MOLECULAR AND PHYSIOLOGICAL INDICATORS OF DROUGHT STRESS IN SOFT WHITE SPRING WHEAT AT THE VEGETATIVE STAGE DEVELOPMENT

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Abstract:

Drought stress has negative effects at all wheat stages and can reduce the total yield by 50%. Two greenhouse were executed one for RNA sequencing and the other one was for chlorophyll fluorescence measurements under drought stress. Transcriptomic analyses via RNA sequencing of two soft white spring wheat at vegetative stage development were achieved under three conditions. The treatments involved well-watered (WW, 100%, 236 ml) as control, moderate stressed (MS, 50%, 118 ml) and severe stressed (SS, 25%, 59 ml). The RNA sequencing datasets from Alpowa contained 690,857; 663,526 and 652,705 reads from WW MS and SS, whereas the Idaho datasets were 523,643; 485,527 and 489,436 reads under WW, MS and SS, respectively. Bioinformatics analysis of the sequence data was performed and in general, Idaho showed 3.1 times more of up regulated and 2.7 times more down regulated differentially expressed genes than Alpowa. The top twenty GO terms were performed for biological processes of up and down regulated transcripts that are differentially expressed in response to MS and SS in comparison to the WW condition. The results suggest that transcription/translation and their associated regulation are the most active biological processes for differential gene expression in vegetative tissues from water limited soft white spring wheat plants. Another two greenhouse experiments for photosynthetic measurements (Long Drought Period vs. Acute Drought Period) were executed. A modulated fluorimeter has been used to compare the effects of long term and acute water limitations imposed on two cultivars in order to determine photochemical differences between soft white spring wheat cultivars, among stress intensities and over several sampling dates. Alpowa and Idaho showed significant differences in terms of Y (II) on May 12 only for all stress intensity treatments (WW, MS, SS for Alpowa and WW, SS for Idaho). The data from Y (II) and Y (NO) suggest that the effect observed on May 12th is not associated with stress imposition but may be the result of leaf maturation and pre-metabolic conversion to complete reproductive function. Both Fv/Fm and Y (II) measure the same response.

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INTRODUCTION

Wheat (Triticum aestivum. L) Is the most human nutritive cereal crop cultivated worldwide. More than 240 million hectares are cultivated, which is considered the largest number of acreage compared to any other crop (Curtis, 2002). Spring white soft wheat grows in the east of the United States, and it is less common than winter wheat. It continually grows from plantings in the spring season until harvesting in late summer or early fall. This wheat has less protein and more complex carbohydrates than hard wheat, and is used primarily for pancakes, cakes, waffles, pastries, and cookies. Wheat flour most closely approximates the density and taste of what's called “white flour”. Soft white spring wheat is high in dietary fiber, protein, manganese, and phosphorus minerals, which are necessary for the body to function suitably. Soft wheat has a high level of protein which is correlated to increased gluten, so those people with intolerance to gluten or celiac disease should avoid it (Overview Soft White Spring Wheat Flour, nd).

Since wheat is cultivated worldwide, environmental stress factors such as drought affect plant growth and cause an increasing threat to economically sustainable wheat agriculture. Drought limits field crop production more than any other environmental stress (Zhu, 2002). The yield loss due to drought stress has a significant impact on the supply of wheat to fulfill demand. Under wheat cultivation in developed countries

drought affects at minimum sixty million hectares, while under wheat cultivation in developing countries about 32% of the 99 million hectares (Rajaram, 2000).

The population of the world will increase approximately by 34 percent by 2050 to just over 10 billion people. This increase in population will require an increase in wheat production, and coupled to climate uncertainty, will be especially difficult (Godfray et al., 2010). Overall climate change is predicted to bring increased temperatures, and reduced water availability in wheat growing areas affecting plant resources, biodiversity and global food security (Ahuja, Vos, Bones, & Hall, 2010).

In spring wheat, the plant biomass is reduced when the plant is exposed to water stress (Farooq, Wahid, Kobayashi, Fujita, & Basra, 2009). Water stress has a significant effect on wheat production, for example, at anthesis, pollination is reduced resulting a reduction in grain yield due to fewer number of grains formed per spike (Ashraf, 1998). The results of Kheyrodin (2015) research showed that water deficit reduced plant height, number of grains per plant, spike weight, 100 grains weight, economic yield, biological yield and harvest index. The reduction in growth ratio is caused by the decrease in radiation change effectiveness when drought imposes at several growth stages, such as, grain filling, booting, tillering, earing, and anthesis, (Ashraf, 1998).

Physiologically speaking, drought stress at the vegetative stage leads to a significant decline in chlorophyll a&b, total chlorophyll, photosystem II efficiency, and results in a significant rise of carotenoid concentration in wheat leaves (Maxwell & Johnson, 2000). Drought stress also leads to smaller plant size, reduced leaf area, limited

leaf extension, decreased leaf count, increased root-to-shoot ratio, diminished photosynthesis and an increase in oxidative stress (Farooq, Wahid, Kobayashi, Fujita, & Basra, 2009). Drought stress may also affect plants in regards to root depth, hormonal composition, osmotic adjustment, the opening and closing of the stomata, inhibiting photosynthesis, cuticle thickness, and reduction in the chlorophyll content and a marked drop in transpiration (Nezhadahmadi, Prodhan, & Faruq, 2013).

Genetically, plants respond to water stress by altering the expression of many genes. Under drought stress, some of the proteins are over or under-expressed in response to the drought (Cattivelli et al., 2008). For instance, proline is an amino acid that is produced constitutively for proteins synthesis, and also, acts as a "molecular guardian" that is able to retain and support protein integrity improving the activities of different enzymes under water-stressed conditions. Also, several studies have described proline as an antioxidant suggesting its character as reactive oxygen species (ROS) scavenger and singlet oxygen quencher (Hayat et al., 2011). With drought tolerance tendencies, plants like wheat when responding to drought produce more proline. In plants, levels of proline have been found to increase by one hundred fold under drought stress (Liang, Zhang, Natarajan, & Becker, 2013). Plants, when exposed to drought stress produce many other compounds. In a study on winter wheat response to drought stress, researchers found that all plants responded to water stress by rising abscisic acid (ABA) concentration. As a stress hormone, ABA induces expression of specific DHNs (dehydrins) which may allow certain cultivars to adapt more successfully with the new establishment water-limited

environment (Vaseva, Grigorova, Simova, Demirevska & Feller, 2010). In addition, ethylene is a hormone that inhibit growth and is driven by environment (Taiz, and Zeiger, 2006). The response of grains to drought includes senescence in older leaf and loss of leaf function. Ethylene might support to advance leaf performance (Young, Meeley, and Gallie, 2004).

Water stress induces a variety of changes in gene expression. Drought-induced genes can be divided into three categories which include regulatory, signaling and functional genes. As an example, Hsdr4 (Hordeum spontaneous dehydration-responsive-unknown function), was identified in wild barley by its differential expression between tolerant and sensitive genotypes under controlled and water-stressed conditions (Wang et al., 2003). The Hsdr4 gene under drought stress was expressed at significantly higher levels which may suggest that Hsdr4 gene plays a role in plant tolerance to drought stress, and which may make it a candidates gene for the engineering or breeding of drought tolerance (Suprunova et al. 2007) in barley and other species. The HVA1 gene which encodes a member of the group 3 late embryogenesis abundant proteins (LEA) is another promising gene. This gene was introduced into spring wheat from barley where Bahieldin et al., 2007 found that the transgenic bread wheat with the gene HVA1 was more tolerant to drought stress. Kasirajan, Boomiraj, and Bansal (2013) have transferred to wheat a stress inducible transcription factor, AtDREB1A cloned from *Arabidopsis*, which showed a positive response to drought stress. The expression of AtDREB1A was correlated with an increased accumulation proline, increased relative water content and lower ion leakage

under drought stress (Bahieldin et al., 2007). Understanding genetic and physiological behavior of plants will contribute to more efficient and effective future crop improvement efforts.

RNA Sequencing

Sequencing technology was invented in the 1970s. The genome for bacteriophage phi X17 was the first complete genome that was sequenced in 1977 (genome size: 5,386 bases) via the Sanger method (Sanger and Coulson 1975; Sanger et al. 1977). The human genome project was completed in 2003 marking a significant milestone in genomic research (Chin et al., 2006). *Arabidopsis thaliana* (a model plant) is the first successfully sequenced plant genome using the Sanger-based approach. The International Wheat Genome Sequencing Consortium (IWGSC) released the first draft genome sequence of bread wheat genome (hexaploid) in July 2014 (International Wheat Genome Sequencing Consortium [IWGSC] 2014). The sequencing of crop genomes have already impacted the improvement of many crops (i.e., concerning critical agronomical traits, discovery of molecular markers, and transfer promising traits into other species) Progress in sequencing has accelerated to the point where the sequencing of whole or partial genomes is performed in a fraction of the time as previously performed. A major development has been the commercialization of what is referred to as next generation sequencing (Bentley et al. 2008) which allows for the sequencing of material at a much faster rate than previously using the Sanger method. Furthermore, these methods can

simultaneously sequence many samples from any type of genomes, including complex genomes. Lately, the progress of high-throughput RNA sequencing has providing a new technique for both quantifying and mapping transcriptome expression. This method is revolutionizing the way by which eukaryotic transcriptomes are analyzed. For instance, Jiang et al., (2017) investigated the transcription factor GmDREB1 in an investigation of salt stress by using RNA-sequence analysis. They compared transgenic plants with wild-type exposed to a range of salt condition. They found that GmDREB1 overexpression had a slight impact on gene expression under normal condition. Also, they discovered that GmDREB1 overexpression caused a transcriptional reprogramming of the salt response. Moreover, lately RNA sequencing was used to study terminal drought responses in (Triticum dicoccoides) wild wheat emmer genotypes contrasting in their yield stability and productivity under water deficit (Krugman et al., 2010). They identified 5,892 differentially regulated transcripts among drought tolerant and drought susceptible genotypes. They found 221 highly abundant transcripts uniquely expressed in the drought-resistant genotype, which make them potential candidates for drought resistance genes. Small transcript reads provide the advantage of giving a complete transcriptome profiling including small regulatory RNAs, which are small, non-coding pieces of RNA that add another level of control to the complex systems that regulate gene expression. (Wang, Gerstein & Snyder, 2009). Thus, RNA-seq methodology will contribute effectively to our understanding of the functional and regulatory changes that may occur in wheat plants exposed to drought stress.

Measurement of drought response by chlorophyll fluorescence

The second part of this research was focused on photosynthetic parameters revealed by chlorophyll fluorescence as an indicator of desiccation stress. Chlorophyll fluorescence is a measure of the light re-emitted by the chlorophyll molecules as they change from their excitatory state to a non-excitatory state. This technique is a commonly used measure by plant physiologists for photosynthetic performance analysis (Maxwell, & Johnson, 2000). The analysis method is based on the simple concept, that once the wheat leaves absorb the light, the light is either used for photosynthesis, converted to fluorescent light, or converted to heat or is reflected back into the atmosphere (Maxwell, & Johnson, 2000). Absorbed light energy is dissipated by the excited chlorophyll through photochemical conversion processes resulting in the emission of heat (which does not quench the photochemical reaction) or fluorescence radiation as a way of losing the excess energy (Maxwell, & Johnson, 2000). As such, any increase or change in one of the three processes will result in a change or a decrease in the other two indicators. Therefore, analyzing the chlorophyll fluorescence yield provides data on changes in photochemistry efficiency as well as the heat dissipation in the photosynthesis process (Burling et al., 2013).

Water stress affects wheat photosynthetic performance inducing a change in yield. In response to drought, wheat plants reduce chlorophyll, alter photosystem I efficiencies, and reduce the relative water content of leaves (Biesaga et al., 2014). However, plant response to water limitation and excess heat energy vary with the intensity of the drought,

the rate and duration of exposure to the drought, and the growth stage of the crop. Many of these effects can be monitored using chlorophyll fluorescence parameters. Fluorescence processes vary with the stage at which the water stress is introduced with maximal impacts evidenced when water stress exposure starts at the stem elongation or the flowering stages in wheat plants. Most investigators focus on the effects of acute stress where water is withheld over a period of days. While valuable there is little information concerning the effect of photosynthetic efficiency over the long term. The major uncertainty in this monitoring process involves a better understanding of the effects drought duration whither as an acute or long term drought, and we use fluorescence to monitor the physiological processes inherent in either case. Here we divide drought imposition into either acute short-term (14 days) compared to long-term (2 months) in our drought imposition.

In summary, the main purposes of this research are:

1 – Identifying the transcriptomic response of two cultivars of soft white spring wheat to two levels of drought stress using RNA sequencing technology.

2 – Characterize the effect of acute and long-term drought stress on fluorescence measurements of plants.

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CHAPTER II

REVIEW OF LITERATURE

The technology of sequencing was invented by Sanger at the late 1970s and is still in use today to sequence single DNA sequences. Sanger differs from NGS (next-generation sequencing) sequencing in that sequencing is performed one sequence at a time, while NGS sequencing is massively parallel, which allows millions of sequences to be sequenced in a single run. These new sequencing technologies allow for sequencing to proceed thousands of times faster and from 25 to 1,000 times less expensive than Sanger-based method. Examples of currently used next-generation sequencing technologies include: Roche 454 pyrosequencing, Illumina HiSeq platforms, Applied Biosystems SOLiD, NanoPore Minlon, and Pacific Biosciences of California (Pac Biosystem) (Brakmann, 2010).

RNA-Seq is used to examine the continually changing cellular transcriptome usually in response to treatment providing an accurate measurement of transcript levels at a given time. DNA or RNA can be sequence from one end to the other (single end) or start from the ends moving inward as an example of paired-end sequencing. Based on the DNA-sequencing technology, most reads are typically 30–400 bp in length (Wang &

Snyder, 2009; Huq et al., 2016). But using more recent third generation sequencing methodology (MinIon and PacBio) are able to produce long sequencing reads with maximum lengths approach 100,000 base- pairs and average fragment lengths of over 10,000 base-pairs.(Giordano et al., 2017). Specifically, RNA-Seq makes it easier to look at gene fusion, alternative gene, mutations, and changes in gene expression over time, spliced transcripts, post-transcriptional modifications, or differences in gene expression in different treatments or groups (Maher et al., 2009) and many other techniques.

Drought Response

Drought stress has negative effects at all stages of wheat growth and development. Reduce plant size, reduced leaf area, and early maturity are all aspects associated with drought response in wheat. In winter wheat, the total yield was reduced by 50% under drought stress (Reynolds, Dreccer, & Trethowan, 2006), whereas the efficiency of water usage defined as the amount of water absorbed per unit of biomass was enhanced (Hawes et al., 2000; Nezhadahmadi et al., 2013). During drought stress, roots and leaves respond by altering their morphology in ways that are clearly visible and indicative of a stress response (Cattivelli et al., 2008). Leaves curl due to reduction in turgor and drop in response to water stress as easy indicators of drought stress. Rucker et al. 1995 declared that drought could reduce leaf area and consequently limited the photosynthesis process. The extension and expansion of the leaf and the rate of growth of

roots during vegetative development is diminished during the drought resulting in a reduction in overall biomass yield (Bernier et al., 2008). Roots will continue to grow in order to explore new soil regions containing water, but the aerial parts of the plant will be limited to root supply of water. In wheat, the flag leaf length and leaf area increased whereas the flag leaf width did not change under water shortage. Drought stress has a particularly severe effect on growth and development at the reproductive and grain filling stages in wheat plants.

Drought stress strongly influences whole plant physiology. In terms of morphological responses under drought stress, wheat alters its morphological characteristics including changes in leaf shape, leaf area, leaf expansion, leaf size, senescence, waxiness, root length, and root density (Dencic, Kastori, Kobiljski, & Duggan, 2000). Physiological responses to drought include a decrease in the activity of photosynthesis, increase in oxidative stress, production of toxic metabolites, and alteration in the integrity of cell wall (Bray, 2002) to name a few. Furthermore, reduction of growth rates, turgor loss and osmotic adjustment, signal recognition of roots, reduction in leaf water potential, reduction of internal CO_2 concentration, and the decrease in stomatal conductance to CO_2 are other responses to drought (Nezhadahmadi, Prodhan, & Faruq, 2013). All these physiological responses have their activity based on certain molecular mechanisms that is accessible to investigation by the new sequencing strategies.

Molecular Response to drought

At the molecular level, the profiling of wheat transcriptome has been studied in response to a wide variety of stresses including drought. However, the gene expression and its regulation under these stresses and its impact on wheat production still needs to be better understood. There are many genes that are differentially expressed during drought stress. Results from RNA sequencing indicates that more than half of the 265 genes detected in spring wheat were involved in responding to water stress during drought (Nezhadahmadi, Prodhan, & Faruq, 2013). Furthermore, analysis of transcriptome by microarray in wheat seed caryopses under water stress or combined with heat, revealed that only 0.5 % of genes under investigation were changed in its expression under drought in durum wheat (Triticum turgidum subsp. durum) indicating the wide range of responses to drought in different parts of the plant (Liu, et al,. 2015).

In polyploid plants, homologous genes could help in providing a diverse set of alleles to enhance tolerance to a diverse set of stresses. Allohexaploid bread wheat is polyploid containing three distinct subgenomes (A, B and D), each subgenome containing a different set of alleles. Under stress the partitioning of gene expression among allelic variants plays an important role in the response to biotic or abiotic stresses (Li et al., 2012; Rizhsky et al., 2004; Johnson et al., 2014). Transcription factors are important for initiating and regulating stress responses. Nearly, 4,375 transcription factors in the wheat genome were identified and 1,328 were stress responsive (Liu et al.,

2015) indicating a robust degree of control. For functional genes, approximately 68.4 % of homologous genes were differentially expressed in response to drought stress (DS), heat stress (HS) or heat and drought combined (HD) (Liu et al., 2015). Below I present a few examples of those genes that were differentially expressed in response to drought stress. Some quantitative trait loci (QTLs) in spring wheat associated with drought and heat tolerance were detected, including dehydrins, glutathione S-transferase (GST), vacuolar acid invertase (Nezhadahmadi, Prodhan, & Faruq, 2013).

One of the major problems associated with drought is the stimulation of reactive oxygen species during drought stress. Reactive oxygen cause much of the damage due to stress in plants. The reduction of reactive oxygen species (ROS) requires antioxidant enzyme systems and redox metabolites (Foyer, & Noctor, 2005). Under normal conditions ROS is reduced to a minimal level but during drought stress energy transduction mechanisms become leaky and transfer pent-up energy to oxygen to form ROS usually in the form of superoxide, hydrogen peroxide, singlet oxygen and the very reactive hydroxide radicle. A certain amount of protection from oxidative damage can also be attributed to the higher osmotic regulators that include small molecules such as soluble sugar, and ions (K+) (Nezhadahmadi, Prodhan, & Faruq, 2013). Antioxidant enzymes are significant in reducing oxidative stress in response to drought and other forms of stress. Some significant enzymes include catalase (CAT), ascorbate peroxidase, superoxide dismutase (SOD) (APX), peroxidase (POD), redox metabolites such as

glutathione and ascorbic acid, and glutathione reductase (GR) (Chelikani, Fita & Loewen, 2004).

One common group of proteins associated with drought stress are the Late Embryogenesis Abundant proteins (LEA), which were first described in wheat and cotton (Cuming, 1999). These proteins are produced in abundance during seed development and are associated with the acquisition of drought tolerance in seeds, and pollen (Amara, et al,. 2014). LEA proteins are classified based on their expression and sequence characteristics. In general LEA proteins are categorized into six families depending on the sequence of their amino acid and corresponding mRNA homology (Hong-Bo, Zong-Suo, & Ming-An, 2005). Many LEA proteins are induced by osmotic stress, cold, abscisic acid, and some are even expressed constitutively (Welin, Olson, Nylander, & Palva, 1994). LEA proteins are thought to function as biomembranes protector under water limitation (Sasaki, Christov, Tsuda, & Imai, 2013).

Dehydrins genes (DHN) are multifamily water-soluble lipid-associating proteins, which accumulate during water deficit conditions. DHN genes are distributed in the cytosol, nucleus, and plasma membrane of plant cells. DHN genes are often associated with critical protective functions during low temperatures, dehydration, salt and osmotic stresses and they play significant roles in mitigating the effects of drought stress (Hanin, et al., 2011). DHN proteins are hydrophilic and thermostable and belong typically to LEA family of proteins mentioned above (Ramya, et al,. 2013). They are typically

characterized by what is termed the K segment protein sequence (EKKGIMDKIKEKLPG) (Malik, et al,. 2017). The mechanisms whereby these proteins protect against drought and other stresses is still open to speculation (Hanin, et al., 2011).

RNA sequencing indicated that HVA1 gene which is also one of the LEA genes (type 3) whose expression is influenced by abscisic acid expression is enhanced. This type of LEA protein has a 11 amino acids motif which are represented in 9 repeats (Bahieldin et al., 2007). On the whole plant level this gene plays an essential role protecting growth of the spring wheat during water stress periods. However, the exact mechanism related to HVA1 in transgenic plants conferring stress tolerance is unknown (Chen, et al., 2015).

The Response to Desiccation gene (RD) also impacts plant response to drought stress (Akpinar, Avsar, Lucas & Budak, 2012). This gene plays a vital role in ensuring that other regulatory genes are activated in response to the water stress. It also enables proteins that are responsible for the protection of the cell against the effects of drought (Akpinar, Avsar, Lucas & Budak, 2012). This gene is divided into two major groups. The first parts includes proteins which directly protect cells from stresses, and the second part includes expression of regulatory gene and signal transduction during the crops' reaction to stress (Nezhadahmadi, Prodhan, & Faruq, 2013).

In rice, drought and salt tolerance genes (DST) have been identified (Huang et al., 2009). In this study they characterized and cloned DST an earlier unknown as factor of

zinc finger transcription. DST controls stomatal closure negatively by straight variation of genes related to H_2O_2 homeostasis. They found that loss of function approaches have revealed that the DST gene decreases stomatal density and enhances stomatal closure and subsequently improve drought and salt tolerance (Huang et al., 2009) and likely water use efficiency. In addition, information about drought-responsive genes is still limited, and their function has not been determined.

DREB2A, drought responsive element binding, are transcription factors involved in stimulating drought-responsive gene expression. They interacts specifically with cis acting desiccation responsive element involved in cold and drought stress-responsive gene expression (Sakuma et al., 2006). Some study suggested that for activation implying DREB2A needs posttranslational modification that, in normal growth conditions, DREB2A does not activate downstream drought responsive genes. They also helps in stabilizing proteins in the nucleus, which are essential for protein activation (Sakuma et al., 2006). Polyamines (PA) are present in almost all living organisms, and are a group of complex aliphatic nitrogen structures. PAs play significant function in many physiological processes, such as respond to environmental stresses and cell growth and development (Gill, & Tuteja, 2010). PAs are known for their cell wall stabilizing abilities and membrane, anti-stress effects, and anti-senescence effects due to their antioxidant properties and acid neutralizing abilities (Gill, & Tuteja, 2010). Under water stress

polyamines increase the growth of the plant during the vegetative stage (Bouchereau, Aziz, Larher, & Martin-Tanguy, 1999).

Other genes that respond to the water stress in wheat include Vacuolar H (+) translocating pyrophosphatase (V-PPase) which functions to transfer metabolites and ions across the vacular membrane, and is a crucial enzyme relative to general cell homeostasis and detoxification, which increase plant growth under abiotic stress. V-PPase genes, TaVP3, TaVP2, and TaVP1, were also identified in wheat. Their results showed that the V-PPase genes were regulated differentially in wheat in response to drought and salt stresses (Wang et al., 2009). How this gene works in association with the vacuole and drought stress is still a matter of intense research.

Guanine nucleotide binding proteins which known as G proteins, are a proteins family that act as molecular transference inside plant cells, and involved in passing signals from a variety of stimuli outside a cell to the nucleus. G proteins are considered as one of the most significant cells signaling cascades proteins known. Proteomics studies discovered that G protein subunits (alpha and beta) were dramatically increased in leaves under drought (Wang et al., 2016). In rice the alpha subunit of the G protein positively plays a role in the regulation of desiccation stress (Wang. et al., 2016). In contrast another study in Arabidopsis found that beta subunit may play a negative role in regulation of drought stress (Xu. et al., 2015).

In transgenic wheat lines (Triticum aestivum L.), the betaine aldehyde dehydrogenase (BADH) gene exhibited an overexpression of glycine betaine under salt stress. Glycinebetaine enhanced the tolerance directly via promoting antioxidant activity, and by the over accumulation of osmolytes, such as soluble sugar, soluble protein, and free proline in order to protect the plants from ion toxicity. Also, glycinebetaine can improve salt tolerance of transgenic wheat plants by regulating osmotic adjustment, scavenging reactive oxygen species (ROS), and regulating ion homeostasis (Liang, et al ,. 2009).

Ribulose bisphosphate carboxylase (Rubisco) is one of the most studied and the most abundant proteins in the world. The enzyme is located in the stroma of chloroplasts. The precise function for this enzyme is in its role in carbon fixation of CO_2 into organic compounds. Severe desiccation is known to lower the amounts of Rubisco in soybean (Majumdar et al., 1991). It has been found under drought stress during anthesis that total Rubisco activity in the flag leaves was reduced by 12%. This decrease was combined with a decline in both chlorophyll and total soluble protein (Holaday. et al., 1992). Kumar, & Singh (2009) in their study about water stress on Rubisco activity in wheat, found that maximum Rubisco activity decreased sharply under severe drought stress. They suggested that the decline in photosynthesis under severe dehydration may be due to decline in Rubisco specific activity rather than the amount of the enzyme, because the supply of CO_2 to Rubisco under drought stress may be limited by stomatal closure. Also,

they found that dehydration did not reduce the amount of Rubisco protein but reduced the total Rubisco activity and the initial. (Kumar, & Singh, 2009). Increasing duration and severity of drought stress decreased total protein content and Rubisco activity in wheat (Kicheva, et al., 1994).

Drought and Other stresses

Drought stress often occurs in combination with heat stress. Tolerance to Heat, drought and their combination were studied in Tobacco, Arabidopsis, Sorghum (Rizhsky et al., 2004). In Arabidopsis, about half of differentially expressed genes under heat or drought stress are related to genes encoding HSPs (heat shock proteins), lipid biosynthesis enzymes, starch, proteases, degrading enzymes. The expression profiles in response to heat and drought combination may differ significantly from those expressed under individual stress treatments. Catalase, dehydrin, oxidase, glycolate respond to drought but not heat. While, Ascorbate peroxidase and thioredoxin peroxidase, respond to heat only (Rizhsky et al., 2004).

Drought and salt stresses are another combination of stressors that affect plant growth in additive ways. According to Munns, (2002) those plants that are exposed to both salt and drought stresses are less viable compared to those that exposed to the drought and salt separately. Drought and salt stress signaling can be splitted into three functional groups: detoxification signaling to repair and control stress damages, osmotic

and ionic stress signal for the restoration of homeostasis of the cell under stress situation, and signaling to regulate cell division and cell extension to levels appropriate for the particular stress situation (Zhu, 2002). The common of drought and salt induced genes seem to perform in damage repair, including a large number of detoxification enzymes, LEA/dehydrin-type genes, osmolyte biosynthesis genes, and ubiquitination-related enzymes, chaperones, and proteases (Zhu, 2001).

RNA seq methodology

The concepts of RNA sequencing have found an important application in analyzing the molecular response of plants to drought stress. RNA sequencing to detect genes that respond to drought stress in spring wheat has also been used to generate drought-tolerant varieties (Poersch, et al,. 2016). RNA sequencing through differentially expressed genes under water stress conditions has played an essential role in providing more information about drought coping mechanism for drought in wheat (Hassan et al., 2015).

The RNA sequencing involves various steps that have to be carried out in order for the process to be successful. One of the first steps in the RNA-seq experiment is in the extraction of RNA from tissue. This is often performed using commercial kits (Kukurba, & Montgomery, 2015) or a phenol chloroform partitioning method such as with the commercial Triazol reagent (Macedo, & Ferreira, 2014). Purified RNA is then

quantitated using spectrophotometric method typically using modern Nano drop spectrophotometers, which are able to determine the average concentrations and the purity of the RNA sample at the same time. (Desjardins, & Conklin, 2010). Once the total amount of RNA is determined then Illumina library development must take place. Illumina library preparation or synthesis of cDNA is also referred to as reverse transcription. There are four steps to prepare RNA for next generation sequencing analysis: fragment the target sequences to the desired length, convert the target sequence to double stranded DNA, attach adapters to the ends of target fragments, and finally determine the quantity of the final library product for sequencing (Head, et al,. 2014). Once complete the DNA is ready for Illumina sequencing.

Bioinformatics process

Once sequence information is obtained it is necessary process it through a number of bioinformatics steps to filter out poor sequences, align it with a reference genome, and determine the number of statistically relevant differentially expressed genes before it can be analyzed effectively. This process for most investigators relies on software that has been specifically designed for this purpose.

Quality filtering of sequences is one of the important aspects that is carried out during the bioinformatics process which removes low-quality sequences (McCarthy, Chen & Smyth, 2012). The Q30 threshold, which is a measure of the PHRED quality

score provides comprehensive information about the reliability of the sequencing information during base calling. After sequencing and filtering sequences are aligned to a reference database for a given species including model organisms. The best database is from the species from which the sequences were obtained. However given the lack of annotation in the wheat sequenced database it may be better to choose a database from another species. The relationship between the sequences can also be obtained through the analysis of the sequence alignment (Ingolia, Brar, Rouskin, McGeachy & Weissman, 2012). A number of alignment programs have been used in the past including: FM-index based aligner Bowtie, Bowtie 2, TopHat, SpliceMap (Lindner, & Friedel, 2012).

Hierarchical indexing for spliced alignment of transcripts (HISAT) is a rapid and sensitive program qualified for mapping RNA-seq reads from RNA sequencing experiment onto the reference genome. HISAT employs an indexing scheme based on the Ferragina-Manzini (FM) index and Burrows-Wheeler transform, using two types of indexes for alignment: a frequent local FM indexes for very fast expansion of alignments and a whole-genome FM index to confirm each alignment (Kim, Langmead, & Salzberg, 2014). HISAT is the fastest program presently available, nearly 12 times faster than GSNAP and 50 times faster than TopHat2, with the same or better precision than any other method and requires less computational memory. HISAT is obtainable as free, open source software from http://www.ccb.jhu.edu/software/hisat.

The Sequence Alignment Map (SAM) format is a general alignment format supporting long and short reads (up to 128 Mbp). SAM stores read alignments in contrast to reference sequences, created by different sequencing platforms. SAM is efficient in random access, compact in size, has a flexible style, and is the format in which alignments from the 1000 Genomes Project have been released (Li et al. 2009). SAMtools apparatuses includes numerous utilities for post processing alignments in the SAM format, like, accordingly affords worldwide tools for processing read alignments indexing, and variant caller alignment viewer (Li et al. 2009).

StringTie is open free source software tools, and is a highly efficient and a fast assembler of RNA sequencing alignments into potential transcripts. StringTie employs an optional *de novo* assembly step, as well as a novel network flow algorithm to assemble and quantify full length transcripts representing multiple splice variants for each gene locus (Pertea, Kim, Pertea, Leek, & Salzberg, 2016). StringTie input can include not only alignments of extended sequences that assembled from those reads, but also alignment of raw reads that used by other transcript assemblers. StringTie's output can be processed by specialized programs such as DESeq2, EdgeR, or other software like Ballgown, Cuffdiff, in order to identify differentially expressed genes between experiments (Pertea, Kim, Pertea, Leek, & Salzberg, 2016).

Ballgown is open free source software tools. Ballgown is found under the Bioconductor package working under R program. Ballgown is used to estimate

differential expression of transcripts, genes, or exons from RNA sequencing experiments. Ballgown is designed to work with the popular transcript assembly software (Frazee et al., 2014). Ballgown handles studies with continuous, allows statistical analysis at the transcript level for a wide variety of experimental designs, and permits adjustment for confounders. Ballgown offers better statistical significance estimates with comparison to the other differential expression tools (Frazee et al., 2014).

Empirical Analysis of Digital Gene Expression Data in R (edgeR) is a Bioconductor software package working under R. EdgeR is used to examine differential expression of replicated count data. This method can be used also with minimal levels of replicates. The software can be used with sequencing or proteomic data (Robinson, McCarthy, & Smyth, 2010).

The Universal Protein Resource (UniProt) is a free available comprehensive resource database for protein functional information and sequencing containing numerous entries being from a variety of genome sequencing projects. It covers a large quantity of information about the biological function of proteins (UniProt, 2017).

Chlorophyll fluorescence

Chlorophyll fluorescence technique has become widespread in plant physiology and ecophysiology studies (Maxwell, & Johnson, 2000). To measure chlorophyll

fluorescence in my research, I used a modulated' measuring system called pulses amplitude modulation fluorimeter (Junior PAM, Walz). In such systems, the light source is modulated to measure fluorescence (switch on and off at high occurrence) and the detector is adjusted to sense only excited fluorescence by the determining light. Hence, in the presence of background illumination, the relative yield of fluorescence can be measured, and most significantly, in the existence of full sunlight in the field. According to the time of measurements, chlorophyll fluorescence parameters are divided into two groups, dark-adapted plants parameters as Fv/Fm, and light adapted plants parameters as NPQ, Fq/Fv, Fq/Fm. Each one of these parameters has a special equation to estimate its value (Junior PAM, Walz). The absorption of light into wheat leaves chemically excites (moves electrons to a higher energy state) the chlorophyll reaction centers within plant leaves (Maxwell & Johnson, 2000). As such, the excited chlorophyll molecules are unstable and cause energy dissipation through several alternate pathways. The absorbed energy molecules may be passed on to a nearby acceptor molecule, which would culminate in photosynthetic electron transport. Alternatively, it may also be released in the form of heat or may eventually be emitted as a lower energy photon (having a higher wavelength) (Maxwell & Johnson, 2000). This is referred to as fluorescence, and it emanates primarily from the photosystem II process. The measurement of chlorophyll fluorescence in the wheat crop samples is done on both uncovered wheat crop as well as those leaves covered with clips. The leaves should be covered or kept in the darkroom for half an hour prior to the fluorescence measurement, and transients are induced with a red

light of 3000 μmol m-2s-1 emitted by an array of light emitting diodes (Almeselmani et al., 2012). Alternatively, measurements under dark adapted conditions can take place just prior to sunrise.

The light is focused on the samples' surface with the aim of measuring the maximum quantum yield of PS II (Fv/Fm) for all the samples (Almeselmani et al., 2012). The PAM fluorometer utilizes a photochemistry analysis method exploring a crop's saturated pulses measuring the different light signals via a fiber optic probe (Murchie, & Lawson, 2013). The modulated beam emitted by the PAM fluorometer is inadequate in stimulating photosynthesis but enhances the fluorescence signal which is modulated into a measuring beam and is measured as it transits through the various filters and electronic devices. This differs greatly in both light exposed, and light deprived tissues hence the need for any researcher to use both lighted and non-lighted environments during their studies (Maxwell & Johnson, 2000).

Wheat Growth and Development under Drought Stress

With changing global climatic conditions, the polygenic stress that accompanies drought heavily impacts crop survival, performance and productivity. Interestingly global warming will result in hotter and drier seasons with observed increases in arid and semi-arid zones. This necessitates a deeper analysis and studies into drought tolerance and survival traits development among wheat cultivars globally (Burling et al., 2013). Drought remains a challenge for agricultural researchers as well as plant breeders, and it is estimated that by 2025 approximately 1.8 billion individuals will experience absolute

water shortage meaning that about 65% of the global population will live under water-stressed surroundings (Nezhadahmadi, Prodhan, & Faruq, 2013). Water stress tolerance is thus an important but complicated parameter among wheat crops and can be evaluated by two means; the plant's ability to avoid drought stress and its capacity to tolerate dehydration (Verslues et al., 2006).

Wheat crops are often grown in arid and semi-arid agricultural zones. Drought is often a major problem in these wheat production zones. Wheat crops exposed to drought in their vegetative stage portray marked in changes in their Chlorophyll fluorescence that are important indicators of the effects of drought on the plant. Desiccation stress causes significant decreases physiological parameters at the vegetative stage such as chlorophyll a, b, and total chlorophyll, and results in a considerable rise of carotenoid concentration in wheat leaves (Maxwell & Johnson, 2000).

Tolerant wheat strains portray insignificant changes in the maximum quantum yield of PSII (Fv/Fm) when exposed to drought stress (Paknejad et al., 2007). As literature indicates, photosynthetic changes that rely on chlorophyll availability and its quantity within in wheat cultivars play an important role in yield formation (Paknejad et al., 2007). Drought stress in wheat crops at their vegetative stage leads to smaller plant size, reduced leaf area, limited leaf extension, decreased leaf count (therefore lesser chlorophyll florescence capacity), increased root-to-shoot ratio, diminishing photosynthesis and an increase in oxidative stress (Mafakheri et al., 2010). Water stress also causes a decrease in stomatal conductance and cell wall integrity changes, as well an

adaptive attempt to develop water use efficiency, and there is an increase in anti-oxidative enzyme secretion, a reduction in rubisco and an overall reduction in chlorophyll content. Modern studies into the photosynthetic performance of different plants exposed to varying field conditions provide quantitative and qualitative information about photosynthetic activity in the chloroplast. It seems that studies lack of this information appear inadequate if they lack data of fluorescence (Rohacek, & Bartak, 1999).

The correlation between water stress and the alterations in secondary fluorescence induction kinetics appears weaker in the first phase of exposing wheat plants to the water stress in their vegetative stage. In a 2013 study, the authors observed that the most robust fluorescence index changes related to drought stress were directly associated with UV-excited blue-to-far-red fluorescence ratios. Leaf shrinkage, reduction in the chlorophyll contents, and increases in the flavone in the epidermis in the wheat crops during drought stress is also associated with a reduction in the UV-induced far-red fluorescence (Burling et al., 2013). Drought resistance among wheat crops explores the root depth, the ability to reasonably utilize the available amount of water and physiological changes in a plant's lifestyle directed at adjusting to using the available water in a more efficient way. Dehydration tolerance involves every plant's potential to dehydrate partially without dying off, and therefore re-grow and prosper when the water is available again (Verslues et al., 2006). These adaptations are crucial to developing newer and improved methods of increasing crop stress tolerance. Several factors including crop genotype, growth stage, the severity of water deprivation and duration of deprivation, physiological processes of

growth, genetic expression patterns, respiratory activity patterns, photosynthetic machinery activity, and environmental factors affect wheat crop's response to drought stress (Nezhadahmadi, Prodhan, & Faruq, 2013).

Drought stress may have various impacts on wheat plant's traits expression and therefore the observation of wheat crop's responses during water stress. Drought stress may also affect plants in regards to osmotic adjustment, hormonal composition, root depth and expression, the opening and closing of the stomata, cuticle thickness, inhibiting photosynthesis, and decrease in the chlorophyll content or marked a reduction in transpiration (Nezhadahmadi, Prodhan, & Faruq, 2013). It may also result in pollen sterility, grain loss, increased abscission in the anthers and spikes among the susceptible strains of a wheat crop. Drought also markedly results in an increase in oxidative stress with the increases in cell wall integrity alterations and the rise in metabolites production of toxins likely to causes wheat cell apoptosis. (Ji, et al., 2010).

Analysis previous studies, researchers have shown prominent effects of drought on wheat crop in various environments. In a study on vegetative growth stage wheat plant's response to water stress, the researchers observed a 54%, reduction in grain yield. They also noted a 45% reduction in the biomass content as well as an 18 % reduction in harvest index as a plant response to water stress. The same study revealed that the wheat crop had a 36% reduction in the number of grains per spike with no significant effects on the weight of the grain (Ardalani et al., 2015).

In another study on Durum spp of wheat in Syria, a relatively arid country, the researchers found that drought was the most influential environmental stress for wheat farmers. It severely impaired plant growth, development and limited the wheat production capacity as well as crop performance (Almeselmani et al., 2015).Through the comparisons in the study, the researchers realized that the effects of drought on all plant traits were eventually transferred to their yielding power and performance. Even though tolerant wheat species showed enhanced physiological performance and had a better yield to their ability to remain stable and physiologically efficient in drought stress, the researchers called for further research into ways of improvement from their analysis of chlorophyll fluorescence studies. The authors recommended genetic cross-breeding between the different subspecies (2-10 in the study) of wheat with Line1, a strain which was evidenced to have better tolerance as seen in its remarkable scores after the chlorophyll fluorescence examination (Almeselmani et al., 2012).

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CHAPTER III

MOLECULAR INDICATORS OF DROUGHT STRESS IN SOFT WHITE SPRING WHEAT AT THE VEGETATIVE STAGE DEVELOPMENT

Introduction

According to the FAO organization about 200 million hectares of land are used for wheat farming worldwide, and about 21% of food consumed by humans comes from wheat (Triticum aestivum) agriculture. With a predicted rise in world population to reach 10 billion by 2050, the demand for wheat is expecting to increase by 60%. To meet this challenge agriculturalists must increase wheat yield per unit of land area. Currently, the wheat yield have been increasing at a rate of 1% per year, but estimates from the United Nations Food and Agricultural Organization indicates to meet the increased demand of a rising population percentage yield increases must expand at a per annum basis of 1.6% (available from http://www.fao.org). Given that wheat is often grown in drought prone areas, our ability to increase productivity will heavily depend on the development of drought resistant cultivars.

Plants naturally are exposed to many abiotic and biotic stresses continuously. Drought is one of the central abiotic stress that threatens wheat productivity. Drought stress leads to reduced leaf area, limited leaf extension, decreased leaf count, increased

root-to-shoot ratio, diminished photosynthesis, an increase in oxidative stress, and eventually productivity reduction (Farooq et al., 2009). As a solution both modern genetics and traditional breeding efforts methods can be used to improve drought tolerance of crop plants (Passioura, 2012).

To improve wheat response to drought stress it is important to understand both the response to drought and how wheat plants can adapt to water limiting conditions. A wide variety of changes affecting cellular metabolism are associated with dehydration stress (Anjum et al., 2011). One of the major cellular events that occurs during drought includes changes in gene expression resulting in controlling of all the biochemical and physiological responses to the stress. Many genes that are involved specifically in stress response have been identified: including a specific class of proteins known as Late Embryogenesis Abundant proteins (LEA) which accumulate under drought stressed conditions, and dehydrins which are a family of proteins that are expressed after exposing to drought stress (Zhu, Choi, Fenton, & Close, 2000). Some of these drought responsive genes may actually function in increasing wheat resistance to drought conditions. Comprehension the molecular and biochemical responses to drought is necessary for a holistic of observation of plant resistance mechanisms under water limitation conditions.

RNA sequencing is a modern technology enabling researchers to monitor changes in transcription that are associated with a given stress with reasonable cost and for many plant species. With regard to next-generation sequencing technology and its advances,

RNA sequencing (RNA-Seq) has been widely used in plant breeding, especially in those lacking complete genomic information. Moreover, results coming from RNA-Seq may facilitate the identification of new proteins and identification of their functional roles in interested traits (Wang et al., 2009). Next generation sequencing commercially adapted technologies most often used include the Illumina and Roche/454 technologies (Berkman et al. 2012; Elshire et al. 2011; Poland et al. 2012; Mwadzingeni et al. 2016). RNA-Seq has been used successfully in wheat to identify differentially expressed genes under drought conditions (Duan, Xia, Zhao, Jia, & Kong, 2012).

There are several types of wheat produced each of which are used for a specific product. The most common form of wheat are the hard spring and winter wheat that are used for bread making. Another type of wheat less commonly investigated by genomic technologies but nevertheless important for wheat agriculture are the soft white wheat that are typically used for producing quick breads products, pancakes, muffins, and pastries. Most work to date on drought stress has been conducted on the hard spring and winter wheat with very little being focused on the soft white wheat. Accordingly, the objective of this study, is to identify the transcriptomic response of two varieties of soft white spring wheat to two levels of drought stress using RNA sequencing technology.

Materials and Methods

The purpose of this experiment is to study genes expression changes in two wheat varieties under drought circumstances. The experiment was planned in complete randomized design two factor ANOVA with the factors being cultivar and water stress intensity treatment levels.

Wheat Growth and Development

Experiments for RNA sequencing were executed at the greenhouse facility in Stillwater Oklahoma during the spring season of 2016. Two spring wheat cultivars (*Triticum aestivum L.*) Alpowa and Idaho were used in this study. Alpowa is a drought resistant soft white spring wheat that was developed by Washington State University in cooperation with the Idaho and Oregon Agricultural Extension Service and the local offices of the USDA-ARS and released in 1994. Alpowa is widely grown in the western United States, and was the leading spring wheat cultivar in Washington State in years 2003, 2004 and 2005 (Lin & Chen, 2007). Alpowa was recommended by the Idaho State breeding program based on its reputed ability to tolerate drought stress. The cultivar Idaho was developed by the Idaho Agricultural Experiment Station, and the University of Idaho breeding program (Montana State University, 2015), and is considered to be more drought susceptible than Alpowa. Idaho is currently used as a check to monitor breeding progress.

Wheat was planted in Treepots (10 cm in width and breadth, and 36 cm in depth (Stuewe and Sons Inc, OR). All pots were sanitized with 70% ethanol, air dried and rinsed with water prior to filling with soil. The soil was mixed in a large scale cement mixer and nitrogen fertilizer in the form of powdered ammonium nitrate was added to a recommended field rate of 67 Kg/ha. After mixing, the soil was evenly distributed across all pots and the process repeated until all pots contain 3.00 Kg of soil. The soil was a Kirtland B sandy loam soil obtained from the Stillwater Agricultural Extension Farm. Initially, three wheat seeds were planted in each pot to the depth of two inches and watered. Upon germination, plants were thinned to one plant per pot. The pots were hand weeded and monitored for insects and when necessary sprayed with Immunox for powdery mildew infestation, and Neem oil for aphid control. Daily, maximum, minimum, average and current air temperature reading were recorded using digital thermometer TMD-52 by Amprobe (Amprobe Test Tools, Everett, WA).

Water Limitation Treatments

Water limitation was imposed on pots containing the two wheat varieties. This was performed by watering plants when the control well-watered plants needed to be watered. Two soil tensiometers per treatment were inserted into randomly assigned control pots in order to measure soil water potential, a direct measure of the availability of water to the plant based on manufacturer recommendation (Irrometer Co. Inc., Riverside, CA). When the control pot readings approached the recommended readings for

watering wheat (50 centibars) water was provided for all pots including the well-watered control (WW, 100%, 236 ml), moderate stressed (MS, 50%, 118 ml), severe stressed (SS, 25%, 59 ml). To avoid acute stress responses the pots were monitored daily, and the average water potential reading was computed. With two varieties, three water treatment levels, and nine replicate plants per treatment the total experiment consisted of 54 pots. For the long term experiment water limitation treatments commenced three weeks after planting approximately when the wheat was at the two-leaf stage of development (Feekes 1).

Plant harvesting

Plants were harvested at Feeke's 10.3 growth stage (boot stage prior to anthesis, (harvesting date 5/17/2016). Each plant was harvested separately by cutting the shoot with scissors at the soil surface, and weighing total shoot weight. The harvested tissues was wrapped in aluminum foil, and dipped in liquid nitrogen, placed in a labeled plastic bag, and then kept frozen at - 4 C^o freezer. After harvest all plant materials were transferred to the main -80 C^o freezer.

RNA isolation, quantification and qualification

Total RNA was isolated from leaves using the TRIzol® RNA Reagent (Invitrogen, Carlsbad, CA, USA). Samples were removed from -80 C freezer and placed on ice at all times. The total sample was ground to a fine powder in liquid nitrogen.

Approximately, 100 mg of the powdered tissue were placed into a chilled ground glass homogenizer and 1 ml of Trizol reagent were added to the homogenizer and ground until all fragments were visibly pulverized. Extracts were poured into a RNAse free 2 ml tube and Centrifuged at 11,000 g at 4 C^o for 10 minutes. The fatty layer was removed from the top of the aqueous layer using a pipette followed by transferring one half of the aqueous phase to a new tube. The extract was incubated for 5 minutes at room temperature and one volume of chloroform was added to the tube followed by vigorous shaking by hand for 15 seconds, and then incubated at room temperature for 3 min. Samples were then centrifuged at 4 C^o at 11000 g for 15 minutes and one half of the upper phase carefully transferred to a new RNase free tube placed on ice. One volume of isopropyl alcohol was added, and the tube was centrifuged at 11000 g at 4 C^o for 15 minutes. The supernatant was poured off leaving the RNA pelleted to the bottom of the tube, and the RNA pellet was washed with 1 ml of 75% ethanol, then air dried for 5 minutes and resuspended in 30 µl of RNase free water. Samples were incubated in water bath at 60 C^o for 10 minutes to dissolve the pellet and then placed on ice. RNA quantification and quality determination was performed using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

RNA Sequencing. Illumina Library Development Illumina Sequencing

Frozen extracts were sent to the Oklahoma Medical Research Foundation (OMRF) for RNA-sequencing using their Illumina HiSeq 3000 instrument. Ribosomal RNA (rRNA) and other RNA species were removed using the RNA depletion procedure

(O'Neil, Glowatz, Schlumpberger, 2013). Most of what is left over after depletion is mRNA and short sequence total RNA. The RNA was converted cDNA and Illumina adaptors were attached. The Illumina library was sequenced using 150 base pair chemistry yielding nearly 300 bp of sequence information per transcript.

According to Oklahoma Medical Research Foundation prior to RNA-seq analysis quality control measures were implemented. Concentration of RNA was ascertained via a Thermo Fisher Qubit fluorometer. Overall quality of RNA was verified using an Agilent Tapestation instrument. Following initial QC steps sequencing libraries were generated using the Illumina TruSeq Stranded mRNA library prep kit according to the manufacturer's protocol. Briefly, mature mRNA was enriched via pull down beads coated with oligo-dT homopolymers. The mRNA molecules were then chemically fragmented and the first strand of cDNA was generated using random primers. Following RNase digestion the second strand of cDNA was generated replacing dTTP in the reaction mix with dUTP. Double stranded cDNA then underwent adenylation of 3' ends following ligation of Illumina-specific adapter sequences. Subsequent PCR enrichment of ligated products further selected for those strands not incorporating dUTP, leading to strand-specific sequencing libraries. Final libraries for each sample were assayed on the Agilent Tapestation for appropriate size and quantity. These libraries were then pooled in equimolar amounts as ascertained via fluorometric analyses. Final pools were absolutely quantified using qPCR on a Roche LightCycler 480 instrument with Kapa Biosystems Illumina Library Quantification reagents. Sequencing was performed on an Illumina

Hiseq 3000 instrument with paired-end 150bp reads. Samples were sequenced to an overall depth of 50 million reads per sample (OMRF).

Sequence analysis and alignment

Bioinformatics analysis of the sequence data was performed by the High-Performance Computing Center (HPCC at OSU) by Dr. Brian Couger. The sequence data was downloaded in Fastq format from the OMRF computers. At first, quality control was achieved by using FastQC using a stand-alone application providing a quick analysis of the reliability of the sequence reads (Andrews, 2010). All Fastq files were screened to the level of Q30. Hisat2 was used to align the genes to the wheat genome (Kim et al., 2015) using the International Wheat Genome Sequencing Consortium (IWGSC) 2014 release for hexaploid bread wheat (International Wheat Genome Sequencing Consortium [IWGSC] 2014). The reference genome in Gene transfer format (GTF annotation) were downloaded and prepared for quantification according to the Hisat2 protocol (Pertea et al., 2016). SAM alignment (Li et al. 2009) file conversion, sorting, and preparation was achieved using the Samtools program (Li, 2011). Quantitative predictions of transcript FPKM levels were produced using the RNA seq software Stringtie. Statistical comparison of all transcripts was achieved using the R package Ballgown (Pertea et al., 2016). Transcripts which showed significant differential expression were annotated using the UniProt database (UniProt, 2017).

Results and Discussion

In order to identify differentially expressed genes related to drought stress in wheat under vegetative/reproductive development, high throughput RNA-sequencing was performed on two wheat cultivars. Two wheat varieties (Alpowa and Idaho) and three levels of water availability (100%, 50% and 25%) were applied in this research to simulate water limited conditions. Both cultivars are genetically distinct having no common ancestors (GRIS, Genetic Resource Information Systems for Wheat and Triticale, http://wheatpedigree.net/about). The RNA-seq datasets from Alpowa contained 690,857 reads from WW control, 663,526 reads from MS, 652,705 reads from SS, whereas the datasets from Idaho were 523,643 reads under WW control, 485,527 reads under MS and 489,436 reads under SS. To understand how the two cultivars responded we identified candidate differentially expressed genes between WW control and stress treated samples (stress intensity comparison, MS-Alpowa, SS-Alpowa, MS-Idaho, SS-Idaho).

In our stress intensity comparisons, up and down regulated differentially-expressed genes were identified under MS and SS conditions compared to a WW control Table 1. There were 12% more up regulated than downregulated genes in Alpowa, while in Idaho the numbers were evenly balanced with only 0.5% more upregulated genes than down regulated genes. Thus up regulation was more active than down regulation in Alpowa. This greater number of up regulated compared to down regulated genes has been

shown before in Bortolon et al, 2016 in response to drought. Overall, Idaho showed 2.1 times more up regulated and 2.3 times more down regulated differentially expressed genes than Alpowa. Thus Idaho appears to be much more transcriptionally active under drought conditions. In contrast, April et al, 2013 in their study on drought and heat in two durum wheat cultivars showed 7,532 differentially expressed genes in Ofanto resistant and 4,212 in Cappelli susceptible. In Alpowa, the number of up and down regulated differentially expressed genes were similar under SS and MS conditions. In Idaho, there were 3.2 times more up regulated genes under MS condition compared to SS, and 1.7 times more down regulated genes in SS than MS conditions. Apparently, Idaho has a much stronger down regulation of gene expression under SS than MS. A minority of genes were common to both MS and SS conditions. Of all the differentially expressed genes only 15% and 21% were common among the two conditions in Alpowa and Idaho, respectively. This means that the transcriptional response to water limitation was very distinct between MS compared to SS in terms of numbers of differentially expressed genes for a given treatment.

Table 1. Up and Down regulated differentially expressed genes in Alpowa and Idaho cultivars as a response to two conditions (Alpowa and Idaho moderate-stress (MS), Alpowa and Idaho severe-stress (SS) against Alpowa and Idaho well-watered (WW) as a control.

Cultivar	Regulation	MS	MS+SS	SS	Total
Alpowa	up	2277	1014	2095	5386
Alpowa	down	2024	548	2223	4795
Idaho	up	1879	3218	5951	11048
Idaho	down	3491	1448	6056	10995

The biological era at the genome-scale has seen accumulated large amounts of biological sequencing data that needs to be put into a functional context. Sequencing genomics made it clear that a large proportion of genes shared by all eukaryotes specify core biological functions. With this in mind a Gene Ontology database whose objective is to apply a planned dynamic and hierarchal vocabulary to all functional roles for a given gene or protein was developed. Gene Ontology provides a key conceptualizations of knowledge domains that simplifies the communication between researchers in terms of gene function. For this purpose, three independent ontologies spanning a wide range of functional attributes have been constructed on the World-Wide Web (http://www.geneontology.org) including: biological process, molecular function and cellular component (GO-EBI and EMBL-EBI 2004). Here in this study we focus on the biological processes Gene Ontology.

Gene Ontology (GO) analysis in this work was performed for the category biological process providing a view into the functional relationships under water limiting conditions. Categories of Gene Ontology (GO) are widely used in this technique to reduce complexity and highlight biological processes in studies of genome-wide expression based on RNA-seq data. The analysis of GO enrichment can facilitates the organization of data from fully or novel annotated genomes to those genomes with limited annotation (Young et al. 2010; Glass &Girvan 20140). GO analysis in wheat is somewhat incomplete give the limited status of the functional annotations in the wheat

genome. The top twenty GO terms for biological processes for up and down regulated transcripts that are differentially expressed in response to MS and SS in comparison to the WW condition are presented in Figure 1. The most frequent GO terms encountered across cultivars and stress intensities involved the up and down regulation of transcription. Over a thousand differentially expressed genes with GO terms associated with DNA-templated regulation of transcription (GO:0006355) and DNA-templated transcription (GO:0006351) were found across stresses and cultivars. Up and down regulation of transcription appears to be more than twice as active in Alpowa compared to Idaho for both terms. Drought stress appears to affect the regulation of transcription and transcription itself more than any other function, and more in Alpowa than Idaho. The changes in transcription are also mirrored in terms of other DNA related processes including DNA integration (GO: 0015074) DNA repair (GO:0006281) and translation (GO:0006412) mechanisms all actively initiated during drought stress. Protein production mechanisms including translation and protein folding (GO:0006557) and glycosylation (GO:0006486 were also strongly enhanced. These transcriptional/translational activities are more upregulated than downregulated in Idaho, while in Alpowa they are more evenly balanced especially under MS compared to SS. One exception to this is DNA repair functions which under MS in Alpowa was more highly down regulated than up regulated. DNA integrative activities which often refer to the insertion of short DNA elements into the genome, such as in the case of transposition are particularly active under water limiting conditions. This activation of "jumping genes" may be part of a

mechanism associated with the generation of mutations under stress conditions in support evolutionary processes. The results suggest that transcription/translation and their associated regulation are the most active biological processes for differential gene expression in vegetative tissues from water limited soft white spring wheat plants.

The next major biological process affected by water limiting conditions were the metabolic associations (GO:0008152) including the child terms carbohydrate (GO:0005975), lipid metabolic process (GO:0006629) and those involved with protein catabolism: ubiquitin-dependent protein catabolic process (GO:0006511). This indicates that carbohydrate, lipid and protein catabolism are strongly affected by water limitation with metabolic processes being more affected than the others. Here again the ratio between up and down regulation was evenly balanced in Alpowa but highly favoring up regulation in Idaho and more pronounced under MS than SS. The only exception is with lipid metabolism in Alpowa under SS where down regulation was more favored than up regulation. Of all the metabolism child terms ubiquitin-dependent catabolism in Idaho showed the most pronounced nearly 16 fold upregulation compared to downregulation indicating a pronounced catabolic degradation of proteins in this cultivar.

Transport processes appear to be altered substantially during drought stress conditions. Biological processes including the general GO term Transport (GO:0006810), and child terms: transmembrane transport (GO:0055085), and vesicle-mediated transport (GO:0016192) all appear to be highly affected by water limitation with transmembrane

transport most affected. Here in Alpowa under MS the balance for intracellular and vesicle mediated transport appears to be more down regulated than upregulated while in Idaho intracellular transport is 21 fold more upregulated compared to down regulated. Thus drought stress appears to alter specific transport mechanisms most likely associated with the golgi apparatus and inclusion of transmembrane proteins into membranes probably destined for the plasma membrane.

Response to stress is also highly affected by drought stress in both cultivars as suggested by the biological process data. In particular response to stress (GO:0006950) and a child terms oxidative stress (GO:0006979) cell redox homeostasis (GO:0006950) appear to be slightly more upregulated in Alpowa and Idaho under MS conditions than down regulated. Furthermore, there were three times as many differentially expressed genes associated with response to stress GO term than in Idaho in both MS and SS conditions. The same is generally true for the child terms as well indicating that Alpowa in general has a much more robust response to stress than Idaho which may be the reason why at least partially Alpowa is more tolerant than Idaho. Response to oxidative stress GO terms involve changes in state or activity of a cell or an organism as a result of oxidative stress, a state often resulting from exposure to high levels of reactive oxygen species, e.g. superoxide anions, hydrogen peroxide (H_2O_2), and hydroxyl radicals or as a result of a disturbance in organismal or cellular homeostasis induced by temperature, humidity, ionizing radiation. Oxidative stress response has been shown to be widely

associated with water limitations in wheat (Devi, Kaur, & Gupta, 2012) and other plants (Sharma, A. Dubey, & Pessarakli, 2012). Oxidative stress under abiotic stresses or senescence processes creates an imbalance in the redox status of plant cells (Das, Nutan, Singla & Pareek, 2015) leading to cell death. The oxidative stress GO: Term above could be considered to be co-occurring term with this particular GO Term. A significant associated GO terms found under cell redox homeostasis included glycerol ether metabolic processes (GO:0006662) and to a lesser degree cellular oxidant detoxification (GO:0098869). Glycerol ether lipid metabolism is often associated with lipid metabolism indicated above, possibly through the peroxisomes (Hajra, Datta, Ghosh, Horie, & Webber, 1986). In plants very little is known concerning the function of glycerol ether lipids, but in animal systems these compounds are known for their effect on immune function (Magnusson & Haraldsson, 2011). Exploratory investigation of genes associated with glycerol ether lipid metabolism under drought stress may provide insight into new and novel mechanisms of drought tolerance or response.

Other significant responses to stress are also noted including: cell wall organization (GO: 0071555), recognition of pollen (GO: 0048544). Cell wall organization was on the whole more downregulated than upregulated in Alpowa under MS and SS but the reverse was true for Idaho. Cell wall organization terms include activities such as the assembly, disassembly, rearrangement and maintaining the shape of the cell wall. Here cell wall organization constituted around 200 members with the

balance associated with down regulation in Alpowa and up regulation in Idaho under both MS and SS conditions. Significant child terms associated with cell wall functional activities included: cell wall biogenesis (GO:0042546) and cell wall modification (GO:0042545) and cell wall macromolecular catabolic processes (GO: 0016998) indicating the modifications of the cell wall in terms of macromolecular catabolism is likely a mechanism associated with drought stress in soft white spring wheat. Recognition of pollen is an odd GO term to be found under water limitations especially given the stage of development at time of harvest. The balance for this GO term favors down regulation in Alpowa and up regulation in Idaho. However given that the wheat plants under study are in the pre-anthesis stage of development it is likely that some activities associated with reproductive stages of development are occurring. This GO term encompasses the establishment of processes where pollen incompatibility interactions are beginning to be established, and this may likely occur prior to pollination. Child terms with differentially expressed functions under drought included mechanisms that promote acceptance of pollen (GO:0060321), mechanisms that insures self-pollination.

Liu et al. (2015), reported that up-regulated gene sets under drought-stress (DS), heat-stress (HS) and heat and drought-stress (HD) treatments included GO terms of stress response, hormone stimulus response and nutrient metabolic processes. Our results showed differentiation in transcripts expression in GO terms related to response to stress

and nutrient metabolic process (GO:0006979, GO:0006950, GO:0045454, GO:0008152, GO:0005975, GO:0006629, GO:0006511 and GO:0042744).. From the stress responsive GO terms mentioned in Liu et al. (2015), two distinct functional categories (RNA processing and epigenetic regulation of gene expression) in up regulated genes in heat and drought-stress (HD) exhibited higher enrichments in comparison to individual stress. These enrichments include epigenetic regulation of gene expression and RNA processing. Our research exhibited the same trend and many GO terms related to regulation to gene expression and translation (GO:0006355, GO:0006351 and GO:0006412) were changed in their expression. In two sorghum genotypes, Fracasso et al., (2016) found that regulation of cell growth by extracellular stimulus" (GO:0001560), regulation of DNA replication" (GO: 0006275), "secondary metabolic processes" (GO:0019748) including "terpenoids biosynthetic process" (GO:0016114), "prereplicative complex" (GO:0005656), "glutathione transferase activity" (GO:0004364) and "cell death" (GO:0008219) considered the most enriched GO terms under drought stress. In the wild barley Hordeum spontaneum, two categories (DNA repair and hydrolase activity) were enriched in the sensitive conditions while about twelve categories (e.g., glycine biosynthetic process, DNA helicase activity and thiol oxidase activity) were enriched in tolerant condition and at least four categories among them are associated with carbon metabolism (Hubner et al., 2015).

GO: functional analysis presents broad areas where differentially expressed genes group under Gene Ontology terms according to their functional specificities. Membership is such groupings is determined by the gene showing a significant change (p value < 0.05) over control adjusted for false discovery rates. Thus gene ontology terms reflect more the numbers of differentially expressed genes rather than the intensity of the change in differential expression, whither up or down regulated.

Gene Ontology Terms		Moderate Stress (50% WW)						Severe Stress (25% WW)					
		Alpowa			Idaho			Alpowa			Idaho		
		Up	Down	U/D	Up	Down	U/D	Up	Down	U/D	Up	Down	U/D
regulation of transcription, DNA-template	GO:0006355	1363	1264	1.08	641	122	5.25	1374	1301	1.06	581	443	1.31
transcription, DNA-templated	GO:0006351	1216	1142	1.06	410	107	3.83	1143	1228	0.93	367	291	1.26
DNA integration	GO:0015074	154	142	1.08	102	41	2.49	156	139	1.12	109	75	1.45
DNA repair	GO:0006281	148	199	0.74	98	13	7.54	171	179	0.96	98	67	1.46
translation	GO:0006412	356	407	0.87	128	23	5.57	450	308	1.46	106	92	1.15
protein glycosylation	GO:0006486	127	115	1.10	72	13	5.54	119	120	0.99	63	37	1.70
protein folding	GO:0006457	114	121	0.94	67	7	9.57	135	99	1.36	58	29	2.00
ubiquitin-dependent protein catabolism	GO:0006511	185	186	0.99	95	6	15.83	198	168	1.18	72	41	1.76
metabolic process	GO:0008152	741	576	1.29	257	77	3.34	638	675	0.95	242	160	1.51
carbohydrate metabolic process	GO:0005975	504	496	1.02	243	52	4.67	484	536	0.90	226	166	1.36
lipid metabolic process	GO:0006629	169	169	1.00	79	13	6.08	140	203	0.69	85	57	1.49
transport	GO:0006810	158	157	1.01	77	6	12.83	154	164	0.94	83	49	1.69
intracellular protein transport	GO:0006886	159	193	0.82	124	6	20.67	165	183	0.90	122	92	1.33
transmembrane transport	GO:0055085	232	232	1.00	107	21	5.10	248	227	1.09	97	73	1.33
vesicle-mediated transport	GO:0016192	93	118	0.79	53	8	6.63	99	111	0.89	56	50	1.12
response to oxidative stress	GO:0006979	243	217	1.12	60	35	1.71	218	240	0.91	63	42	1.50
response to stress	GO:0006950	142	117	1.21	44	25	1.76	131	123	1.07	41	58	0.71
cell wall organization	GO:0071555	175	207	0.85	76	28	2.71	169	221	0.76	69	45	1.53
recognition of pollen	GO:0048544	146	179	0.82	84	9	9.33	139	182	0.76	64	46	1.39
cell redox homeostasis	GO:0045454	180	149	1.21	57	11	5.18	182	144	1.26	44	51	0.86

U/D
<0.85
0.85-1.0
1.0-1.15
>1.15

Figure 1: Gene Ontology Terms for differentially expressed genes from two soft white spring wheat cultivars (tolerant Alpowa, and susceptible Idaho) treated under well-watered (WW), moderate stress (MS), and severe stress (SS) conditions. Gene Ontology Terms are presented along with their GO numbers and the total membership in terms of differentially expressed genes that are up or down regulated. The GO Terms are sorted based on shared function such as DNA processes, metabolism, transport etc. The ratio of upregulated to downregulated is given as U/D for each GO term, cultivar and stress intensity. U/D ratios are color codes in blue for predominately down regulated and red for predominately up regulated

To determine the genes that show the greatest shifts in gene expression we identified the top 20 up and down regulated genes Figures 2 and 3, respectively. The top up regulated differentially expressed genes are categorized into 9 functional categories (unknown, proteins, photosynthesis, hormonal, drought stress, pathogen defense, transport, cell wall and gene evolution) (Figure 2) with more than one differentially expressed genes comprising the first five categories. The functional categorization was based on UniProt classifications and definitions (UniProt, 2017). The most up regulated differentially expressed genes include: an uncharacterized protein (225-fold MS-Alpowa), an ethylene responsive transcription factor (217-fold SS-Alpowa), photosystem I subunit 7 (213-fold MS-Alpowa), and a 50S ribosomal protein 6 (188-fold MS &SS-Alpowa). There were two functionally unknowns at the upper end of the top 20 list indicating a need for further functional classification efforts and presenting an opportunity for further exploration into the molecular unknowns. The top differentially expressed gene was highly expressed across MS and SS in Alpowa, but not in Idaho. Protein functional categorization included 4 differentially expressed genes including two involved in protein catabolism (Zn-dependent exopeptidase, and a predicted Ion protease) and two in protein synthesis or being a synthesis product (50S ribosomal protein, and glutenin gene). The exopeptidase was most highly expressed under SS in Idaho while the Ion protease in SS in Alpowa. The protein synthesis genes associated with translation that is typically nonregulatory and the glutenin gene a seed storage protein were also up regulated in almost all instances. It is interesting the seed storage gene was upregulated

prior to anthesis in leaf tissue suggesting that this typical seed storage gene may have additional functionality in leaf tissue compared to its seed storage function typically associated with seed tissue.

There were four genes broadly associated with photosynthesis. These included two apparently structural proteins, one from photosystem I (subunit VII) and the other from photosystem II (psbP protein). The subunit VII is highly expressed in MS-alpowa while the PS II protein in SS-Idaho. The photosystem I protein is an iron containing apoprotein that functions in electron transfer to ferredoxin. The photosystem II protein function is not entirely clear but there are suggestions that it may be involved in strigolactone synthesis- a hormone involved in root morphological changes (Roose, Frankel, & Bricker, 2011). The other two chloroplast proteins involved in photosynthesis include a protoporphyrin IX methyl transferase associated with chlorophyll synthesis and amidophosphoribosyl transferase (APRT) an enzyme associated with purine synthesis from glutamate essential for chloroplast biogenesis. The APRT enzyme was highly upregulated under MS and SS in Alpowa but only so in SS in Idaho. These results indicate that chloroplast function and photosynthesis activities are highly upregulated under water limitations.

Enzymes associated with hormonal activities were highly upregulated including those with jasmonic acid synthesis, ABA synthesis and a transcription factor responsive to ethylene and drought. All these hormones are known to be intimately involved in plant

drought responses (Wang, & Irving, 2011). Two highly upregulated genes were associated with jasmonic acid synthesis. Jasmonic acid is known to increase under drought conditions (Gonzalez, Keller, Chan, Gessel, & Thines, 2017); (Du, Liu, & Xiong, 2013) but its precise role is still not well understood and in fact may be a negative regulator of drought stress response (Dhakarey et al., 2017). These two gene expression pattern were highly similar to each other suggesting that they act in concert and may actually be isozymes. The zeaxanthin dioxygenase was likely along with the psbP domain protein referred to above was involved with strigolactone synthesis, a hormone that inhibits tillering and shoot branching (Booker et al., 2004) and functions as a microbial signal in the rhizosphere of various species (Xie, Yoneyama, & Yoneyama, 2010). The last hormone associated differentially expressed gene encodes for one of 12 possible ethylene responsive transcription factor that are known to be previously induced by osmotic and salt stress (Zhu et al., 2010) and possibly pathogen defense (Buttner & Singh, 1997). This particular gene was highly upregulated under SS-Alpowa.

A total of three upregulated differentially expressed genes appeared to be associated with pathogen defense. These were genes involved in leaf rust resistance (Lr1), a gene encoding for an enzyme chalcone synthase, and a LURP-one protein. Lr1 is one of the oldest genes associated with leaf rust resistance known. The Lr1 gene has been localized and is associated with the NBS-LRR type gene that are good candidates for transmembrane pathogen recognition and signal transduction proteins (Cloutier et al.,

2007). The dramatic upregulation of this gene may suggest either a significant pathogen load or an alternative function. Chalcone synthase is a key enzyme in the flavonoid/isoflavanoid pathway typically involved in phytoalexin production which has strong links to pathogenesis reactions (Dao, Linthorst, & Verpoorte, 2011). The LURP protein is known to be strongly induced by infection with the oomycete *Hyaloperonospora parasitica* in *Arabidopsis*. The expression of all three of these categorized pathogen defense proteins, and the two jasmonic acid synthesis proteins are very similar across stress intensity and cultivar suggesting that they are co-regulated and likely function together in terms of pathogen defense.

The remailing upregulated genes with disparate functions include a TolB protein associated with transport, 1,3 beta glucanosyltranferase associated with cell wall synthesis and a gene involved in retrotransposon integration into the wheat genome. The TolB protein is known from studies in gram negative bacteria where it acts as a transmembrane channel across the periplasm. The gene is not exclusive to bacteria but is also predicted to exist in rice and *Brachypodium distachyon.* Recent work has shown that an apoplastic space protein found in Lupinus luteus L. and regulated by ABA and salt stress also contains the TolB motif (Demidenko et al., 2015). This gene was highly upregulated in MS and SS-Alpowa and SS-Idaho but barely so in MS-Idaho. It would be interesting to determine the intercellular location of such a protein and whither this protein is associated with the plasma membrane or with the chloroplast to any significant

degree. It is likely to be involved with transport mechanisms based on the work with bacteria (Lazzaroni, Germon, Ray, & Vianney, 1999). The enzyme 1, 3-beta glucanosyltransferase is a protein that is found in bacteria and fungi, but not known in plants, but gene predictions algorithms have in fact predicted it to be present in rice. The enzyme is known to function in the cell walls of fungi to facilitate cell wall repair under elevated temperatures (Zhao, Li, Liang, & Sun, 2014). Most recently an alternative function has come forward suggesting that this enzyme may actually function either in chromatin remodeling and gene silencing (Koch & Pillus, 2009) or as part of DNA damage response (Eustice, 2014). The last upregulated protein was one with significant similarity to a rice retrotransposon protein (E value, 8E-31) and was found under salinity stressed conditions (unpublished) by S. Kumari. Retrotransposon are short DNA elements that can multiply extensively within a given genome. About 68% of the wheat genome consists of these kinds of repeats, so it is highly likely that a very active retrotransposition mechanisms should exist (Li, Zhang, Fellers, Friebe, & Gill, 2004). The fact that the retrotranposition protein was strongly upregulated under drought stress may suggest a highly active mutational mechanisms (Alzohairy et al., 2014). The increase in mutations under stress may actually be a mechanism associated with further evolutionary development of the wheat genome under adverse environmental conditions. The strong similarity in expression patterns with the pathogen defense may also suggest that transposition may be co-regulated with biotic stress as well.

Upregulated Diffentially Expressed Genes or Proteins	Functional Categories	Alpowa		Idaho	
		MSA	SSA	MSI	SSI
		fold change			
uncharacterized protein	unknown	225	224	ND	60
uncharacterized protein	unknown	ND	150	6	45
Zn-dependent exopeptidases	protein catabolism	3	46	21	141
lon protease	protein catabolism	ND	158	21	45
50S ribosomal protein 6, chloroplastic	protein synthesis	188	188	28	45
glutenin	protein synthesis	ND	139	ND	45
photosystem I subunit VII	photosynthesis	213	72	21	45
psbP domain-protein 5	photosynthesis	58	55	28	174
magnesium protoporphyrin IX methyltransferase	chlorophyl synthesis	166	64	25	55
amidophosphoribosyltransferase	chloroplast biogenesis	149	148	21	113
4-coumarate--CoA ligase-like 9	jasmonic acid synthesis	144	68	21	45
4-coumarate--CoA ligase	jasmonic acid synthesis	137	56	21	45
zeaxanthin 7,8(7',8')-cleavage dioxygenase	ABA synthesis	ND	54	144	45
ethylene-responsive transcription factor	drought stress	3	217	ND	59
Triticum aestivum Lr1 disease resistance gene	pathogen defense	142	54	21	65
chalcone synthase	pathogen defense	144	58	21	43
protein LURP-one-related 8	pathogen defense	144	72	21	60
TolB protein	transport	179	178	6	145
1,3-beta-glucanosyltransferase	cell wall	ND	44	ND	178
retrotransposon	gene evolution	138	44	21	107

>200
150-200
100-150
50-100
1-50
ND

Figure 2: The top 20 up regulated genes with the greatest fold change by treatment from control in soft white spring wheat cultivars (tolerant Alpowa, and susceptible Idaho) treated under well-watered (WW), moderate stress (MS), and severe stress (SS) conditions. Top upregulated differentially expressed genes along with a functional categorization and the fold change for each cultivar and stress intensity treatments are presented. The genes are sorted based on functional categorization. The fold change are color coded in decreasing hues of red with decreasing fold changes as outlined in the figure legend

The top 20 down regulated differentially expressed genes under different stress intensities and for the two soft white spring wheat cultivars of are reported in Figure 3. Down regulation suggest that the activities of these genes were curtailed in response to stress. Wheat plants at this time were in the pre-reproductive stage when presumably the plant was preparing for reproductive processes. As with the upregulated genes, the differentially expressed genes were functionally categorized into 10 distinct categories. The most down regulated genes included a unknown chloroplast protein, 18S rRNA gene and an ethylene-responsive transcription factor RAP2-13. The unknown chloroplast protein showed dramatic down regulation (248 fold) in MS-Idaho but no change in MS-Alpowa. The 18S rRNA gene was dramatically downloaded under SS in Alpowa and to a lesser extent in Idaho. This transcript response was almost completely restricted to severe stresses. Finally the ethylene responsive transcription factor was dramatically reduced in MS and SS-Alpowa. This transcript in particular could be one of a family of 11 transcriptions factors. It is interesting that within the same family there was found an ethylene responsive transcription factor that was upregulated especially in MS-Alpowa. Given that the genes responded differently with stress it is highly likely that the transcription factors are different genes.

Transcripts associated with unknown functions are becoming less common in wheat given the most recent efforts to sequence and functionally annotate the wheat genome (Mayer et al., 2014). Here among the top 20 downregulated genes there were

three with unknown functions including a chloroplast protein which was highly downregulated under MS-Idaho.

Down regulated transcripts associated with proteins synthesis, catabolism, oligomerization and modification numbered 6 out of the top 20 down regulated genes. These include two involved in transcription coding for ribosomal proteins, ion proteases associated with protein catabolism, heat shock protein associated with protein oligomerization and a U-box domain protein associated with ubiquitin protein modifications. The two ribosomal proteins corresponding to the small and large ribosomal subunits are usually known to be constitutively expressed (Kundu, Patel, & Pal, 2013) but here the 18S rRNA gene in pre-reproductive wheat tissues showed dramatic down regulation in SS-Alpowa while the 28S rRNA gene shows downregulation in MS-Idaho. This dramatic down regulation of these two translational genes suggest the translation may actually be reduced in leaves under drought stress. The two Ion protease genes associated with protein catabolism include an Ion protease ARASP2 gene which according to UniProt functional annotation may be associated with intramembrane proteolysis. The 16.9 kDa heat shock protein was shown to be more downregulated in Idaho than in Alpowa. This gene is thought to be associated with thermal tolerance serving as a protein chaperone and regulating enzyme activities associated with antioxidant and ABA signaling in response to severe heat stress. The reason why this protein should be downregulated across treatments and cultivars is

unknown and seemingly contradictory. The U-box domain protein 4 transcript was found to be highly down-regulated in MS, SS-Alpowa compared to Idaho. This protein is known to function in terms of protein ubiquitinization leading to protein transport or catabolism (Zeng, Park, Venu, Gough, & Wang, 2008).

A total of three proteins were categorized as being involved in secretion or intracellular transport systems. These include the golgin protein which was found to be downregulated only under SS in Alpowa and much more so in Idaho. The golgin family of proteins are associated with maintaining Golgi apparatus structure in their role in the secretory pathway (Munro, 2011). These proteins are known to contain RAB GTPases binding sites to associate with RAB coated membranes. Rab protein, presumably a RAB GTPase enzyme was also down regulated in a pattern that is similar but not identical to the Golgin transcript, the obvious exception being with MS-Idaho where the golgin protein was not differentially expressed and the rab protein dramatically downregulated. Rab proteins are associated with membrane trafficking and vesicle movement along the cytoskeleton network of filaments helping to facilitate the functions of the secretory pathway. The activities of these two proteins are also correlated with the last protein in the secretion classification, the transmembrane 9 superfamily member 12 transcript. This protein according to UniProt annotation may be associated with the Golgi apparatus or the endosomes which may suggest transport to either lysosomes or vacuole.

A number of other transcripts with significant downregulation were classified as senescence, detoxification, water transport, flavonoid metabolism, signal transduction, RNA editing and tetrapyrrole binding proteins. A MARD1-like protein was found downregulated primarily in MS-Alpowa and SS-Idaho. This protein has an unknown function and is found to be upregulated during senescence in *Arabidopsis* (He et al., 2001). The patter of expression of the MARD1-like protein was closely matched by a glutathione-S-transferase protein. Glutathione-S-transferases constitute a family of enzymes that are well known as detoxification enzymes for xenobiotics. Recent research has uncovered significant endogenous roles in oxidative stress metabolism, light signaling, phytochrome A regulation, and negative association with drought and stress tolerance (Chen et al., 2012). If the negative association with drought tolerance is definite, then a down regulation of this gene would seem to support tolerance in plants. Another gene that was significantly downregulated especially in SS-Idaho and to a lesser extent in SS-Alpowa is an aquaporin TIP1-2. This protein is well known as a water channel protein that participates in water transport across the tonoplast membrane of the vacuole (Pih et al., 1999). Aquaporins function in osmotic adjustment across cell membranes including the plasmamembrane and the tonoplast. Experiments in bean has indicated that aquaporins are significantly downregulated under drought conditions, presumably a mechanisms to help cell retain water under osmotic stress conditions by restricting free movement of water across membranes (Zupin, Sedlar, Kidric, & Meglic, 2017). Bisdemethoxycurcumin synthase is an enzyme that functions in flavonoid

synthesis. This little known enzyme has been shown to be antiinflamatory, antioxidant and a scavenger of nitric oxide (Sreejayan & Rao, 1997). Its exact role in plants is open to speculation, but here it appears to be highly downregulated under SS-Idaho treatment more so in Idaho than in Alpowa. The CBL-interacting protein kinase showed pronounced down regulation in MS- Alpowa compared to the other treatments. This signal transduction protein has been shown to be important for potassium uptake in plants (Lee et al., 2007) and presumably associated with stomatal closure as well. The pentatricopeptide repeat protein was highly downregulated in MS and SS-Alpowa and to a lesser extent SS-Idaho. This protein is associated with RNA editing mechanisms (Hayes, Giang, Berhane, & Mulligan, 2013) and in the establishment of leaf venation patterning in Arabidopsis (Petricka, Clay, & Nelson, 2008). Lastly the tetrapyrrole-binding protein was downregulated most in MS-Idaho. This gene also known as GUN4 is associated with plastid to nucleus signaling and with chlorophyll biogenesis regulation (Larkin, Alonso, Ecker, & Chory, 2003).

Downregulated Diffentially Expressed Genes or Proteins	Functional Categories	Alpowa		Idaho	
		MSA	SSA	MSI	SSI
		fold change			
unknown chloroplast protein	unknown	ND	59	248	50
unknown	unknown	141	138	ND	95
uncharacterized protein	unknown	ND	140	56	61
18S rRNA gene	protein synthesis	ND	208	1	57
28S rRNA gene	protein synthesis	ND	51	170	51
lon protease	protein catabolism	ND	65	139	51
Ion protease ARASP2	protein catabolism	160	49	ND	47
16.9 kDa class I heat shock protein 1	protein oligomerization	5	55	101	138
U-box domain-containing protein 4	protein modification	132	125	41	45
ethylene-responsive transcription factor RAP2-13	stress	191	204	ND	62
golgin #2 isoform X2	secretion, intracellular transport	ND	51	ND	179
rab protein	secretion, intracellular transport	12	65	120	167
transmembrane 9 superfamily member 12	secretion	ND	51	136	130
MARD1-like protein	senescence	141	79	3	104
glutathione-S-transferase	detoxification	147	71	ND	45
aquaporin TIP1-2	water tranport	ND	56	17	158
bisdemethoxycurcumin synthase	flavanoid metabolism	ND	46	101	157
CBL-interacting protein kinase 16	signal transduction	152	61	ND	45
pentatricopeptide repeat protein DOT4	RNA editing	150	136	ND	109
tetrapyrrole-binding protein	chlorophyl synthesis regulation	1	71	139	52

>200
150-200
100-150
50-100
1-50
ND

Figure 3: The down regulated genes with the greatest fold change by treated from control in soft white spring wheat cultivars (tolerant Alpowa, and susceptible Idaho) and treated under well-watered (WW), moderate stress (MS), and severe stress (SS) conditions. Top downregulated differentially expressed genes along with a functional categorization and the fold-change for each cultivar and stress intensity treatments are presented. The genes are sorted based on functional categorization. The fold change are color coded in decreasing hues of blue with decreasing fold changes as outlined in the figure legend

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CHAPTER IV

PHYSIOLOGICAL INDICATOR OF DROUGHT STRESS IN SOFT WHITE SPRING WHEAT AT THE VEGETATIVE STAGE DEVELOPMENT

Introduction

Wheat is one of the most substantial cereal crop contributing more protein and calories to the world food than any other cereal crops, and it is a staple food for more than one-third of the population worldwide (Abd-El-Haleem. et al., 2009). Drought is the major cause of the significant reduction in growth and productivity of plants, and the most severe abiotic stress facing wheat production (Ludlow and Muchow, 1990). Drought creates significant alterations in plant biochemistry and physiology. With a short period of desiccation, the first line of plant defense is using stomatal closure in trying to save water inside the plant. When the drought is extended then other physiological change will occur as increased root growth, reduced leaf, and stem growth, and decline photosynthesis. As biochemical changes include a production of stress proteins such as dehydration responsive element binding proteins (DREBs). Up-regulation of antioxidants, such as proline which is an amino acid that is used in the biosynthesis of many proteins, and an accumulating of osmoprotectants or compatible solute, which are small molecules that act as osmolytes and help plants survive extreme osmotic stresses, such as: glycine betaines (Lisar, Motafakkerazad, Hossain, & Rahman, 2012).

Under water stress condition, the crucial aspect of the plant tolerance mechanisms depends on the photosynthetic system, which is very sensitive to be damaged. The primary processes affected by drought are in fact cell growth and this is directly related to photosynthesis (Chaves, Flexas, & Pinheiro, 2009). However, drought stress has a negative effect on photosynthesis in most plants including wheat by altering the structure of its organs and the concentration of various metabolites and pigments involved in this procedure (Bhushan et al., 2007). Wheat cultivar vary widely in their response to drought in terms of their photosynthetic performance. To evaluate photosynthetic performance, under water deficit, there are several methods that can be used. Therefore, analyzing how drought can affect the photosynthetic machinery of wheat is a very powerful tool to increase our understanding about wheat photosynthetic activities under stresses. For example, through taking several measurements of different fluorescence parameters of photosynthesis, we can easily find out the degree of damage that drought has caused to the wheat crop (Mouron et al., 2016). Under water stress, changes in the fluorescence induction parameters can be used to characterize tolerant wheat cultivars.

Many researches used acute drought to study chlorophyll fluorescence under water limitation, usually by withholding irrigation treatment for as much as 10-14 days. Few of these researches focus on long term effects of drought over a period of months. This is especially true with the soft white spring wheat in comparison to their more studied hard spring and winter wheat. The objective of this study, therefore, is characterizing the effect of acute and long term drought stress on fluorescence parameters

of plants in two cultivars of soft white spring wheat that differ in their response to drought.

Materials and Methods

Two greenhouse experiment; for photosynthetic measurements (Long Drought Period vs. Acute Drought Period) were executed at the greenhouse facility in Stillwater Oklahoma during the spring season (planting date 3/22/2016). Two cultivars of spring wheat (Triticum aestivum L.) Alpowa and Idaho were used. Alpowa is a resistant soft white spring wheat that was released in 1994 and was developed by Washington State University in cooperation with the Idaho and Oregon Agricultural Extension Service and the local offices of the USDA-ARS. Alpowa is widely grown in the western United States, and it was the leading spring wheat cultivar in Washington State as of years 2003, 2004 and 2005 (Lin & Chen, 2007). While Idaho: is a drought susceptible spring wheat with a high yield, that was developed by the Idaho Agricultural Experiment and the University of Idaho and Station (Montana State University, 2015). Idaho is currently used as a control to monitor breeding progress. This study uses the same plant materials for examining differential gene expression using RNA-seq as described in chapter III.

Wheat Plants were planted in single Treepots (Treepots, 4 inches in width, 14 inches in depth (Stuewe and Sons Inc, OR). All pots were sanitized with 70% ethanol and air dried and rinsed with water. The soil was mixed to a recommended nitrogen rate of 67 Kg/ha by the addition of ammonium nitrate fertilizer that has been ground to a fine powder. After mixing, the soil was evenly distributed across all pots and the process repeated until all pots contain 3 Kg of soil. The soil was a sandy loam (Kirtland B) obtained from the Stillwater Agricultural Extension farm. Initially, three wheat seeds

were planted in each pot to the depth of two inches and watered. Upon germination, plants were thinned to one plant per pot. The pots were hand weeded and monitored for insects and sprayed with Immunox in case of powdery mildew infestation, and Neem oil for aphid infestation. Daily, maximum, minimum, average and current temperature reading were recorded using digital thermometer TMD-52 by Amprobe (Amprobe Test Tools, Everett, WA).

Water Limitation Treatments:

Water limitation was imposed on pots containing the two wheat cultivars. This was performed by watering plants when the control well-watered plants needed to be watered. Two soil tensiometers per treatment were inserted into randomly assigned control pots in order to measure soil water potential, a direct measure of the availability of water to the plant based on manufacturer recommendation (Irrometer Co. Inc., Riverside, CA). When the control pot readings approached the recommended readings for watering (50 centibars) water of varying volumes was provided for all pots including the well-watered control (WW, 100%, 236 ml), moderate stressed (MS, 50%, 118 ml), severe stressed (SS, 25%, 59 ml). To avoid acute stress responses the pots were monitored daily, and their average was computed. With two varieties, three water treatment levels, and nine replicate plants per treatment the total experiment consisted of 54 pots. For the long term experiment water limitation treatments commenced three

weeks after planting approximately when the wheat was at the two-leaf stage of development (Feekes 1). For the acute drought treatment when plants reach Feek's stage 10, then two water treatments were imposed, one set of plants was maintained under well-watered conditions and the other set water was withheld (stopping irrigation) as an acute stress treatment.

The experiment was designed in complete randomized design two factor ANOVA. Two factors were used in this experiment; cultivars and water stress intensity treatments for four distinct treatments. Nine replicates were used for each treatment ending with the sum of 36 pots (experimental units for the experiment). After collecting fluorescence measurements, plants were harvested (harvesting date 5/17/2016) and then following physiological measurements were recorded: total plant weight, total green weight after removing dry leaves, number of tillers, number of spikes, and spike weight.

Fluorescence measurements

The main objective of these experiments was to test chlorophyll fluorescence measurements on detecting wheat responses to long-term and acute drought stress durations. Chlorophyll fluorescence measurements were collected using pulses amplitude modulation (Junior PAM, Walz) Fluorimeter. Fluorescence data was collected as indicated above after 4, 8, 12 days corresponding to Feeke's growth stages (10, 10.1, 10.3). Coupled to the instruments WinControl-3 software measurements were collected for both light and dark-adapted plants. For light adapted plants measurements were taken

at 3 PM., which is the time where photosynthetic activities are maximized. For dark-adapted plants measurements, were taken around 3 AM before dawn, which is the proper time to insure photosynthetic inactivity and depletion of previous photosynthetic substrates. Non-photosynthetic green light was used for workplace illumination under dark illumination. Nine replicates per the treatment were taken, one measurement per the plant by selecting the second leaf from the top.

The following parameters were taken*:

Light adapted plants:

F': Fluorescence yield shortly before onset of a strong light pulse.

Fm': Maximal fluorescence yield when a strong light pulse closes photosystem II reaction centers.

Y (II): Photochemical quantum yield of photosystem II; derived from F' and Fm' measurements.

Y (NO): Quantum yield of non-photochemical fluorescence quenching other than that caused by downregulation of the light harvesting function.

Dark adapted plants:

Fo: Basic fluorescence yield (relative units) recorded with low measuring light intensities.

Fm: Maximal chlorophyll fluorescence yield when a strong light pulse (relative units) closes photosystem II reaction centers.

Fv/Fm= (Fm-Fo)/Fm; maximum photochemical quantum yield of photosystem II.

*(Heinz Walz GmbH, 2007).

Data Analysis

Two factor Analysis of variance (ANOVA) was used to carry out using JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989-2007 to determine the significance of variation (p value ≤ 0.05) for all the traits measured for this study.

Results and Discussion

Chlorophyll fluorescence has been used to probe the mechanisms of photochemical reactions ever since Kautsky in 1960 observed changes in photosynthetic fluorescence in response to light (Maxwell & Johnson, 2000). Since then fluorescence measurement has developed into a highly sophisticated technology that is capable of nondestructively examining the inner workings of the photosynthetic apparatus yielding information on photosynthetic performance, adaptation and impairment. To accomplish these goals sophisticated fluorimeters capable of flashing short bursts of saturating light over a precisely choreographed timeline, and detectors capable of measuring the resulting fluorescence signals have been developed by variety commercial sources to allow investigators to use this technology in the laboratory and field setting (Maxwell & Johnson, 2000). However there is a steep learning curve for the novice investigators which constitute the majority of users inevitably resulting in errors and misunderstandings. Nevertheless, the technique provides a powerful capability that with time and effort becomes more accessible to those intent on mastering the details.

When light strikes the light harvesting centers contained within the thylakoid membranes, the light energy is quickly funneled to a reaction center where it is used to boost the energy of an electron. The energy then can either be utilized or decay following three competitive pathways: 1) use for photochemistry, 2) release as light of longer wave lengths in a process known as fluorescence and 3) release as heat (Ritchie, 2006). The

idea behind all chlorophyll fluorescence technology is to use fluorescence kinetics in such a way that allows for a determination of the status and capability of photochemistry and nonphotochemical processes. Photochemical processes transfer the energy to electron transport acceptors which function to pass hydrogen ions into the thylakoid lumen eventually contributing the development of a pH gradient across the thylakoid membrane. This gradient drives the reduction of ADP to ATP via thylakoid bound ATPases. The ATP generated is used in the reduction of carbon dioxide in the Calvin cycle. Thus a direct line between photochemistry and carbon reduction is anticipated in many cases (Kramer, Cruz, & Kanazawa, 2003). Nonphotochemical processes are in competition with photochemical processes leading to a release of energy as heat or fluorescence. Investigators have uncovered two types of nonphotochemical processes, those that are regulated and those that are not regulated (Ruban, 2016). The regulated nonphotochemical processes are typically associated with a build-up of a pH gradient across the thylakoid membrane or based on the activities of the zeaxanthin cycle (Ruban, 2016). The nonregulated processes are usually associated with the spontaneous emission of heat or fluorescence (Demmig, 2016). Fluorescence measurements can also be used to determine the quantum yield of photosystem II as well as the reduction in quantum yield associated with nonphotochemical processes in terms of the fractions of quanta utilized for photochemistry and nonphotochemical reactions. Some of the parameters measure similar attributes but differ on the underlying biophysical model and assumptions from which they were derived concerning the light harvesting centers (Ritchie, 2006). For

instance, the older puddle model assumes that the light harvesting centers funnel all the energy into a single reaction center which is independent of other similar reaction centers (Kramer, Johnson, Kiirats, & Edwards, 2004). The newer Lake model suggests that the reaction centers are interconnected in some way or another. Both parameters estimate the contributions to photochemistry but the Lake model parameters are considered more accurate (Kramer, Johnson, Kiirats, & Edwards, 2004). Investigators monitor fluorescence under both dark and full sunlight conditions. Dark adaptation is necessary to normalize the background fluorescence so that accurate results can be obtained and an estimation of the current status of the photosystem II machinery ascertained. However since photosynthesis takes place in the light there is a major advantage in conducting these measurements under full sunlight conditions. For this reason investigators developed the light modulated fluorimeter. The modulated fluorimeters provide ultra-short bursts of saturating light stimulating a rapid burst of fluorescence that is used to measure overall fluorescent yield. Initial fluorescence is measured based upon the fluorescence level prior to the flash. Absolute background levels can be measured using a short burst of far-red light which stimulates PS I activity relieving the backlog and opening up energetic reactions centers bringing fluorescence down to a basal level. All fluorescence parameters are derived from fluorescence response and decay curves over short period of time (few seconds) and after an initial burst of light (Ritchie, 2006). Decay also called quenching can be the result of photochemistry or nonphotochemical reactions. By providing a saturating light signal the technique closes all photochemical

reaction centers allowing the resultant fluorescence to serve as a measure of nonphotochemical processes. Commonly used parameters for quantum yield measurements include Fv/Fm (dark adapted photochemical) and Y(II) (photochemical), and Y(NO) nonphotochemical quenching nonregulated and Y(NPQ) regulated by thylakoid pH gradients or zexanthin cycles. Other parameters associated with photochemical fluorescence quenching include qP (puddle model) and qL (lake model) and non-photochemical quenching both regulated and unregulated as measured by qN and NPQ. Fluorescence measurements have been extensively used to monitor the response of the photosynthetic machinery to a variety of stresses including: heat (Sharma, Andersen, Ottosen, & Rosenqvist, 2012), drought (Paknejad, et al., 2007), photosynthesis acting herbicides (Varshney, Hayat, Alyemeni, & Ahmad, 2012), salt (Oyiga, et al., 2016), cold (Ya-nan, et al., 2013). Under stress conditions photochemical parameters are known to decrease over a period of time possibly indicating damage to the photosystem II due to photooxidation (Hasanuzzaman, et al, 2013) or other processes.

Here we use a modulated fluorimeter to compare the effects of long term and acute water limitations imposed on two cultivars in order to determine photochemical differences between soft white spring wheat cultivars, among stress intensities and over several sampling dates. Any differences will then be traced to specific aspects of the photochemical physiology. We are particularly interested in the differences between acute and long term stresses. Most chlorophyll fluorescence studies focus on short term acute drought imposition based on water withholding from plants over a period of 4 to 12

days. Acute drought responses have shown significant changes in the fluorescence parameters Fv/Fm in dark adapted tissues indicating an impairment of the photosystem II machinery and a reduction in overall quantum yield (Baker, 2008). Most researches detail the response of plants acute stress response over a limited period of time (Havaux & Lannoye, 1985). In contrast, very few long term studies where plants have been exposed to drought over a period of months (Paknejad, et al., 2007) have been conducted. During this time of long term stress it is reasonable to infer that a certain amount of adaptation can occur. The differences between long term and acute response may be a reliable measure of the degree of adaptation and may actually be a significant parameter for distinguishing cultivars for water limitation adaptation. Here we expose soft white spring wheat to either a two month long term water limitation treatment at three levels of intensity (well-watered (WW, 100% stress free), moderate stress (MS, 50% of well-watered) and severe stress (SS, 25% of well-watered), and an acute stress of 12 days where water is withheld and compared to the well-watered state.

Table (2) Fluorescence parameters accessible to the PAM-Junior fluorimeter, the equation and indicator along with original reference.

Parameter	Equation	Indicator	Original reference
Fv	Fm-Fo	Variable fluorescence	
Fm		Maximal fluorescence (dark adapted)	
F'm		Maximum fluorescence (light adapted)	
F'		Steady state fluorescence (light adapted)	
Fo		Minimal fluorescence (dark adapted)	
Fv/Fm	(Fm-Fo)/Fm	Maximal photochemical quantum yield for PSII: fraction of absorbed quanta used for PSII photochemistry (dark adapted)	(Kitajima and Butler, 1975)
Y(II)	(F'm-F')/F'm	Photochemical quantum yield of PS II: fraction of absorbed quanta used for PSII photochemistry (light adapted)	(Genty et al., 1989)
Y(NO)	Y(NO)=1-(Y(II)	Quantum yield of basal non photochemical fluorescence quenching: regulated and nonregulated.	(Kramer et al. 2004)

Soft white spring wheat were treated with three levels of water limitations (WW, MS, and SS) over a two month period from Feekes 2 to Feekes 10 and then monitored by fluorescence at three measurement dates (May 5, 9, and 12) and harvested on May 17th. As expected the long term stress treatment resulted in a significant 57% and 76% reduction in shoot biomass under MS and SS conditions, respectively (Figure 4). Examining the differences between cultivars: resistant Alpowa on average across stress treatments had 17% greater overall shoot biomass than susceptible Idaho indicating a moderate differential in terms of tolerance to water limitation in terms of biomass yield.

While being numerically higher the differences were not significant based on the Tukeys HSD (p value <0.05). These differences were readily apparent under WW and MS but not SS where biomass was very close to being identical indicating that tolerance to water limitations may be more restricted to moderate stress induced mechanisms.

Acute stress showed a similar response compared to long term stress impositions. Here again an acute stress (complete water withholding) resulted in a 69% reduction in overall biomass across both cultivars (Figure 4). This large reduction is likely due to dehydration of the tissues under stress imposed conditions compared to well-watered control. With respect to cultivars, here again there were no significant difference between cultivars, although under WW conditions Alpowa numerically out yielded Idaho by 15%. Thus the long term and acute water limitation treatments were effective in reducing overall biomass yield. These results are in line with those obtained by Anjum et al. (2011), and Yeganehpoor et al. (2016). Water stress may decreases water absorption and flow from the root to the leaves, impacting cell division, and elongation and overall vegetative growth and yield characteristics (Abdelaal et al., 2017). One of our main objectives in this paper is to determine whether we can detect differences in photosynthetic performance as judged by fluorescence technology between acute and long term stress, stress intensity levels, cultivars and measurement dates.

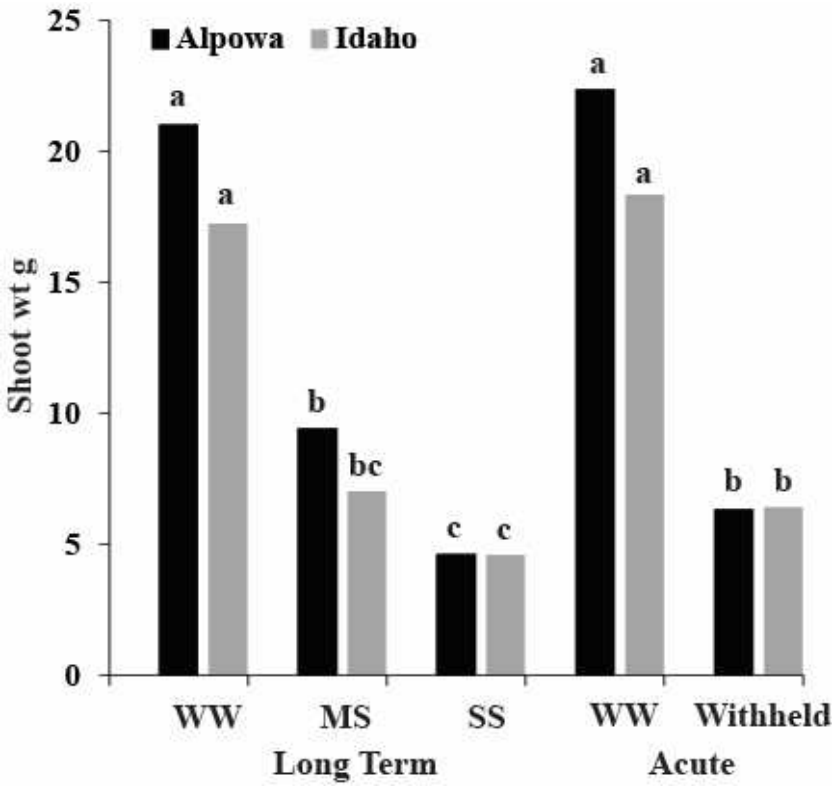

Figure (4) Shoot weights for soft white spring wheat cultivars Alpowa and Idaho treated under long term and acute water limitation. Long terms stressed plants were either WW (well water, 100% control), MS (moderate stress, 50% of control), and SS (severe stress, 25% of control) and acute stressed plants were maintained under WW conditions up until Feekes stage 10 and then water was withheld from treated plants, but not the control WW plants. Values differing in letter beside measurement indicate a statistically significant difference (p value $\leq$ 0.05, Tukeys HSD). The longterm and acute stress experiments were independent of each other.

Fluorescent measurements can be used to ascertain the status of the light associated portion of photosynthesis associated with photosystem II including light harvesting, light absorption, and electron transport. One of the most common parameters used in this kind of assessment is Fv/Fm a measure of photosynthetic efficiency, a sensitive indicator of plant photosynthetic performance. Fv/Fm is the ratio of variable fluorescence (Fv), which is the difference between maximum fluorescence (Fm) minus minimum fluorescence (Fo) divided by Fm (Fm-Fo/Fm). The value reflects the overall amount of fluorescence stimulated under saturating light when all reaction centers are closed divided by total fluorescence. In healthy plants under the best conditions, this parameter takes on values ranging from 0.80-0.84, and values lower than this are indicative of stress reactions ("Common Parameters - Hansatech Instruments", 2018). The values suggest that in healthy tissue more than 80% of the energy of the photons are utilized by photosystem II for photosynthesis. This parameter is most reliably measured after a period of darkness where previous photosynthetic substrates have been depleted leading to a baseline level of minimum fluorescence (Fo). For consistency, all measurements were made on the same leaf, the leaf below the flag leaf.

Plants were grown under long term water limitations (Figure 5A) or acute short term water stress (Figure 5B) followed by photosynthetic quantum yield measurements (Fv/Fm) using the PAM fluorimeter under dark adapted conditions. Soft white spring wheat cultivars (Alpowa and Idaho) were treated with three levels of water limitation (WW, MS, SS) over a 12 day period following watering in late spring of 2016.

Measurements were taken on penultimate leaf at the Feekes 10.1 to 10.3 stage of growth on the 5th, 9th, and 12th day of May before watering. Average values across stress intensities, cultivar and duration for long term treatments was 0.82 indicating little overall impact on the whole by water limitations. A three way analysis of variance with interactions indicated that measurement date, stress intensity and an interaction between stress intensity and measurement date showed significant differences (Tukeys HSD, pvalue < 0.05). The differences were found exclusively at the latter date (May 12th) and only under severe stress for both Alpowa and Idaho. Moderate stress while reduced was not significantly different from WW across date and cultivar. There were no significant differences between cultivars in terms of Fv/Fm.

Under an acute stress condition, soft spring wheat were raised under WW conditions up through Feekes 10 and then subjected to water withholding for 12 days (Figure 5B). Plants were also treated with an acute exposure by withholding water for 12 days during which Fv/Fm measurements were taken on May 5, 9 and 12. Three way analysis of variance indicated that all three effects showed significant impact on quantum yield (P value < 0.05, Tukeys HSD). As with the long term stress experiment no significant differences was apparent until the May 12 treatment in both Alpowa and Idaho. In contrast to long term treatments, the effect is much greater under acute conditions with average values across cultivar and stress intensity nearing 0.72, significantly different from control (0.82).

The parameter Fv/Fm is termed maximal photosynthetic quantum yield and is considered to represent the proportion of quanta used for photosynthesis for photosystem II. While it is clear from the shoot biomass data that the stress imposition did impact biomass yield significantly, those aspects affecting yield were not apparent in terms of quantum yield values for photosystem II at the time of measurement except on May 12th under severe stress conditions where quantum yields were significantly lower (0.73 to 0.75) compared to well-watered conditions (0.82) in both cultivars. Moderate stress conditions were not significantly impacted by water limitation in terms of maximal quantum yield. Under acute stress conditions where water is withheld for up to 12 days we see a similar response where significant differences only occur after day 12 in the water withheld treated plants. Thus the long term stress imposition pattern matches closely the acute stress imposition pattern in terms of quantum yield. However the degree of impact was much more severe under the acute severe stress than under long term severe stress indicating a limited measure of adaptation during the long term stress imposition. Curiously the quantum yield for Idaho was numerically greater but not significantly different than that of Alpowa under long term stress imposition. Only under acute stress cultivar differences were significantly different from each other. Thus an acute imposition of stress in susceptible Idaho appeared to be less able to adapt the photosynthetic machinery to stress imposition than Alpowa.

This marginal impact of water limitation is likely indicative of an adaptive response of the photochemistry to the water limiting conditions. When adaptation takes

place during the stress period is still too early to say. It must be remembered that site of measurement was the leaf below the flag leaf which at the time of long term stress initiation existed as leaf initials, and at time of measurement was still early in its maturation process. Thus, the penultimate leaf were present only as leaf initials during much of the long term stress period. The fact that the acute stress pattern was very similar to the long term patters also suggests that a very limited level of adaptation may be occurring during the 2 month treatment span. The fact that the two cultivars did not differ significantly in Fv/Fm values after a long term stress suggest that the non-significant but numerically greater differences in biomass shoot yield may not be associated with quantum yield photosystem II chemistry. The fact that Alpowa and Idaho differed significantly in response to acute stress in terms of quantum yield suggests that the level of tolerance may be associated more with short term phenomenon. Lower yield in Idaho may actually reflect its response to a single or multiple short term stresses which are frequently experienced by wheat plants in the field. Repetitive treatments of both cultivars to multiple short term stresses may be a better way of evaluating stress response and cultivar adaptation using fluorescence measurements.

Maximal quantum yield measurements (Fv/Fm) are best measured under dark adapted conditions and are a reflection of the potential quantum yield. Dark adaptation is often imposed by using a short 30 minute dark period during which photochemistry is eliminated and the backlog of photochemical energy dissipated. An alternative is to perform the test late after a prolonged state of darkness as was performed in this

experiment. However, it must be remembered that these measurements do not take place during the day in full sunlight and that they represent potential quantum yield. With our PAM modulated fluorimeter it is possible to measure actual photochemical quantum yield, a measurement very similar to Fv/Fm, in the light. Accordingly, measurements were taken at 3 PM under full sunlight using the PAM fluorimeter with wheat plants treated as indicated above. Parameters that estimate the quantum yield for photochemistry Y(II) and nonphotochemical processes Y(NO) are presented in Figures 6 and 7 for both acute and long term stresses.

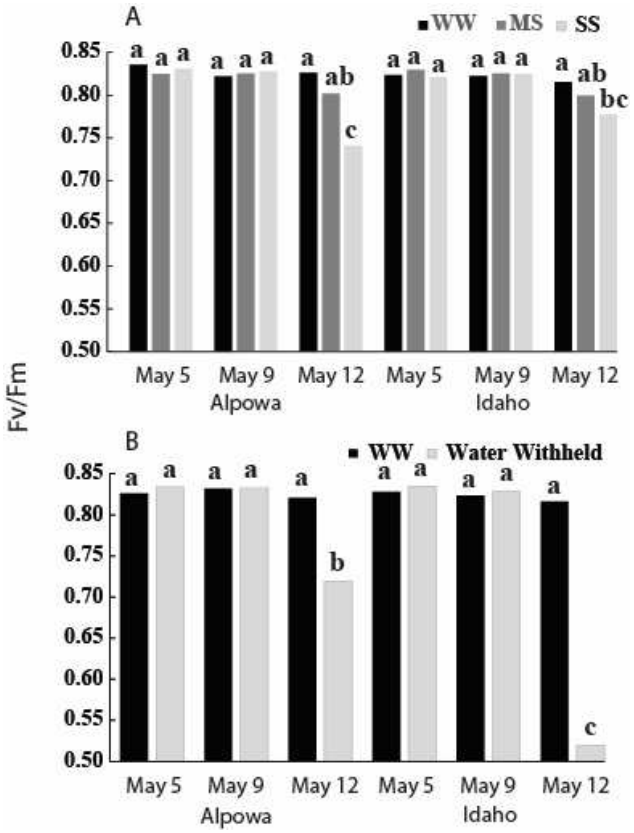

Figure (5) Photosynthetic efficiency as measured by Fv/Fm parameter in resistant Alpowa and susceptible Idaho under dark adaptation conditions over a 7 day period under long term (A) and acute water limitations (B). Longterm stress intensity treatments were 100% well-watered control (WW) 50% of well-watered (moderate stress, MS) and 25% of well-watered, (severe stress, SS). Acute stress intensity treatments occurred at Feekes 10 where water was withheld for 12 days followed by fluorescent measurements. Treatments were well watered control (WW) compared to Water Withheld treatments. Columns with different lettering are significantly different from each other based on a p value ≤ 0.05, Tukeys HSD.

The results for long term photochemical quantum yield (Y (II)) were very similar to those obtained for Fv/Fm under dark adapted conditions for long term and acute stresses with one important exception. Alpowa and Idaho showed significant differences in terms of Y(II) on May 12 only for all stress intensity treatments (WW, MS, SS for Alpowa and WW, SS for Idaho). This contrasts with the Fv/Fm measurements where WW was not significantly different from measurements derived from earlier dates. Here Y(II) showed a significant reduction across all stress intensity measurements indicating that the differences were not associated with stress imposition but may be associated with leaf age and condition (Figure 6). At May 12 under long term stress conditions the penultimate leaf is a little older and is also most likely undergoing significant pre-senescence activities related to the oncoming reproductive stages where wheat physiology shifts from vegetative growth to reproduction and grain filling. During reproduction and grain filling the penultimate leaf would experience significant degradation of the metabolic machinery and conversion to transportable metabolites for export to the growing grain. It may be likely that the reduction in Y(II) is associated with the beginning phases of this activity and not to the long term stress imposition. While there were differences in the long term response the acute response for Y(II) was very similar to that of Fv/Fm where only the water withheld treatments showed significant differences and Idaho response was much more severe than Alpowa.

Nonphotochemical processes also sap quantum yield as visualized by the quenching of fluorescence. Y(NO) a measured parameter that indicates both

fluorescence, heat dissipation, pH differential across the thylakoid, and the zeaxanthin cycle activities are in competition with Y(II) photosynthetic processes, summing to unity. Thus a reduction in Y (II) will result in an increase in Y (NO) (Figure 7). Here we see just that taking place. The only differences associated with Y(NO) is with May 12 sampling date where Y(NO) at all levels of stress intensity was significantly different from the other dates for both Alpowa and Idaho, the inverse of Y(II). Thus while photochemical processes were reduced, this reduction is due to an increase in nonphotochemical effects. The same comparison can be said for the acute treatment where only the water withheld treatment differed from the WW treatments in both Alpowa and Idaho with Alpowa being numerically less than Idaho. These data from Y(II) and Y(NO) suggest that the effect observed on May 12th is not associated with stress imposition but may be the result of leaf maturation and pre-metabolic conversion to complete reproductive function. Both Fv/Fm and Y(II) measure the same response. The fact that the pattern of Fv/Fm response differs from those of Y(II) suggest that metabolism may be adapting during the dark adaptation phase prior to Fv/Fm measurement where WW appears to be normal, but with Y (II) it appears to be reduced.

The PAM fluorimeter is capable of measuring a wide array of fluorescence parameters not presented here in this research. These include qL, qP, Y(NPQ), qN and NPQ. However these parameters require the accurate determination of Fo which requires that far red light be turned on after the saturating pulse to reduce fluorescence to background readings as explained above. At the time of the experiment we did not fully

understand the usage of this important function and were thus limited to the measurement of Fv/Fm, Y(II), and Y(NO). Inclusion of the far red light in the experiment would have allow us to differentiate between regulated and non-regulated nonphotochemical processes. This would have added an additional piece of information for our evaluation.

In conclusion water limitation in soft white spring wheat cultivars Alpowa and Idaho results in a significant reduction in processes that contribute to yield. These processes appears to affect Idaho more than they do Alpowa resulting in a numerically lower level of shoot weight. However caution must be used here because the differences were not statistically significant. The differences in biomass between the two cultivars was not the result in their impairment in quantum yield in photosystem II processes or in nonphotosynthetic processes under long term stress, but may have to do with the response of Idaho to short term stresses that are frequently experienced by wheat plants growing in the field.

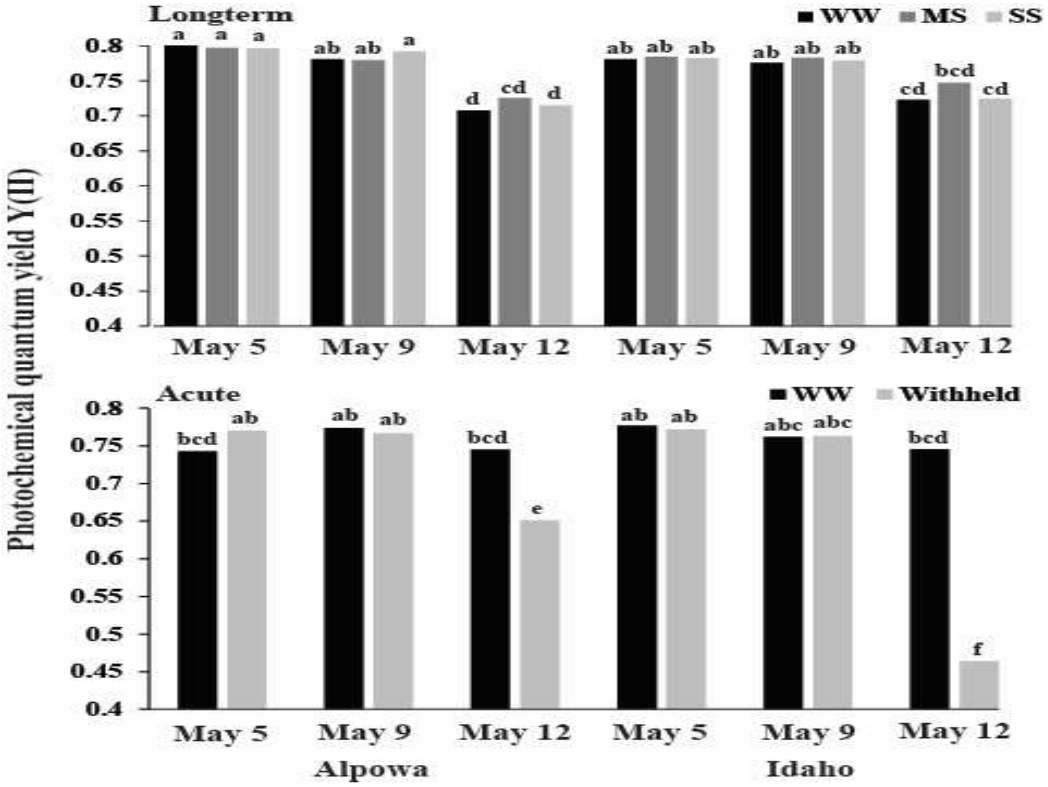

Figure (6) Photochemical quantum yield of photosystem II as measured by Y(II) parameter in resistant Alpowa and susceptible Idaho under light adaptation conditions over a 7 day period under long term, and acute water limitations. Longterm stress intensity treatments were 100% well-watered control (WW) 50% of well-watered (moderate stress, MS) and 25% of well-watered, (severe stress, SS). Acute stress intensity treatments occurred at Feekes 10 where water was withheld for 12 days followed by fluorescent measurements. Treatments were well watered control (WW) compared to Water Withheld treatments. Columns with different lettering are significantly different from each other based on a p value ≤ 0.05, Tukeys HSD.

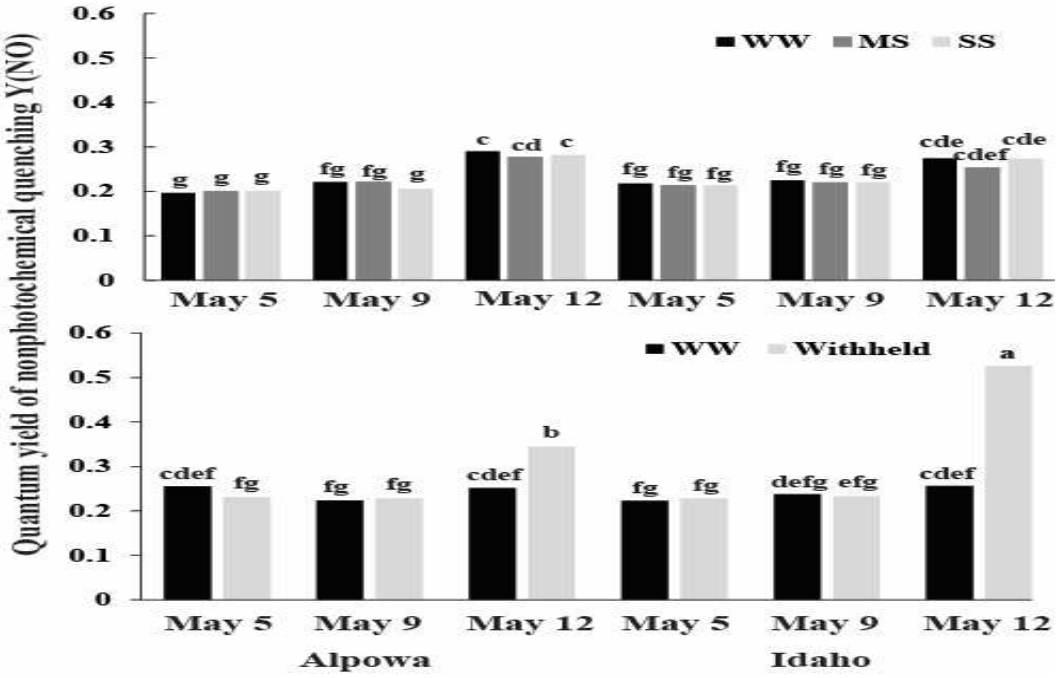

Figure (7) Quantum yield of non-photochemical fluorescence quenching as measured by Y (NO) parameter in resistant Alpowa and susceptible Idaho under light adaptation conditions over a 7 day period under long term, and acute water limitations. Long term stress intensity treatments were 100% well-watered control (WW) 50% of well-watered (moderate stress, MS) and 25% of well-watered, (severe stress, SS). Acute stress intensity treatments occurred at Feekes 10 where water was withheld for 12 days followed by fluorescent measurements. Treatments were well watered control (WW) compared to Water Withheld treatments. Columns with different lettering are significantly different from each other based on a p value ≤ 0.05, Tukeys HSD.

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