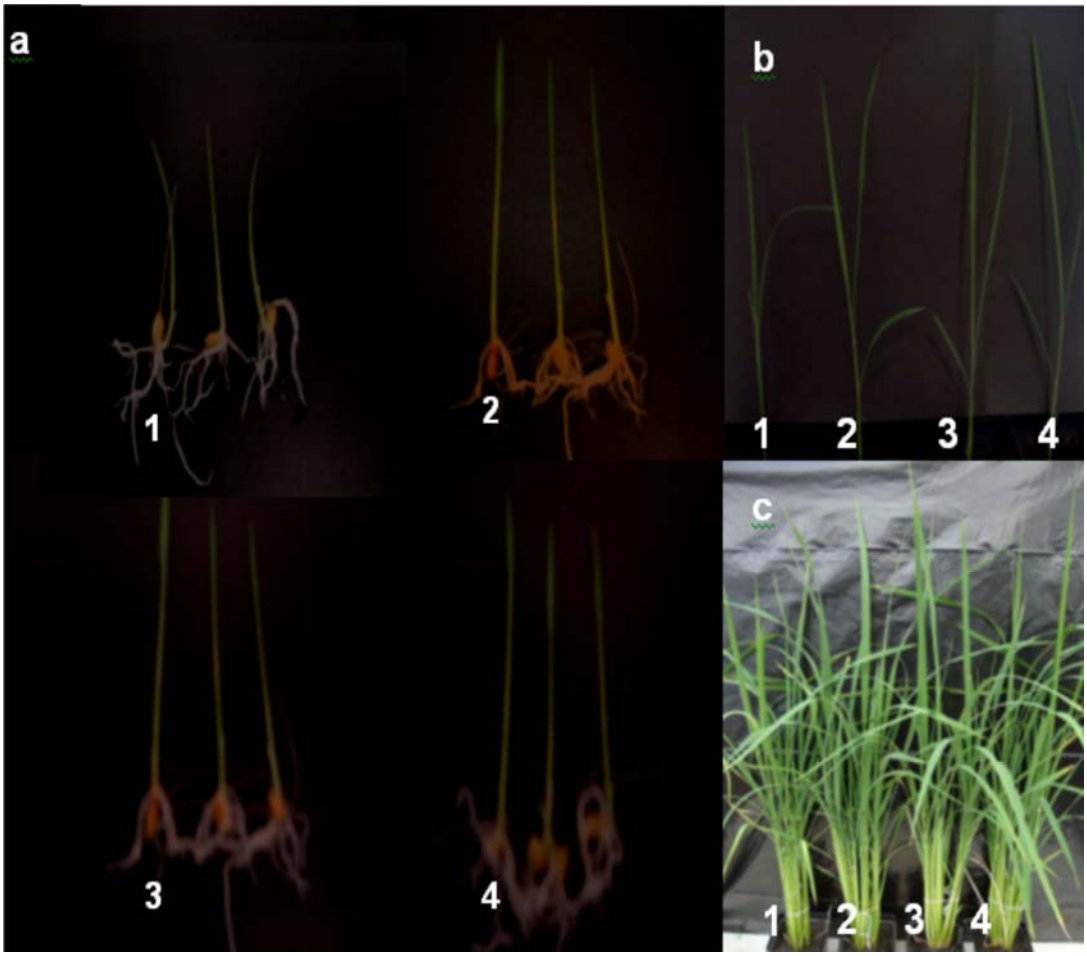

Figure 4.3. The morphology of well-watered HYR-transgenic plants compared to the wild type (WT): a. one week after planting; b. two weeks after planting; c. ten weeks after planting. (1 = WT, 2 = HYR-2, 3 = HYR-4, 4 = HYR-16).

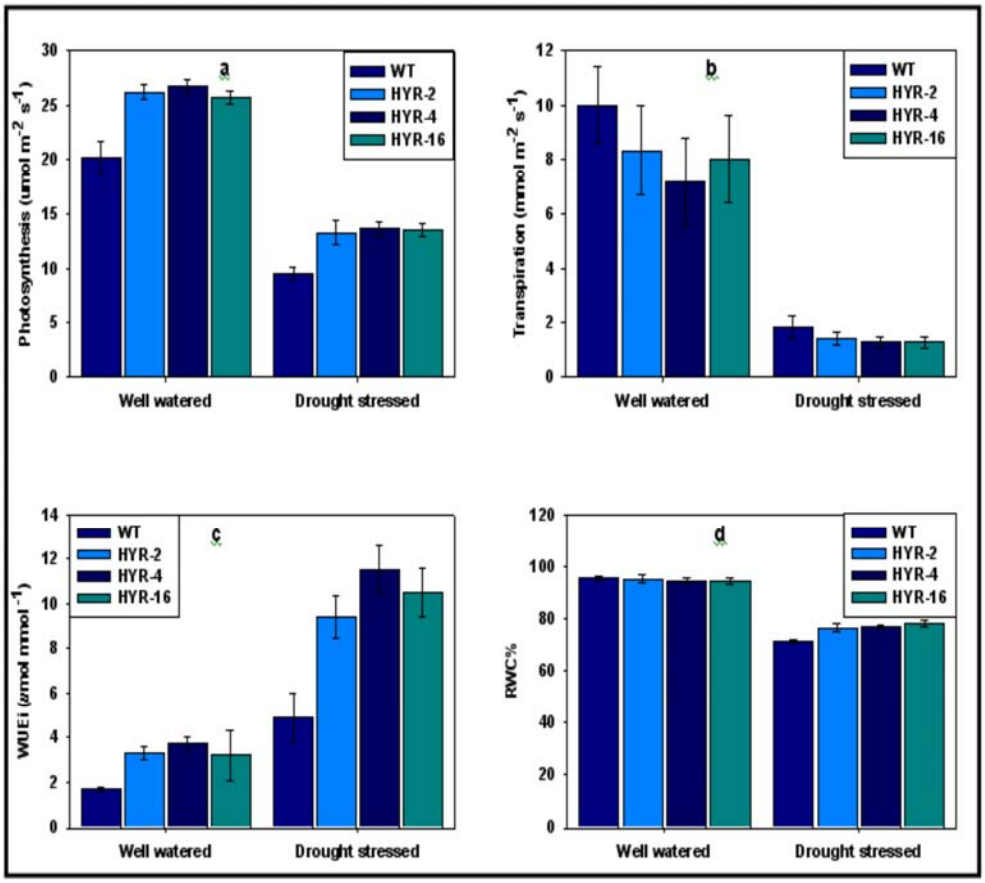

Figure 4.4. Gas exchange analysis and leaf water status of wild type (WT) and transgenic HYR lines under well-watered and drought-stressed conditions: a, Photosynthesis (Pn); b, transpiration (E); c, instantaneous water use efficiency (WUEi); and d, relative water content (RWC%). Error bars are standard error of the means, n = 4.

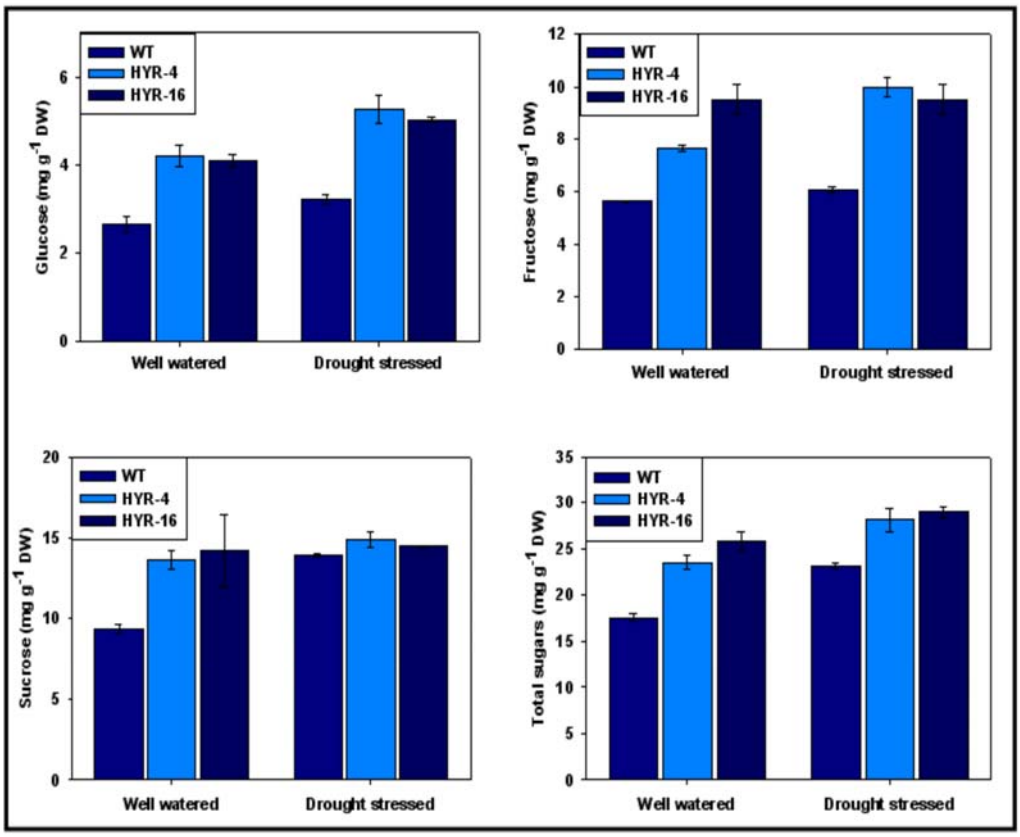

Figure 4.5. Analysis of soluble sugars: glucose, fructose, sucrose, and total sugars in WT and HYR lines under well-watered and drought-stressed conditions. Errors bars are standard error of the means. For each data point, n = 4.

Table 4.1. Segregation of hygromycin resistance gene in self progeny of HYR-transgenic plants

Genotype	Seed Number Tested	Number Hygromycin Resistant	Number Hygromycin Sensitive	hpt Segregation Ratio
WT	20	0	0	0
HYR-2	20	15	5	3:1
HYR-4	20	15	5	3:1
HYR-12	20	14	6	2.3:1
HYR-16	20	16	4	3.5:1
HYR-45	20	15	5	3:1

Seeds from putative transformants were dehusked and germinated in Murashige and Skog medium supplemented with 50 mg/L hygromycin. Ten seeds were used for an experiment and this was repeated. Seeds that grew on a hygromycin-supplemented medium were scored as resistant (Figure 4.1).

Table 4.2. Analysis of shoot biomass and yield components in wild type (WT) and three HYR-transgenic plants.

Genotype	Spikelet Number (SP)	Plant Biomass (g/plant)	Grain Number (grain/plant)	Grain Yield (g/plant)	Single-Grain Weight (g)	Harvest Index (HI)	Spikelet Fertility (SF)
WT	381^{b}	7.32^{c}	351^{b}	7.34^{b}	0.019^{c}	0.50^{a}	0.92^{a}
HYR-2	424^{ab}	9.33^{ab}	395^{a}	8.74^{a}	0.023^{b}	0.48^{a}	0.93^{a}
HYR-4	430^{ab}	9.54^{a}	396^{a}	8.81^{a}	0.025^{ab}	0.48^{a}	0.91^{a}
HYR-45	448^{a}	8.57^{b}	420^{a}	9.80^{a}	0.024^{a}	0.51^{a}	0.92^{a}
LSD(0.05)	45.75	0.91	30.4	0.84	0.003	0.04	0.05
P Value	0.04	0.004	0.001	0.004	0.012	0.60	0.48

Plants were grown under well-watered conditions through the R9 stage (grain maturity).Means within columns followed by the same letter are not significantly different at P=0.05. For each data point, n = 4.

Chapter 5. General Discussion and Conclusions

Drought stress often limits productivity of economically important plants. The impacts of drought have been felt in essentially all parts of the world, including the most productive areas for many of the most important crops. This suggests that drought stress should be addressed by all means possible. Classical plant breeding has addressed drought through selecting for resistant genotypes, and this has led to improvement in some crops. However, this has not been backed up by research on the molecular basis underlying resistance or susceptibility to drought. Availability of sequenced genomes for *Arabidopsis thaliana*, rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and maize (*Zea mays*) and their expressed sequence tag (EST) resources can be used to identify genes and processes, and this approach can shed light on a molecular basis of drought resistance in these plants and related species.

Research in this dissertation has confirmed for the cultivar of particular interest ('Nipponbare') the often-observed sensitivity of rice to drought stress during reproductive phases. Brief periods of drought stress just before anthesis inhibited photosynthesis as determined by chlorophyll fluorescence and ultimately reduced grain yield. Analysis of yield components revealed reductions in spikelet number (SP) and spikelet fertility (FS). Drought stress reduced SP due to abscission of already-formed spikelets (depending on the timing of drought), possibly as a result of higher abscisic acid levels (ABA) or perhaps as a result of spikelet tissue death caused by reactive oxygen species (ROS). Reduction in FS indicates that the processes of pollination and fertilization and/or fruit set were inhibited. In rice, drought stress can inhibit development of both the male and female gametophytes; or, in cases where fertilization has occurred, abortion of fertilized ovules can still take place.

A drought-induced reduction in photosynthesis at a critical stage may also indirectly affect reproductive development, in that sucrose export from the leaves and utilization in the florets might be affected. The carbon assimilation capacity of the plant can also be affected by drought, as parts of or whole leaves senesce (become fired). Leaf firing, can arise from drought-induced accumulation of ABA, which can activate production of ROS. When antioxidant systems of plant cells are overwhelmed by these species, there can be programmed cell death in the affected tissues, which will be indicated by significant tissue death. The affected tissue can be leaves, whose photosynthetic ability will be compromised; and ultimately post-drought-stress development of the plant affects its ability to support the reproductive parts. This perhaps explains the reduction in the harvest index (HI) after drought. Rice reproductive development has been known to be drought sensitive.

The results in the physiological studies done in this work reaffirm past findings about the responses of rice and maize to drought and confirm that the treatments imposed were indeed causing the plant to demonstrate symptoms of drought stress. Those responses could then be correlated with transcriptomic data.

In order to examine the genomic response of rice tissues to drought, panicle (reproductive) and leaf (vegetative) tissues were compared by microarray analyses. Induced and repressed genes were identified in each type of tissue. Some of these up- and down-regulated genes are associated with key metabolic processes, transport, signal transduction, and protein biosynthesis. The diversity of biological processes identified in this study suggests a high level of regulation of cell processes in mitigating the effects of drought. The biological processes are diverse in the sense that cellular metabolic pathways, communication between cells, and processes involved in stress protective roles were affected by drought stress.

Analyses of the rice transcriptome revealed that vegetative and reproductive organs responded differentially to drought stress. Those differences in responses to drought could hypothetically stem from two causes, either or both of which may be inherent in plants: 1) organ-specific regulation in response to drought, mediated by organ-specific transcription responses; 2) unequal sensing of the stress between the two organs. The two hypotheses need further investigation at both physiological and molecular levels. While the results shed some light on drought stress in rice, many of the genes observed to be up- or down-regulated in response to drought code for proteins of unknown function. These genes need to be studied further in order to establish their role in drought responses and their possible participation in drought resistance. Drought-responsive genes, especially those coding for transcription factors, could be primes candidates for improving crops' drought resistance through transgenic approaches.

Comparative studies between related species have established some shared molecular responses between those genomes. In this dissertation, rice as a model cereal was used in a comparative genomic study with maize to identify possible conserved genes and biological processes that may be important for drought resistance. Exposure of maize to acute drought stress at silking/anthesis caused reductions in photosynthesis (chlorophyll fluorescence) and the number of seed produced. The seed-reduction response could be for similar reasons that drought affected yield performance in rice, i.e., reduced numbers of ovules (or spikelets in the case of rice), poor fertilization or abortion of fertilized ovules, and/or indirect effects of altered photosynthesis on reproduction. Previous studies with maize have shown that the crop's reproductive development is sensitive to drought. The results from this work confirm this, and in addition they can be used to correlate the extent of drought and genomic responses.

Although the soil moisture potential imposed in maize studies was lower than in rice, maize maintained a higher water status as measured by relative water content. Therefore, at a cellular level, the two plants may have been experiencing different levels of drought stress. Despite this, comparative genome analysis between rice and maize established that there are indeed drought-responsive genes and processes shared between the two – putatively conserved from some common ancestor. This was perhaps not particularly surprising for maize and rice, since the two species belong to the same family. However, identification of orthologous genes between related species can unravel the level of gene conservation between species, which is an important criterion in determining the extent to which comparative knowledge can be applied across species.

The conserved drought-responsive genes included some believed to be directly involved in stress protection and some involved in broader regulation of gene expression, e.g., transcription factors. Among other generalizations, the data show that, at the biological process level, both plants switched from protein synthesis to degradation in drought. This may be a consequence of some general signal for reduced growth and/or of processes resulting from tissue death.

Tissue death (seen visibly as leaf "firing") could be a result of the cells' inability to protect themselves from reactive oxygen species, of high levels of abscisic acid induced during drought, or of other causes. Reactive oxygen species are likely at least partially involved; in the vegetative tissues, genes and processes that deal with oxidative stress were up-regulated, presumably to minimize oxidative stress and damage associated with it. The senescent/necrotic tissues could also have undergone starvation, because photosynthesis, which supplies sugars necessary for metabolism, was highly down-regulated in both rice and maize. While it is well

established that drought stress represses photosynthesis, these studies show that both the light and Calvin cycle reactions are targets for stress at a molecular level. The light reactions were the most affected when looking at the number of genes affected. The down-regulation of photosynthesis was accompanied by up-regulation of some elements of carbohydrate catabolism. The plant's ability to acquire hexoses from sucrose might be compromised due to inhibition of the enzymes involved. The up-regulation of carbohydrate catabolism could be the cells' response to acquire glucose from other carbohydrates in order for primary metabolism to proceed in a drought situation. Genes identified in these analyses are likely to be valuable candidates for transformation studies

A transcription factor herein named HIGHER YIELD RICE (HYR) was found to be induced by drought stress in rice reproductive tissues. This is a transcription factor of the AP2/ERF family. Transcription factors are regulatory genes that activate cascades of genes believed to act together in enhancing tolerance toward stress. As evidenced by the many processes affected, drought stress is complex. Transferring a single gene encoding a single protein might not be expected to have much impact in maintaining myriad cellular functions and making plants more tolerant to stress. Conversely, constitutive expression, or overexpression, of transcription factors that might switch on multiple downstream drought-responsive genes can hypothetically enhance drought tolerance dramatically. Indeed, such an approach has been shown previously in Arabidopsis and rice.

In the next phase of this dissertation work, the HYR transcription factor gene was constitutively expressed in transgenic plants of the rice cultivar Nipponbare. Several HYR lines had increased shoot biomass, photosynthesis, sugar levels, and water use efficiency (WUE) under both well-watered and drought-stressed conditions. We did not test for yield under drought

stress, but well-watered HYR lines were, 19 to 32% higher yielding than the wild type (hence the HIGHER YIELD RICE name). The enhanced photosynthesis and other downstream events likely contributed to the yield increases observed in the transgenic plants. In a yield-components analysis, the HYR lines (compared to the wild type) produced more spikelets per panicle with the same level of spikelet fertility (hence, more total grains per panicle) and also heavier grains (higher single-grain weights). Collectively, the higher grain number and larger grain size constituted an increased sink capacity (or demand). Although many factors, such as nutrition and sink capacity, are important for increased grain yield, we suggest that promoting photosynthetic source activity contributed to greater grain development, i.e., higher grain yield.

We do not know the exact mode(s) of action of HYR in boosting processes as diverse as phototsynthesis, water use efficiency, and yield. Overexpression of the HYR transcription factor could have led to structural changes in the leaves, such as increased mesophyll cells in thicker leaves, and therefore increased the activity of photosynthetic machinery per unit area. Targets for the HYR transcription factor could include genes involved in assimilation of carbon, affecting the biochemistry of photosynthesis. It follows that plants with higher levels of photosynthesis might be expected to produce higher yields as shown in this dissertation. In addition, the higher sink activity of HYR plants could also be such that sucrose, which is produced in higher amounts by the transgenic plants, is more efficiently unloaded into the developing panicles and seeds. Perhaps the increased sink size minimized putative feedback inhibition of photosynthesis and partially accounted for higher rates of photosynthesis observed.

The leaves of the drought-stressed HYR-overexpressing plants also maintained higher relative water content (RWC). The maintenance of higher photosynthesis and water use efficiency and especially of higher RWC by the transgenic plants can be viewed as drought-

resistance mechanisms. Several hypotheses can be put forward to explain these phenotypes. The higher photosynthesis was perhaps due to the improved water status in the HYR-transgenic plants (as indicated by their higher RWC). A possible cause for the improved water status could be reduced stomatal apertures in the transgenic plants; but this was not the case, as stomatal conductance and transpiration were similar between the wild type and transgenic plants.

An alternative explanation for the higher RWC in HYR genotypes could be that the transgenic plants achieved greater water uptake from the soil. Adaptive physiological mechanisms for enhanced water uptake could be through structural or functional changes in the roots and/or through osmotic adjustment. No attempt was made to measure root development, but we can speculate from their larger shoot biomass that the transgenic plants had superior root biomass as well, which could allow them to take up more water. The HYR plants might also be able to take up more water at low soil water potential due to osmotic adjustment in their leaves and the roots. Osmotic adjustment could be indicated by the higher accumulation of sugars, especially sucrose, in the transgenic plants. Higher sugar accumulation was correlated with osmotic adjustment in previous studies. In some species, drought tolerant plants, with high sugar levels also have higher proline content. Perhaps the transgenic plants also accumulated other solutes such as proline, which is important for osmotic adjustment. Possible changes in the roots or the plants' ability to accumulate solutes could also be in addition to the ability of the HYR transcription factor to affect many other downstream genes that affect myriad processes.

Whatever its precise mode of action when overexpressed, the HYR transcription factor appears to impart qualities or processes to transgenic rice plants that allow them to be more productive in well-watered and especially in drought-stressed environments. The research presented in this dissertation demonstrates the application of newer technologies, such as

microarrays, to identify genes, such as transcription factors, and to demonstrate or validate their use for imparting drought resistance to a crop plant.