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Published Version Information

Citation: Reddy, S. K., Liu, S., Rudd, J. C., Xue, Q., Payton, P., Finlayson, S. A., … Lu, N. (2014). Physiology and transcriptomics of water-deficit stress responses in wheat cultivars TAM 111 and TAM 112. Journal of Plant Physiology, 171(14), 1289-1298.

Digital Object Identifier (DOI): doi:10.1016/j.jplph.2014.05.005

Publisher's Link: http://www.sciencedirect.com/science/article/pii/S0176161714001394

Physiology and transcriptomics of water-deficit stress responses in wheat cultivars TAM 111 and TAM 112

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Abstract

Hard red winter wheat crops on the U.S. Southern Great Plains often experience moderate to severe drought stress, especially during the grain filling stage, resulting in significant yield losses. Cultivars TAM 111 and TAM 112 are widely cultivated in the region, share parentage and showed superior but distinct adaption mechanisms under water-deficit (WD) conditions. Nevertheless, the physiological and molecular basis of their adaptation remains unknown. A greenhouse study was conducted to understand the differences in the physiological and transcriptomic responses of TAM 111 and TAM 112 to WD stress. Whole-plant data indicated that TAM 112 used more water, produced more biomass and grain yield under WD compared to TAM 111. Leaf-level data at the grain filling stage indicated that TAM 112 had elevated abscisic acid (ABA) content and reduced stomatal conductance and photosynthesis as compared to TAM 111. Sustained WD during the grain filling stage also resulted in greater flag leaf transcriptome changes in TAM 112 than TAM 111. Transcripts associated with photosynthesis, carbohydrate metabolism, phytohormone metabolism, and other dehydration responses were uniquely regulated between cultivars. These results suggested a differential role for ABA in regulating physiological and transcriptomic changes associated with WD stress and potential involvement in the superior adaptation and yield of TAM 112.

Keywords: Abscisic acid, TAM 111, TAM 112, transcriptomics, water-deficit stress, wheat.

Abbreviations: *A* **–** photosynthesis, ABA – abscisic acid, BR – brassinosteroid, C_i/C_a – ratio of intercellular CO_2 to ambient CO_2, CK – cytokinin, *E* – transpiration, ET – ethylene, FC – fold change, GA – gibberellin, g_s – stomatal conductance, SGP – Southern Great Plains, WD – water deficit.

Introduction

Wheat is the second most important cereal crop in the United States and winter wheat comprises 70% of the cultivated wheat area (http://www.nass.usda.gov/). The Southern Great Plains (SGP) of the United States produces over half of the total US winter wheat crop but is often plagued by severe to extreme drought. Breeding for drought tolerance under such low-yielding environments with high evaporative demand is a complex process. When breeding for drought tolerance and sustained yield gains, it is necessary to understand the physiological and molecular basis of stress tolerance and employ an integrated approach (Araus et al., 2002; Reynolds et al., 2012).

Plant performance and yield responses to water-deficit (WD) stress conditions have been extensively studied (Blum, 2009; Chaves et al., 2003; Lawlor, 2013). Crops may employ a variety of strategies for successful production under water-limiting environments such as resistance, tolerance, avoidance, survival, and escape, or combinations thereof (Chaves et al., 2003; Passioura, 2007). Phenotypic adaptation to WD stress at the whole-plant level includes alterations in canopy architecture, root-to-shoot ratio, plant height, above ground biomass, tiller and spike number, grain number, and grain weight. The terminal phenotypic traits are mainly driven by photosynthesis and associated carbon allocation and partitioning, and competition between the sink tissues. WD conditions have been shown to limit photosynthetic rate and affect yield; however, the causal mechanisms are complex owing to inherent differences in photosynthesis, efficiency, and interactions with the dynamic environment (Lawlor and Tezara, 2009).

WD stress responses at the cellular level induce osmotic adjustment, reduced transpiration, varied CO_2 diffusion, and decreased photosynthesis due to stomatal closure (Lawlor and Tezara, 2009). WD induced changes at the molecular level are mediated by numerous signals. The key responses and core signaling components include photosynthetic byproducts such as sugars and reactive oxygen species (ROS), phytohormones, transcription factors, amino acids, and other metabolic signals (Gong et al., 2010; Lawlor, 2013; Pinheiro and Chaves, 2011). The phytohormone abscisic acid

(ABA) has been proposed to regulate a reduction in photosynthesis and mediate photosynthate remobilization between source and sink tissues, while other hormones also play key roles (Pinheiro and Chaves, 2011; Wilkinson et al., 2012).

Transcriptomic, proteomic, and metabolomic studies have increased the understanding of WD stress responses at the molecular level (Harb et al., 2010; Padmalatha et al., 2012; Payton et al., 2009; Seiler et al., 2011). Gene expression profiling of wheat subjected to biotic and abiotic stress has been conducted previously (Aprile et al., 2009; Ergen et al., 2009; Krugman et al., 2010; Mohammadi et al., 2007; Reddy et al., 2013b; Szucs et al., 2010; Xue et al., 2006). However, these studies were not conducted in widely planted commercial wheat cultivars with distinct adaptation mechanisms. The results of "omics" studies targeting commercial varieties may support breeding efforts to increase grain yield and sustain productivity (Langridge and Fleury, 2011; Tester and Langridge, 2010).

The hard red winter wheat cultivars TAM 111 and TAM 112 (Lazar et al., 2004; Rudd et al., 2014), are widely cultivated on the SGP and continue to gain popularity among producers. The germplasm resources of these cultivars are used by breeding programs in the US and around the world to improve drought tolerance in arid and semi-arid production regions. TAM 112 had high yield stability and performed better in most environments including low yield potential environments, while TAM 111 is superior in high input environments (Battenfield et al., 2013). Studies also suggested the cultivars show altered growth responses to variable WD conditions and potentially distinct adaptation mechanisms (Xue et al., 2013). However, the physiological and molecular basis of their drought tolerance mechanisms remains unknown. The wide adaptability, distinct drought tolerance responses, and their popularity among scientific and farming communities make TAM 111 and TAM 112 ideal cultivars for studying important traits like drought tolerance.

We hypothesized that the distinct stress adaptation observed in the TAM cultivars is a resultant of altered responses at gene and whole-plant level, and can be explained using relevant transcriptomic and physiological studies. The results suggested a key role for the phytohormone ABA in altering gene expression and photosynthetic parameters and generated several hypotheses for further investigation.

Materials and methods

Plant materials, growth conditions, and treatments

Hard-red winter wheat cultivars TAM 111 and TAM 112 were used. The cultivars partially share pedigree and were developed by Texas A&M AgriLife Research. The pedigree for TAM 111 (TAM 107//TX78V3620/CTK78/3/TX87V1233) includes TAM 107, Centurk, and Texas experimental lines, while TAM 112 (TX98V9628=U1254-7-9-2-1/TXGH10440) is derived from TAM 110 sib, TAM 200, and Kansas experimental lines. Greenhouse experiments were conducted at the Texas A&M AgriLife Research and Extension Center at Bushland, Texas USA (35° 11' 31" N / 102° 3' 53" W). Seeds were planted in LC1 potting mixture (Sun Gro Horticulture Canada Ltd.). After emergence, the seedlings were vernalized for seven weeks at 4 °C in a growth chamber. Seedlings were then transplanted into two gallon pots filled with Calcined Fullers Earth (PrimeraOne®, Ohio, USA) and moved into a greenhouse. Osmocote Controlled Release fertilizer (19-6-12; 100 ppm N) was thoroughly mixed in the soil and another 100 ppm N of Miracle-Gro (24-8-16) plant food was added in four intervals along with irrigation water during early seedling establishment. To emulate natural conditions, greenhouse temperatures were periodically adjusted. The day/night temperatures were initially set to 18/10 °C. At the start of the WD stress treatment the temperatures were increased to 22/14 °C, and increased further to 26/18 °C during grain filling.

Five seedlings were transplanted into each pot and rubber mulch was added to minimize evaporation. At 14 days after transplanting (DAT), the pots were thinned to retain three healthy seedlings. Prior to the WD stress treatments, the pots were maintained at 100% gravimetric water content (GWC) for seven weeks. After WD treatments started (50 DAT; jointing stage), during each irrigation event (three times a week) the control pots were brought back to 90% GWC and the WD treatment were brought back to 50% GWC. A confounded factorial design was used in the study with three replicates for each cultivar, irrigation treatment (wet and dry), and sampling stage (heading, grain filling, and physiological maturity). Sampling stages were treated as blocks within the replication and each block included a randomized arrangement of cultivar and irrigation treatments.

Gas exchange, chlorophyll, and other physiological measurements

Measurements of net photosynthesis (A), stomatal conductance (g_s), transpiration (E), and the ratio of intercellular to ambient [CO_2] (C_i/C_a) were made on flag leaves of primary shoots using a Licor 6400XT (Licor, Lincoln, NE, USA). Key physiological and molecular measurements were made on flag leaves to develop methods for trait characterization, given the role of flag leaves in determining grain yield in wheat (Guoth et al., 2009; Hui et al., 2008). All measurements were made at saturating light levels (1800 μmol m^{-2} s^{-1}), ambient growth [CO_2] (385 μl l^{-1}), mid-day growth temperature (29 °C), and a leaf vapor pressure deficit ranging from 0.7 to 2.4. Measurements were made twice a day at 3 (AM) and 8 (PM) hours after the start of the photoperiod. Three and six plants were measured for each genotype and treatment during the 2012 and 2013 studies respectively at the grain filling stage when the GWC of most pots was between 70-80 % and 40-50 % for wet and dry treatments, respectively. Trends in photosynthetic parameters were similar across the years. Results from 2013 are presented and discussed in the manuscript, while data from 2012 can be accessed in Supplementary Fig. S1.

Chlorophyll content of flag leaves of the primary shoots was measured using a Konica Minolta SPAD 502 meter. Cumulative transpiration, tiller number, plant height, above ground biomass (AGB), and below ground biomass (BGB) were measured at different developmental stages. Stems, leaves, roots, and spikes were harvested separately at physiological maturity to determine the grain yield and other yield parameters.

Tissue sampling, RNA extraction, and microarray assay

Transcriptome profiling was conducted on flag leaves at heading (79 DAT) and grain filling stages (100 DAT). The flag leaves of primary shoots from four individual plants were harvested together and pooled to make one sample. Samples were harvested into liquid N_2 and stored at -80 °C until processing.

RNA was extracted from flag leaf samples collected during the 2012 study using a Qiagen – RNeasy Mini kit. Labeled cRNA was hybridized to the Affymetrix wheat

GeneChip Genome array (Affymetrix, Santa Clara, CA, USA), consisting of 61,127 probes representing 55,052 transcripts. More information about chip design can be accessed at, http://www.affymetrix.com/catalog/131517/AFFY/Wheat-Genome-Array#1_1. Probe synthesis, hybridization and data extraction were performed at the Kansas State University Integrated Genomic Facility in Manhattan, Kansas, USA. Microarray data was submitted to the National Center for Biotechnology Information - Gene Expression Omnibus data base repository (http://www.ncbi.nlm.nih.gov/geo/info/linking.html).

Microarray data analysis

GeneSpring software v.12.5 (Agilent Technologies, Santa Clara, CA, USA) was used for data analysis. Principle component analysis (PCA) and correlation coefficients used to visualize the complex data and assess the variability among samples indicated that replicates within treatment/genotype combinations were similar (Supplementary Fig. S2). Normalized data were subjected to analysis of variance (ANOVA, $P < 0.01$) with levels resulting from all combinations of the three factors (cultivar, treatment, stage). Following ANOVA, the Benjamini and Hochberg false discovery rate algorithm (FDR, $q < 0.01$) was employed to identify 5,754 differentially expressed genes. Differential expression was then defined as ≥ 2 fold difference. The different ANOVA models tested on the microarray data yielded a negligible number of genes in the interaction groups, hence main effects were pursued for downstream analysis.

Functional classification using MapMan

Functional classification of the differentially expressed transcripts was performed using MapMan v.2.2 (Thimm et al., 2004; Usadel et al., 2009). The built-in mapping file for Affymetrix wheat GeneChip genome arrays was used for visualization of functional classes and pathways organized into hierarchical BINs and sub-BINs based on the putative functions. Transcripts with multiple putative functions were placed in the best matched BINs. Following MapMan analysis, a manual search was applied to identify transcripts annotated with putative functions in hormone metabolism and the transcripts were grouped accordingly.

Phytohormone measurements

The flag leaf tissue used for gene expression profiling was also used for quantifying abscisic acid (ABA) using methods previously described (Reddy et al., 2013a) with minor modifications. Thirty milligrams of finely ground flag leaf tissue was used for each analysis. Modifications included adjusting the pH to 8.0 and 6.0 for the first and second phase partitioning steps during extraction and analyzing the samples in negative chemical ionization mode on the gas chromatograph-mass spectrometer with methane as the reagent gas and source and quadrupole temperatures at 150 °C. Two sets of ions were monitored (260, 266, 278, 284 m/z), and the larger fragments were used for quantification.

qRT-PCR Analysis

The gene expression levels observed in microarray experiment were validated by quantitative real-time polymerase chain reaction (qRT-PCR) using the SYBR Green I detection method. Subsets of RNA samples used for microarray hybridizations were used to run qRT-PCR analysis as well. Primers for eight genes of interest including a reference gene were designed with the help of Primer3 software (primer combinations along with annotation information are provided in Supplementary Fig. S8A). Primer efficiencies were determined using serial dilution analysis and seven genes showing efficiencies from 94% to 100% were selected for validation. The analysis was performed on an Applied Biosystems® 7500 Real Time PCR system following cDNA synthesis by the Omniscript reverse transcription kit (Qiagen, Catalog # 205113). Changes in gene expression were determined using the methods previously described by Reddy et al. (2013b). Pairwise relative expression differences similar to microarray analysis were also estimated for qRT-PCR data and the results are presented in Supplementary Figs. S8B and C.

Statistics

Statistical procedures used for microarray data analysis are described above. For all other data, SAS software (v 9.2) was used with PROC GLM procedure and LSMEANS

statement. Pots with weak or unhealthy plants were removed from statistical analysis, resulting in variable numbers of data points. Multiple mean comparisons were performed with least square means using Tukey – Kramer's approach on cultivar and water treatment differences. Due to statistical similarity in the physiological parameters measured at the heading stage (79 DAT), the time point is eliminated from the results and discussion section.

Results

Whole-plant growth and yield

In response to WD stress, both cultivars demonstrated similar trends in growth and phenotypic responses, although TAM 112 performance differed slightly from TAM 111. The cumulative transpiration, plant height, tiller number, tiller-spike number (Fig. 1), AGB, BGB, and SPAD chlorophyll content (Supplementary Fig. S3) determined at the grain filling stage suggested that TAM 112 responded differently than TAM 111 to altered WD conditions. Differences also existed in cumulative transpiration, total grain yield, tiller-grain yield and harvest index determined at physiological maturity (Fig. 2). Significant differences (P <0.01) occurred in all of the parameters described above between water treatments, while fewer traits were different between the cultivars within treatments. TAM 112 transpired more water (only at $P < 0.1$; Fig. 1A), was shorter (Fig. 1B), and produced more tillers (Fig. 1C) and tiller-spikes (Fig. 1D) compared to TAM 111 under WD through the grain filling stage (P <0.05). Sustained WD stress through physiological maturity did not affect transpiration (Fig. 2A), but resulted in higher grain yield in TAM 112 (Fig. 2B), with tiller spikes making a substantial contribution (Fig. 2C). TAM 112 also had a greater harvest index under WD stress conditions compared to TAM 111 (Fig. 2D).

Leaf-level photosynthetic measurements

Photosynthetic parameters including net assimilation (A), the ratio of intercellular CO_2 to ambient CO_2 (C_i/C_a), stomatal conductance (g_s), and transpiration (E) were determined in flag leaves under both full and deficit-irrigated conditions at the grain

filling stage. Both cultivars generally showed reduced gas exchange parameters (*A*, C_i/C_a, g_s, and *E*) under WD compared to well watered conditions (Fig. 3), although the differential water treatment had no effect on *A* and C_i/C_a in TAM 111 during the early hours of the day or on *A* during afternoon hours. WD through the grain filling stage resulted in significantly lower *A*, C_i/C_a, g_s, and *E* in TAM 112 compared to TAM 111 at the AM time point (Fig. 3A-D) and *A* and *E* at the PM time point (Fig. 3E and H). Well watered TAM 111 also had greater g_s and *E* at the AM time point compared to TAM 112 (Fig. 3C-D). Overall, the photosynthetic parameters *A*, *E*, C_i/C_a, and g_s, were lower in TAM 112 compared to TAM 111, especially under WD at the grain filling stage.

Gene expression profiling

Transcriptome profiling of flag leaves in response to WD stress in TAM 111 and TAM 112 at different stages identified 5,754 differentially expressed genes (Supplementary Fig. S4). Based on the physiological data, the grain filling stage in TAM 111 and TAM 112 was selected for detailed functional classification and analysis (Fig. 4). WD through the grain-filling stage resulted in altered expression of more genes in TAM 112 (3,197 transcripts) compared to TAM 111 (2,131 transcripts). A joint analysis indicated that 1,657 transcripts were commonly altered in both cultivars, while 474 and 1,540 transcripts were unique to TAM 111 and TAM 112 respectively (Fig. 4A). qRT-PCR analysis on selected candidate genes confirmed the gene expression patterns observed in microarray data (Supplementary Fig. 8). The unique genes differentially expressed in response to water deficit stress suggested distinct molecular responses. A detailed investigation of these genes was conducted to investigate the mechanisms underlying the drought responses.

Functional classification using MapMan

Genes responsive to WD stress at the grain filling stage that were unique to TAM 111, TAM 112, and common to both cultivars were functionally classified for overview (Fig. 4). Genes were grouped into 32 BINs (out of 36 BINs defined in MapMan) with putative functions (Fig. 4). BIN 35, not presented in the figure, comprised transcripts not assigned to any functional group due to lack of annotation information. More than

half of the genes unique to TAM 111 (271 transcripts), TAM 112 (821 transcripts), and common between the cultivars (866 transcripts) were assigned to BIN 35 and were not used further.

Out of 474 transcripts with altered expression only in TAM 111, 209 transcripts were classified into 24 functional BINs (Fig. 4B). Nearly 83% of these genes fell into 10 major groups (with ≥ 8 genes). WD stress induced changes in the expression of genes unique to TAM 111 ranged between -13.2 to 16.2 FC (Fold Change). TAM 112 had 1,540 unique genes responding to WD stress at the grain filling stage, and 729 of these genes were classified into 32 BINs with putative functions (Fig. 4C). Eleven BINs, (with ≥ 20 genes) contained about 79% of the genes. The expression responses of unique TAM 112 genes to WD stress ranged from -6.0 to 22.8 FC.

Of the 1657 genes responsive to water deprivation in both cultivars, 813 genes were categorized into 31 BINs with trends similar to TAM 112 (Fig. 4D). Among the genes with putative functions, 701 (86%) were grouped into 14 BINs (with ≥21 genes). The expression responses to WD stress of commonly expressed transcripts ranged from -72.8 to 229.3 FC.

Metabolism overview

The overview analysis implied that gene expression responses to WD leads to altered metabolism, which was then visualized using metabolism overview graphs (Supplementary Fig. S5). Expression values of photosynthesis-related transcripts, including those involved in light capture, electron transport, ATP synthesis, and the Calvin cycle were altered between -28.5 to 4.5 FC. Under WD at the grain filling stage TAM 112 suppressed more photosynthesis-related transcripts with greater expression differences compared to TAM 111 (Supplementary Figs. S5 - S6). While 34 transcripts showed similar patterns in both cultivars subjected to WD stress, TAM 112 responded with 30 additional genes related to photosynthesis. Transcripts similar to photosystem (PS) II - light harvesting complex (LHC) genes were down-regulated, potentially impacting light absorption and energy transfer to the reaction center in both cultivars (Supplementary Fig. S5). WD stress at the grain filling stage in both cultivars resulted in mixed responses of genes involved in other photosynthetic reactions, including starch

and sucrose metabolism, which may indicate a dynamic reprogramming of carbohydrate and other metabolic pathways (Supplementary Figs. S5 - S6).

Hormone related transcripts

The transcriptome data indicated that phytohormones might modulate WD stress responses in both TAM 111 and TAM 112 (Fig. 5). The data identified 123 genes putatively associated with the biosynthesis and signal transduction of seven phytohormones, including auxin, ABA, brassinosteroid (BR), cytokinin (CK), ethylene (ET), gibberellin (GA), and jasmonic acid (JA). In addition to 58 commonly expressed hormone-related transcripts, 13 and 52 transcripts were uniquely altered in TAM 111 and TAM 112, respectively (Fig. 5). Most of the hormone-related transcripts differentially expressed in response to WD stress at the grain filling stage were associated with ABA (Fig. 5A), auxin (Fig. 5C), and ET (Fig. 5E).

Transcripts encoding ABA biosynthesis enzymes were up-regulated in both cultivars, but greater numbers and expression differences occurred in TAM 112 (Fig. 5A). An increase in ABA activates downstream stress-associated genes via ABA responsive *cis*- and *trans*-acting elements/factors. WD stress induced a variety of transcripts involved in ABA signal transduction, including protein phosphatases and transcription factors, in both cultivars at the grain filling stage (Fig. 5A). A few ABA associated transcripts were down-regulated in TAM 112 alone (Fig. 5A). Two transcripts similar to *PLEOTROPIC DRUG RESISTANCE 12* (*PDR12*, encoding an ABA transporter) were up-regulated in TAM 112 at greater levels (Fig. 5A) compared to TAM 111, while a third gene unique to TAM 111 was suppressed (Fig. 5A). Transcripts involved in auxin (Fig. 5C) and ethylene (Fig. 5E) biosynthesis and signal transduction were promoted in response to WD stress in both cultivars, but more strongly in TAM 112 as opposed to TAM 111. CK related genes were down regulated in both cultivars, and greater numbers of genes related to CK biosynthesis and signaling were suppressed in TAM 112 compared to TAM 111 (Fig. 5D).

WD stress responsive transcripts

Transcripts mediating WD stress responses encode various proteins such as dehydrins, late embryogenesis abundant (LEA) proteins, aquaporins, heat shock proteins and redox and other abiotic stress signaling related proteins (Supplementary Figs. S7 and S8). DEHYDRIN and early responsive to dehydration (ERD) transcripts were up-regulated in both cultivars (Supplementary Fig. S7A). Most aquaporin related transcripts were down-regulated. In addition to commonly expressed dehydration responsive transcripts, TAM 112 uniquely induced the expression of transcripts similar to *LEA*, *ERD1* and others. Many heat shock protein related genes (Supplementary Fig. S7B) and transcripts classified under drought/salt stress (Supplementary Fig. S7C) were also up-regulated in both cultivars in response to WD stress. A variety of stress-related transcription factors showed complex expression patterns in response to water deficit stress in both TAM 111 and TAM 112 at the grain filling stage (Supplementary Fig. S7D). Redox associated transcripts were commonly up-regulated in both cultivars, and the expression of 13 additional redox transcripts was modified in TAM 112 (Supplementary Fig. S7E).

Flag leaf ABA abundance

The expression data indicated that ABA could be playing a differential role in WD stress responses in TAM 111 and TAM 112 at the grain filling stage. The abundance of ABA was determined in the flag leaves of plants exposed WD stress at the grain filling stage. ABA levels were low, and similar in both cultivars under well watered conditions. Water deficit stress caused ABA levels to increase dramatically, with significantly greater accumulation in TAM 112 compared to TAM 111 (Fig. 6).

Discussion

The development of wheat cultivars with better adaptation to arid and semi-arid climates is necessary for the U.S. SGP to sustain agricultural and economic viability. The winter wheat cultivars TAM 111 and TAM 112 are characterized by distinct adaptation mechanisms to the water-limited environments of the SGP. However, the physiological and molecular basis of these adaptation mechanisms remains unknown. In light of previous reports of differences in the WD stress responses, the basis of

physiological responses at the whole-plant level and gene expression, and ABA analysis at flag-leaf levels under WD treatment were explored.

Physiological responses

Increasing transpiration efficiency (TE) in water-limited environments has been pursued as means to increase crop yield (Blum, 2009; Sinclair, 2012). The current study suggests that cumulative transpiration was not the sole determinant of yield and that tiller/ spike numbers, carbon metabolism, allocation, and partitioning may be involved. Some of the observed yield increases in TAM 112 under WD stress can be attributed to increase in tillers/ spikes and harvest index (Figs. 1 and 2). Increased tillering and spikes in TAM 112 was also observed in field studies, specifically under WD stress (Xue et al., 2013). The number of fertile spikelets on the primary spike was not influenced by WD in either cultivar, but WD stress led to more sterile spikelets in TAM 111 compared to TAM 112 (data not shown). Studies from other labs also indicate that yield and yield stability in cereals may be improved by increasing grain numbers via elevated grains/spike, spikelet fertility, and/or number of reproductive tillers (Dolferus et al., 2011; Sreenivasulu and Schnurbusch, 2012).

The distinct yield responses to WD stress may result, at least in part, from altered photosynthesis and stomatal conductance. However, interpreting the reduction in photosynthesis in response to WD stress in the context of grain yield is complex (Lawlor and Tezara, 2009). Under WD stress, plants generally decrease stomatal conductance leading to reduced photosynthesis. Our results showed that under WD stress TAM 112 produced higher grain yield, despite reduction in the gas exchange parameters, A, g_s, E, and C_i/C_a compared to TAM 111(Fig. 3). The increase in final grain yield could be the result of a combination of several phenotypes and processes including spike number (Xue et al., 2013), dry weight of the spike at anthesis, efficient resource partitioning (Fischer, 2011; Foulkes et al., 2011), sink strength (Ji et al., 2010; Marcelis, 1996), radiation use efficiency (Parry et al., 2011), ear photosynthesis (Maydup et al., 2010), and overall photosynthetic capacity and efficiency (Fischer, 2011; Parry et al., 2011; Sreenivasulu and Schnurbusch, 2012). However, details about the specific processes remain unknown.

The reduction in flag leaf photosynthesis and stomatal conductance in TAM 112 during grain filling may indicate a distinct strategy of resistance and/or tolerance to survive under WD stress. Increased ABA levels in response to either short term or sustained WD stress and maintenance of ABA homeostasis has been linked to adaptability and grain yield (Sreenivasulu et al., 2012). ABA levels increased in various tissues under WD stress resulting in massive transcriptional reprogramming, reduced photosynthesis (Guoth et al., 2009; Ji et al., 2011; Seiler et al., 2011; Thompson et al., 2007) and a reduced precursor pool for chlorophyll biosynthesis (Sreenivasulu et al., 2012). The reduction in photosynthesis and related parameters (Fig. 3) in TAM 112 under WD may be partially explained by elevated ABA (Fig. 6). ABA application has also been shown to increase carbohydrate accumulation and remobilization to the grain in wheat, rice, and barley (Seiler et al., 2011; Tang et al., 2009; Travaglia et al., 2007; Yang and Zhang, 2006). Thus, elevated ABA might cause a reduction in photosynthesis, but could also provide a means for maintaining grain yield.

Global transcriptomic responses

Physiological data showed that TAM 112 responded to WD by reducing photosynthesis and stomatal conductance, and increasing ABA content, potentially as a result of more robust gene expression responses compared to TAM 111 (Fig. 4). WD stress triggered transcriptomic changes initiated by TAM 112 comprised more diverse functional classes of genes with greater expression differences. In addition to commonly regulated transcripts in both cultivars, the expression of additional transcripts related to photosynthesis, carbohydrate metabolism (Supplementary Fig. S6), phytohormones (Fig. 5), and other dehydration induced transcripts (Supplementary Fig S7) were uniquely modified in TAM 112 compared to TAM 111. Previous studies have identified a role for ABA in triggering dramatic changes in the transcriptomes of different crops (Krugman et al., 2010; Seiler et al., 2011; Thompson et al., 2007; Xue et al., 2006), and it is possible that at least some of the differences observed in the current study are due to elevated ABA. ABA-responsive transcripts, encoding dehydrins, LEA proteins, and heat shock proteins, and transcription factors such as MYB, NAC, WRKY, etc. were differentially expressed between the cultivars in response to WD

(Supplementary Figs. S7 and S8). Similar changes in ABA-related transcripts with WD stress have been reported previously (Krugman et al., 2010; Seiler et al., 2011; Zhu et al., 2011).

In addition to ABA, our results identified major changes in transcripts related to auxin and ethylene, and minor responses for BR, CK, GA and JA related transcripts (Fig. 5). Expression of the majority of auxin and ethylene related transcripts contrasted with expression of CK related transcripts; however specific roles in dehydration responses were not clear. Auxin levels were increased in cucumber (*Cucumis sativus* L.) leaves in response to dehydration stress (Pustovoitova et al., 2004), while there was an ambiguous relationship in wheat at the grain filling stage (Xie et al., 2003). Ethylene has also been associated with dehydration stress responses, though independently or in a complex relationship with ABA (Tanaka et al., 2005; Wilkinson and Davies, 2009). CK regulation of dehydration responses also involves cross-talk with other phytohormones including ABA (Peleg and Blumwald, 2011; Vysotskaya et al., 2009).

A meta-analysis studying the metabolic connections between drought and photosynthesis identified interactions between sugar, starch, hormone, and ROS signaling pathways (Pinheiro and Chaves, 2011). The production of ROS during reduced photosynthesis under WD stress has been studied and components mediating the responses have been identified (Saibo et al., 2009). Transcripts involved in carbohydrate, lipid, amino acid, nucleotide and hormone metabolism, and redox regulation/signaling showed greater differences in expression in WD stressed TAM 112 compared to TAM 111 (Fig. 5 and Supplemental Figs. S5 – S7). It seems likely that ROS also plays an important role in integrating multi-process responses to WD stress, however further studies are required to elucidate the mechanisms.

WD responses in wheat – physiological and molecular connections

The mechanistic basis of adaptation to WD may vary among crops as a result of differences in genetic composition, environmental conditions and management practices. Nonetheless, these adaptations are triggered at the level of the transcriptome and lead to proteomic and metabolic changes. The whole-plant data is perhaps most relevant from a production standpoint, while the leaf-level data provide insights into the

mechanisms involved. Although the documentation of differential gene expression is rather straightforward, the interpretation in terms of potential physiological mechanisms is complex. Dehydration responses are mediated by multiple genes at a given developmental stage and vary with the severity of stress treatment and genetic background.

Phytohormones have been shown to regulate stem carbon reserves, spike dry weight at anthesis, efficient carbon allocation, and partitioning between and among sink tissues to determine final grain yield (Dreccer et al., 2009; Fischer, 2011; Sreenivasulu et al., 2012; Wilkinson et al., 2012). The differential accumulation of ABA in the flag leaves of TAM 112 and TAM 111 (Fig. 6) could be linked to the altered transcriptomic changes and phenotypes under WD stress. A dehydration induced surge in ABA resulted in stomatal closure and a reduction in photosynthesis (Tanaka et al., 2005; Thompson et al., 2007), and triggered vast transcriptional reprogramming (Fujita et al., 2011; Seiler et al., 2011).

Regulation of stomatal movement under WD is not solely regulated by ABA; evidences also suggests a role for ethylene (Dodd, 2003; Vysotskaya et al., 2011). Ethylene may potentially modulate dehydration responses by acting in concert with ABA to regulate photosynthesis and/or carbon metabolism. Furthermore, increasing CK biosynthesis by genetic modification delayed senescence and increased the yield of WD stressed rice (Peleg et al., 2011). WD induced suppression of CK associated genes in flag leaves at grain filling could reflect decreased flag leaf sink strength to increase carbon allocation to the grain. Increased grain yield in TAM 112 also suggests a greater sink strength (higher tiller yield) and resource allocation under WD conditions. ABA accumulation and its association with altered grain yield under WD conditions have been studied previously in rice, barley, and tomato (Ji et al., 2011; Seiler et al., 2011; Thompson et al., 2007).

In summary, both cultivars used different strategies to adapt to WD conditions; in contrary to TAM 111, TAM 112 responded with distinct sink strength, gas exchange, and source-sink relationships via robust transcriptomic modifications and elevated ABA content. Recent studies have highlighted the importance of phytohormone balance and ABA homeostasis in gas exchange, resource mobilization and determination of grain

number and eventual grain yield (Acharya and Assmann, 2009; Ji et al., 2011; Peleg and Blumwald, 2011; Seiler et al., 2011; Sreenivasulu and Schnurbusch, 2012). The contribution of phytohormones in adaptation to WD stress makes the manipulation of their homeostasis and signaling promising breeding targets to improve yield, efficiency, and performance (Wilkinson et al., 2012). Further studies are required to elucidate the potential roles of phytohormones in determining grain yield and source-sink relationships under WD stress.

Acknowledgements

We are grateful to Texas A&M AgriLife Research staff Maria Fuentealba, Cody Shachter, Serina Nelson, and Peihua Yan for their help in greenhouse studies; ChorTee Tan and Li Paetzold for help with qRT-PCR; Kirk Jessup for assistance in gas exchange measurements; and Yanni Lun from Integrated Genomics Facility at Kansas State University for array hybridization. The work was supported by the Texas Wheat Producers Board, Texas A&M AgriLife Research, and the USDA-ARS Ogallala Aquifer Initiative. The authors thank William A. Payne and Yiqun Weng for their contribution during the initial wheat project funding application to USDA-ARS Ogallala Aquifer Initiative.

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Fig. 1.

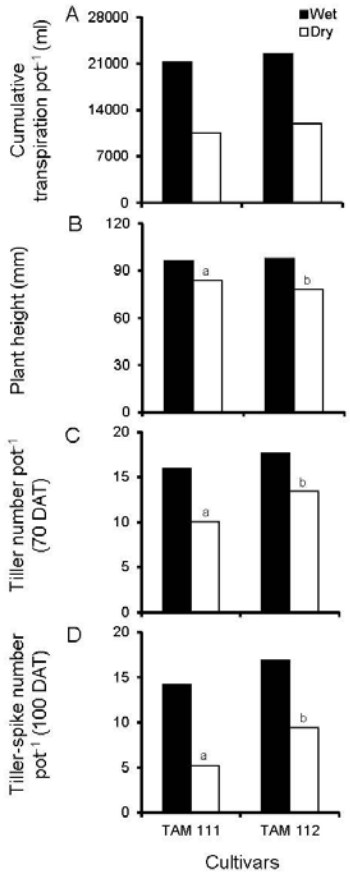


Fig. 1. Cumulative transpiration (A), plant height (B), tiller number (C), and tiller-spike number (D) in TAM 111 and TAM 112 in response to WD through the grain filling stage. Data are means of 7-11 pots. All the comparisons between water treatments within a cultivar were significantly different at $P < 0.05$. Bars with different letters indicate significant differences between the cultivars within the water treatment at $P < 0.05$; bars without letters are not significant.

Fig. 2.

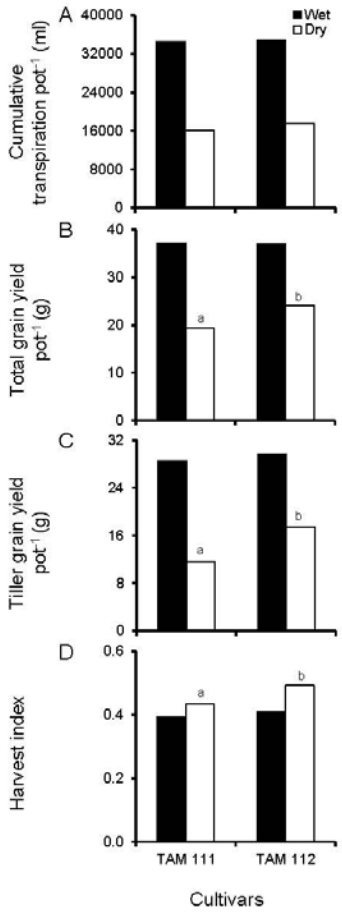


Fig. 2. Cumulative transpiration (A), total grain yield (B), tiller grain yield (C), and harvest index (D) in TAM 111 and TAM 112 in response WD through physiological maturity. Data are means of 7-11 pots. All the comparisons between water treatments within a cultivar were significantly different at $P < 0.05$. Bars with different letters indicate significant differences between the cultivars within the water treatment at $P < 0.05$; bars without letters are not significant.

Fig. 3.

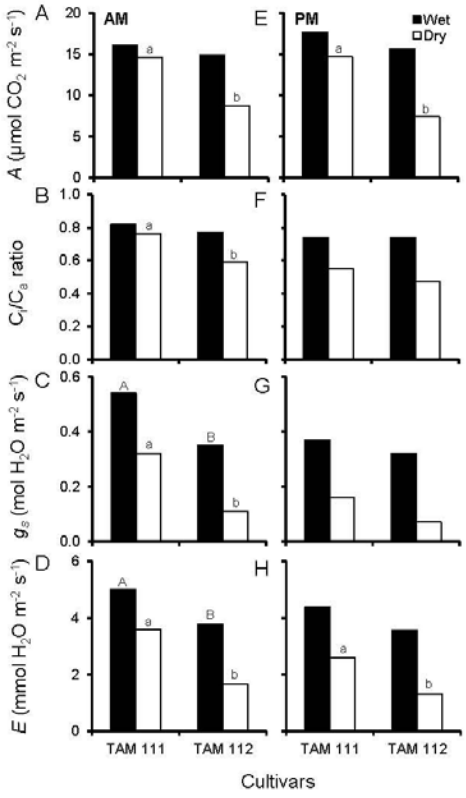


Fig. 3. Flag leaf photosynthetic CO_2 assimilation (A); ratio of intercellular CO_2 to ambient CO_2 (C_i /C_a); stomatal conductance (g_s); and transpiration rate (E) measured during morning (AM; A-D) and afternoon (PM; E-H) during the grain filling stage. Data are means with n=6 plants. All the comparisons (except, A under both AM and PM time points and C_i/C_a during AM time point) between water treatments within a cultivar were significantly different at P <0.05. Bars with different letters indicate significant differences between the cultivars within the water treatment at P <0.05; bars without letters are not significant.

Fig. 4.

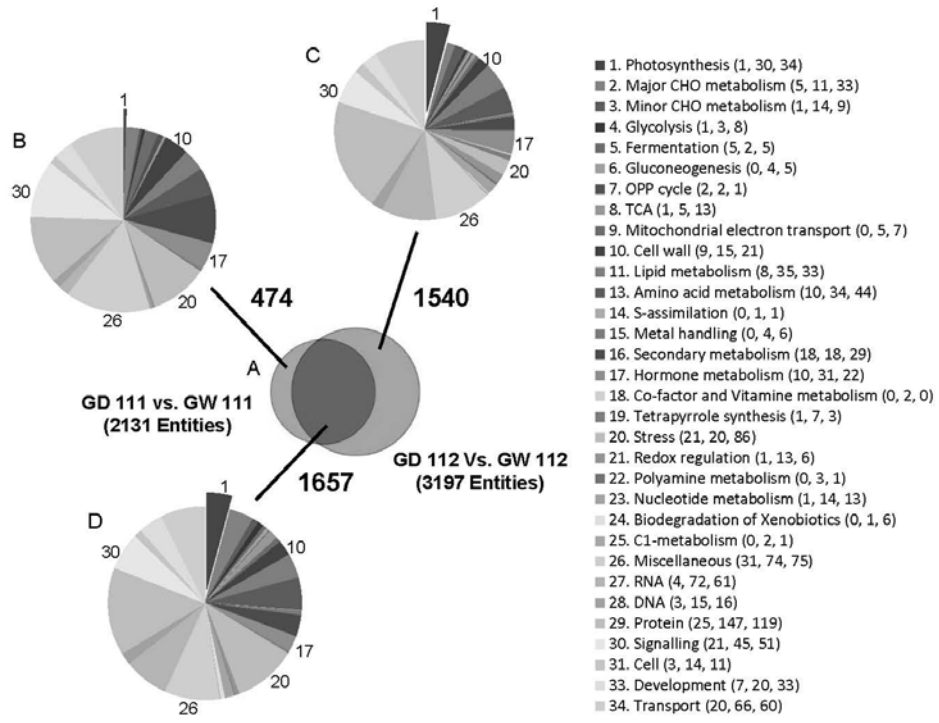


Fig. 4. Composite illustration of joint analysis (A) and MapMan overview analysis (B, C, and D) on the resulting groups. Joint analysis on the differentially expressed transcripts under WD in TAM 111 (2131 entities) and TAM 112 (3197 entities) identified 474 transcripts specific to TAM 111 (B), 1540 genes specific to TAM 112 (C) and 1657 genes common to both TAM 111 and TAM 112 (D); MapMan overview analysis presents the respective functional classifications. The legend provides the parent BIN number, BIN name and the gene number in parentheses; as a reference, some BIN numbers are also presented as data labels in the respective pie charts; GD: grain filling stage, dry treatment; GW: grain filling stage, wet treatment; 111: TAM 111; 112: TAM 112.

Fig. 5.

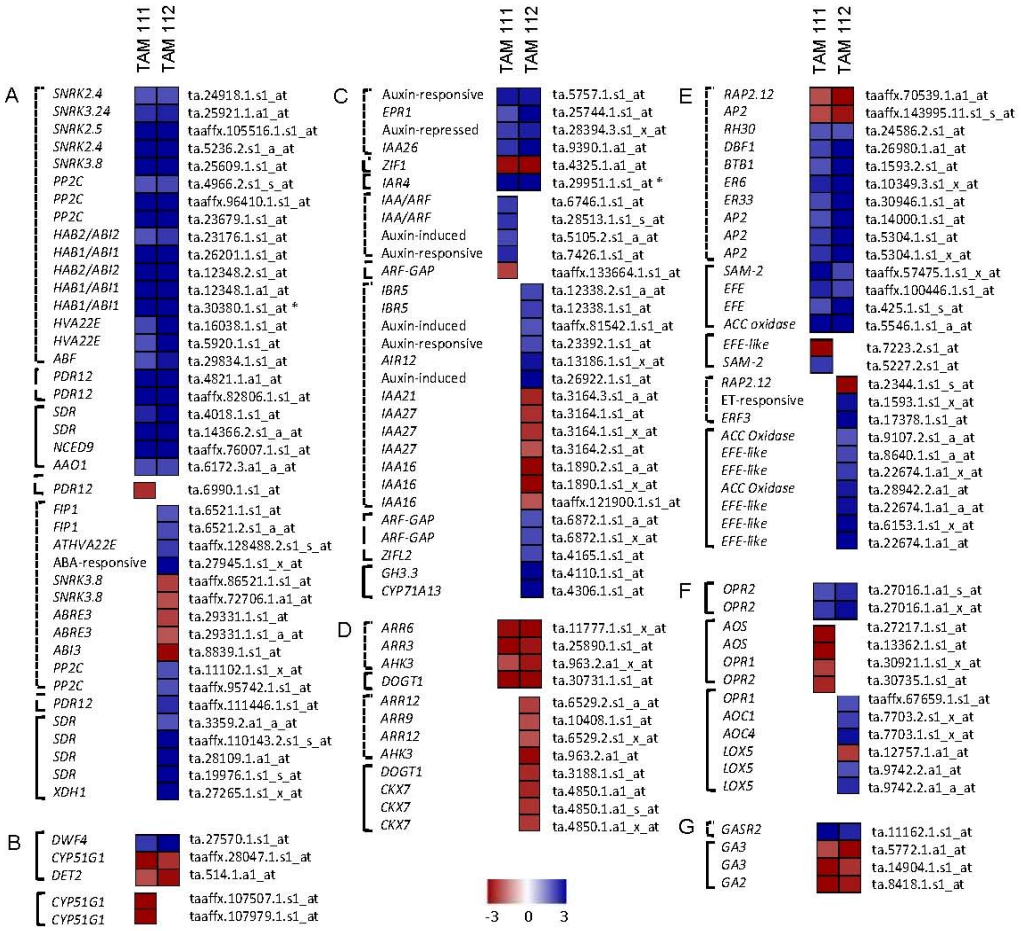


Fig. 5. Heat map of transcripts putatively associated with the biosynthesis (bracket with line), transport (bracket with long dashes) and signal transduction (bracket with small dashes) of abscisic acid (A), brassinosteroid (B), auxin (C), cytokinin (D), ethylene (E), jasmonic acid (F), and gibberellin (G). The color scale represents the expression values of transcripts under WD stress compared to control where red represents down-regulation and blue represents up-regulation. Probe IDs marked with an asterisk were used for qRT-PCR analysis.

Fig. 6.

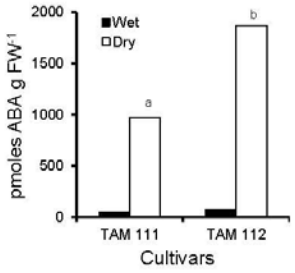
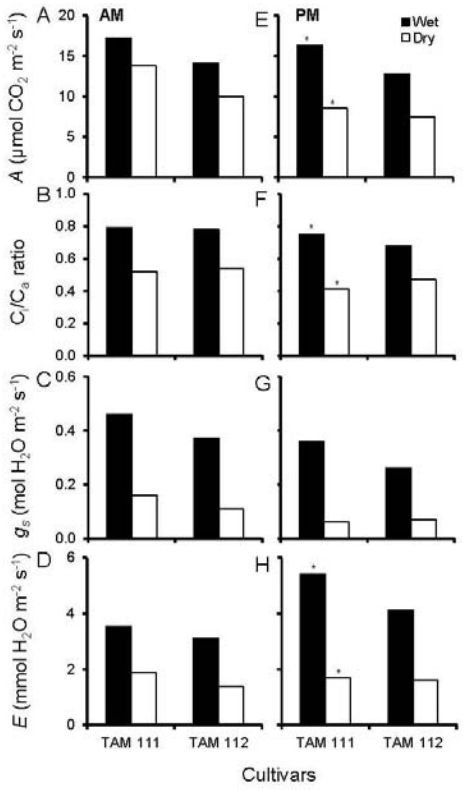
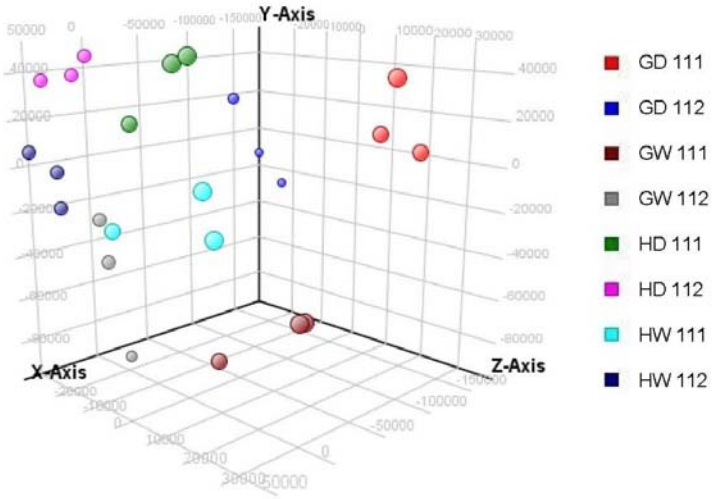


Fig. 6. Abscisic acid levels in flag leaves of TAM 111 and TAM 112 in response to wet and dry treatments during the grain filling stage. Data are means with n=3. All the comparisons between water treatments within a cultivar were significantly different at P <0.05. Bars with different letters indicate significant differences between the cultivars within the water treatment at P <0.05; bars without letters are not significant.



Supplementary Fig. S1. Flag leaf photosynthetic CO_2 assimilation (A); ratio of intercellular CO_2 to ambient CO_2 (C_i /C_a); stomatal conductance (g_s); and transpiration rate (E) measured during morning (AM; A-D) and afternoon (PM; E-H) during the grain filling stage in the 2012 study. Data are the means with n=3 plants. Multiple comparisons were performed using the Tukey – Kramer approach with least square means (LSM) for both water treatment and cultivar differences. None of the comparison between the cultivars within a water treatment were significantly different at P <0.05. The bars with asterisk indicate significant differences between the water treatments within a cultivar at P <0.05; bars without letters are not significant.

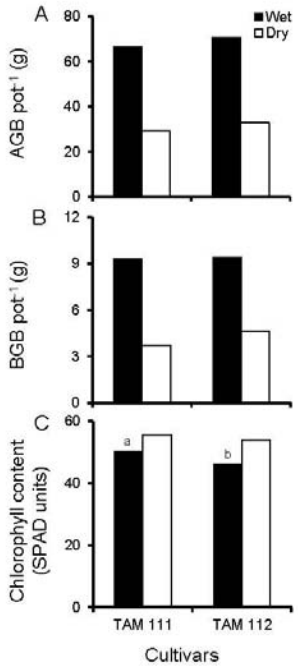
A



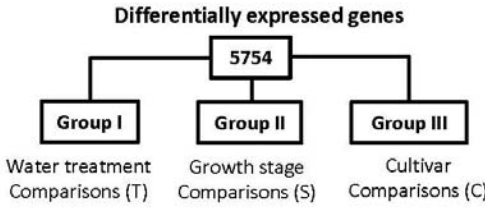
B

Array	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	Treatment
S1	-																								HW 111
S2	0.99																								HW 111
S3	0.98	0.99																							HW 111
S4	0.99	0.97	0.97																						HD 111
S5	0.99	0.98	0.98	0.99																					HD 111
S6	0.98	0.97	0.97	0.97	0.99																				HD 111
S7	0.99	0.99	0.98	0.98	0.99	0.98																			HW 112
S8	0.97	0.99	0.99	0.97	0.97	0.96	0.99																		HW 112
S9	0.98	0.98	0.98	0.98	0.99	0.98	0.99	0.99																	HW 112
S10	0.97	0.96	0.96	0.99	0.99	0.99	0.98	0.96	0.98																HD 112
S11	0.98	0.97	0.97	0.99	0.99	0.99	0.98	0.97	0.99	0.99															HD 112
S12	0.98	0.97	0.97	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.99														HD 112
S13	0.97	0.98	0.99	0.96	0.97	0.96	0.97	0.98	0.97	0.95	0.96	0.96													GW 111
S14	0.95	0.97	0.97	0.93	0.94	0.94	0.95	0.97	0.95	0.93	0.93	0.94	0.98												GW 111
S15	0.97	0.98	0.98	0.96	0.96	0.96	0.97	0.98	0.96	0.95	0.95	0.96	0.99	0.99											GW 111
S16	0.93	0.93	0.93	0.94	0.94	0.94	0.93	0.92	0.92	0.95	0.93	0.93	0.94	0.93	0.94										GD 111
S17	0.94	0.94	0.94	0.95	0.95	0.95	0.93	0.93	0.93	0.95	0.94	0.94	0.94	0.93	0.95	0.99									GD 111
S18	0.96	0.95	0.95	0.97	0.97	0.97	0.95	0.94	0.95	0.97	0.96	0.96	0.95	0.94	0.96	0.99	0.99								GD 111
S19	0.93	0.95	0.95	0.91	0.92	0.92	0.94	0.96	0.93	0.92	0.91	0.92	0.96	0.99	0.97	0.92	0.92	0.93							GW 112
S20	0.97	0.98	0.98	0.97	0.98	0.97	0.98	0.99	0.98	0.97	0.97	0.98	0.99	0.98	0.98	0.94	0.94	0.96	0.97						GW 112
S21	0.96	0.98	0.98	0.96	0.97	0.96	0.98	0.99	0.97	0.96	0.96	0.97	0.99	0.99	0.99	0.93	0.94	0.95	0.98	0.99					GW 112
S22	0.86	0.87	0.86	0.86	0.86	0.86	0.86	0.86	0.85	0.88	0.86	0.86	0.86	0.88	0.87	0.96	0.96	0.93	0.88	0.88	0.87				GD 112
S23	0.88	0.88	0.87	0.88	0.88	0.88	0.88	0.87	0.87	0.90	0.89	0.88	0.88	0.89	0.88	0.97	0.97	0.94	0.89	0.89	0.88	0.99			GD 112
S24	0.94	0.94	0.93	0.95	0.95	0.95	0.94	0.93	0.94	0.96	0.95	0.95	0.93	0.92	0.93	0.98	0.99	0.98	0.92	0.95	0.94	0.96	0.97	-	GD 112

Supplementary Fig. S2. Principal component analysis (A) and correlation coefficients (B) for each sample used in microarray analysis. S1 - S24: samples 1 through 24; HW: heading stage, wet treatment; HD: heading stage, dry treatment; GW: grain filling stage, wet treatment; GD: grain filling stage, dry treatment; 111: TAM 111; 112: TAM 112.

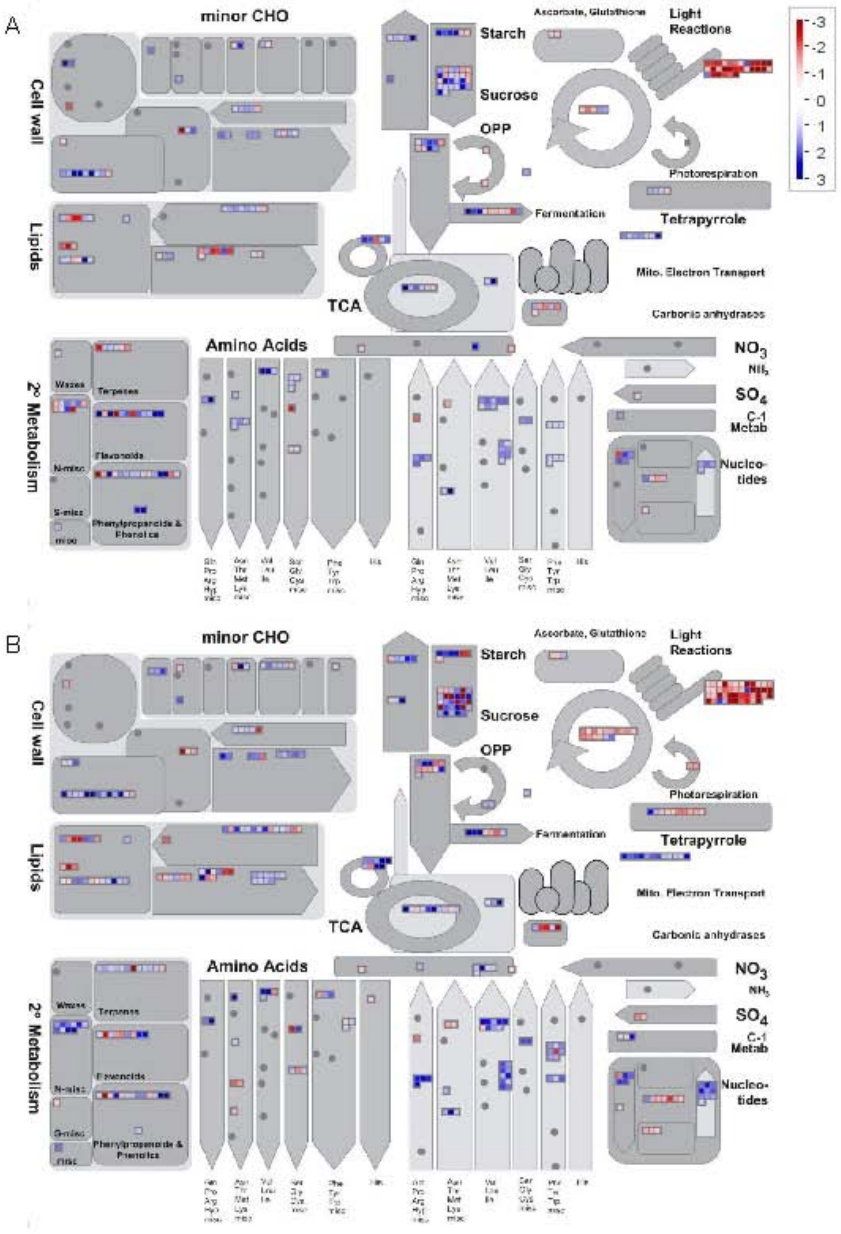


Supplementary Fig. S3. Above ground biomass (AGB, A), and below ground biomass (BGB, B) and SPAD chlorophyll content (C) determined at the grain filling stage (100 days after transplanting) across water treatments in TAM 111 and TAM 112. The multiple comparisons were performed using Tukey – Kramer approach with least square means (LSM) for both water treatment and cultivar differences. All the above comparisons between water treatments in a cultivar were significantly different at $P < 0.05$. The bars with different letters indicate significant differences between the cultivars in the water treatment at $P < 0.05$; bars without letters are not significant. Data is based on least square means of 7-11 pots across three replications for combination of each cultivar and water treatment.

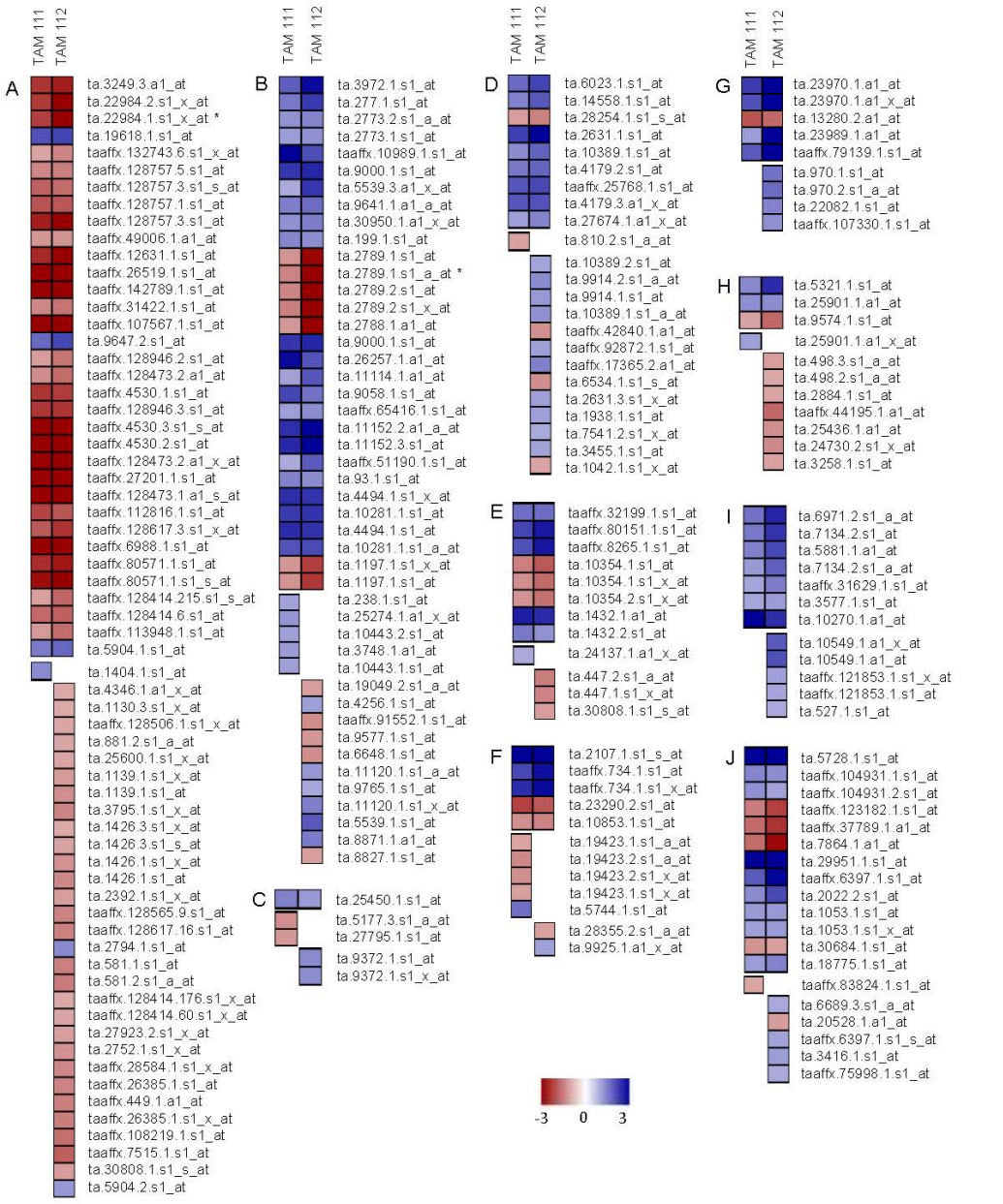


#	Comparison	Total	Up	Down	
T1	GD 111 vs. GW 111	2131	1131	1000	Group I
T2	GD 112 vs. GW 112	3197	1866	1331	
T3	HD 111 vs. HW 111	756	217	539	
T4	HD 112 vs. HW 112	539	307	286	
S1	GD 111 vs. HD 111	1558	1053	535	Group II
S2	GW 111 vs. HW 111	471	231	240	
S3	GD 112 vs. HD 112	3246	1730	1516	
S4	GW 112 vs. HW 112	598	277	321	
C1	GD 111 vs. GD 112	1130	619	511	Group III
C2	GW 111 vs. GW 112	686	465	221	
C3	HD 111 vs. HD 112	579	320	259	
C4	HW 111 vs. HW 112	663	428	235	

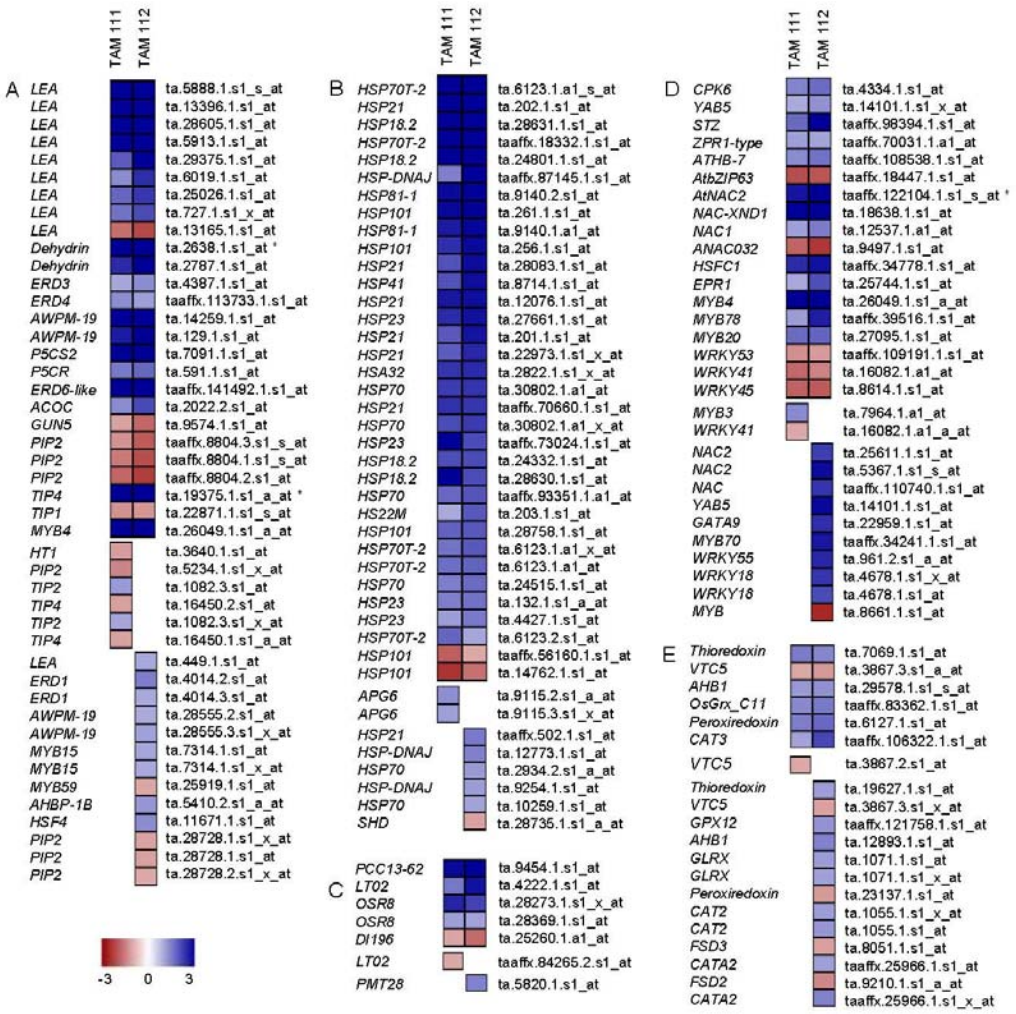
Supplementary Fig. S4. Organization of differentially expressed transcripts (5754) based on treatment groups for functional classification. Four biologically meaningful pairwise comparisons within each treatment group were constructed to classify genes accordingly. In the current manuscript, comparisons between water treatments within TAM 111 (T1) and TAM 112 (T2) in Group I (highlighted rows) were considered for detailed functional analysis. GD: grain filling stage, dry treatment; GW: grain filling stage, wet treatment; HD: heading stage, dry treatment; HW: heading stage, wet treatment; 111: TAM 111; 112: TAM 112; Up: number of up regulated genes; Down: number of down regulated genes.



Supplementary Fig. S5. MapMan metabolism overview of drought responsive transcripts in TAM 111 (A) and TAM 112 (B) at the grain filling stage. The color scale represents the expression intensities of transcripts under WD stress compared to control where red represent down-regulation and blue represents up-regulation.



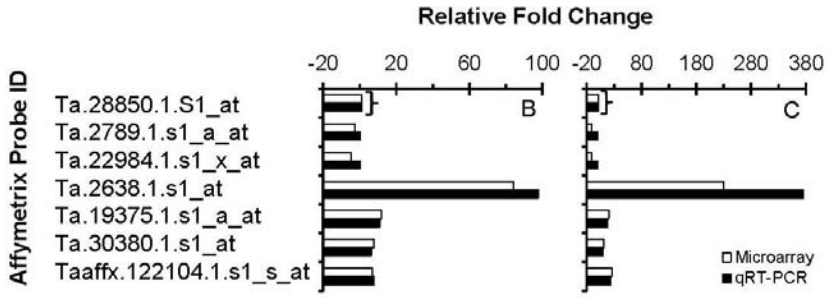
Supplementary Fig. S6. The heat map of transcripts putatively associated to mediate photosynthesis (A) major carbohydrate metabolism (B), TCA cycle (C), minor carbohydrate metabolism (D), glycolysis (E), fermentation (F), gluconeogenesis (G), tetrapyrrole synthesis (H), mitochondrial electron transport (I), and OPP electron transfer (J). The color scale represents the expression values of transcripts under water-deficit stress compared to control where red represent down-regulation and blue represents up-regulation. Probe IDs marked with asterisk were used for qRT-PCR analysis.



Supplementary Fig. S7. Heat map of transcripts that are putatively dehydration responsive (A), heat shock proteins (B), abiotic stress – salt/drought associated (C), dehydration-related transcription factors (D), and redox reaction associated (E). The color scale represents the expression values of transcripts under WD stress compared to control where red represent down-regulation and blue represents up-regulation. Probe IDs marked with asterisk were used for qRT-PCR analysis.

A

Affymetrix Probe ID	Annotation	Forward	Reverse
Ta.28850.1.S1_at	AAA-superfamily of ATPases	CAAATACGCCATC AGGGAGAACATC	CGCTGCCGAAACCA CGAGAC
Ta.2789.1.S1_a_at	Sucrose:fructan 6-fructosyltransferase	TCGTTCTATGATCC GGCGAA	TCCTTGGAACTGAC TGAATTGA
Ta.22984.1.S1_x_at	chlorophyll A-B binding protein	GACCCTGTCAACA ACAACGCAT	TATGTGTGCAGTTC ACCGCA
Ta.2638.1.S1_at	DHN4/ Dehydrin	ACTTCTGAGAGTG GAGGTGC	CGCAAGTGAGCTAG GTGAAC
Ta.19375.1.S1_a_at	TIP4-2/ Aquaporin	TGATTTGTGCGTG ATGGGTC	ACGGGGAGGCCTTA TACACTTAC
Ta.30380.1.S1_at	HAB1/ Protein phosphatase 2C	GCAATGTCACGGT CAATCGG	AGTCATGTTGGGAC TCGTGG
TAffx.122104.1.S1_s_at	NAC domain transcription factor	ATGCTGATCCATC CGTTCCT	GCAAGCTAGATCCC TGCCTA
Ta.29951.1.S1_at	IAR4/ Pyruvate dehydrogenase E1 α	CCAGGAGCTCAAG GACATGG	GGACAGGAAGGTG GTGAGAA
SYBR-Actin		ACCTTCAGTTGCC CAGCAAT	CAGAGTCGAGCACA ATACCAGTTG



Supplementary Fig. S8. qRT-PCR validation of selected transcripts from microarray results. Primer combination and annotation information of genes used for validation process (A). Relative fold change values under WD treatment compared with control condition in TAM 111 (B) and TAM 112 (C) at the grain filling stage. Reference gene used in the tests is represented with a flower bracket.