CHANGES IN GENE EXPRESSION UNDER WATER STRESS IN THE FLAG LEAF AND SEED HEAD DURING THE GRAIN-FILLING STAGE OF TWO SPRING WHEAT CULTIVARS

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Abstract: Drought poses severe limitations on crop production. Therefore, drought tolerance mechanisms need to be better understood to select, modify and recommend certain cultivars of wheat. The grain filling stage of wheat development is particularly sensitive to water stress conditions. This research studied biological response to water stress under, well-watered (WW- 220 ml), moderate stress (MS- 110 ml), and severe stress (SS- 55 ml) conditions, with cultivars of soft white spring wheat (Alpowa and Idaho) during the early grain-filling stage of wheat development (Feekes 11.2) and under two duration of stress treatments (5, and 8 days), with a particular focus on the flag leaf and seed head. The flag leaf weights averaged across durations were 91%, 62% and 68%, 41% of well-watered under MS and SS in Alpowa and Idaho, respectively, compared with the well-watered control. The differential expressed genes were 2.32 and 3.9 fold more up and down-regulated genes in Alpowa compared to Idaho, respectively. Shared transcripts between stress intensities MS and SS constituted only 3 to 17% of the overall differentially expressed transcripts in both cultivars. Most of the top GO terms were predominantly down-regulated with a ratio of less than 1.0 under both stress intensities. The most abundant GO terms are related to transcriptional biological processes and to a lesser extent metabolic, transport, and translational activities. Seed head weights did not differ statistically among any of the water limitations treatments, cultivars, or durations. Idaho had 5.5 times the number of DE transcripts compared to Alpowa (338 in Alpowa, and 1843 in Idaho) with only 53 transcripts shared between the two cultivars in their response to water stress. The top GO terms for the biological process was predominantly down-regulated especially in Idaho under both conditions (MS and SS) while in Alpowa a moderate tendency towards down-regulation was detected. The top twenty up and down-regulated transcripts were examined for flag leaf and seed head.

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CHAPTER I

INTRODUCTION

Wheat production uses more land than any other crop. It is also an essential food grain for human nutrition (Curtis, 2002). The food products made from wheat are a staple diet for most people in the world. In addition, wheat is one of the most nutritious grains produced agriculturally containing a wide assortment of minerals, vitamins, carbohydrates, and proteins. Estimates show that by 2050, the world population will reach 9.37 billion which is 35% higher than today's population, (FAO, 2017). With this increase in population, production of sufficient wheat will continue to be a major issue in feeding the planet. World wheat production increased dramatically after leveling off at 592 million tons in 1990. After 1990, the production has shown a gradual increase, and recently, the annual global wheat production has been estimated at 735 million tons (FAO, 2015). However, a projected 786 million tons of wheat will be required annually for human use by the year 2025, highlighting the need for rapid and continuous increasing levels of production (Curtis, 2002). Currently, the average wheat yield worldwide is predicted to rise from 2.8 to 3.8 metric tons/ha from 2011 to 2050 in response to accelerated demand and increased population counter balanced by increased production efficiencies (Alexandratos & Bruinsma, 2012). At the same time that yield must increase, climate scientists suggest that plant water availability will decrease as a function of intense and less frequent rainfall

events and increase in temperature, leading to greater evapotranspiration and greater soil water deficits. However, the total amount of wheat land farmed has been in decline, and producers are faced with the challenge of producing more crop from a smaller parcel of land. For this reason, the development of a water stress tolerant and high yielding varieties of wheat is critical to maintaining food security for much of the world's population.

Wheat is often planted in dry or semi-arid areas. Projected changes in the earth's climate will exacerbate water stress conditions for agricultural crops, resulting in increased pressure on producers to adapt to limitations in water availability and quality. If rainwater is less available during the traditional growing season, wheat may replace or supplement other the traditional crops currently planted– increasing the importance of wheat as a global dietary staple. Currently, agriculture uses 70 percent of the world's available freshwater (Pimentel et al., 2004). While advances in irrigation delivery systems, plant water use efficiency, and drought mitigation have increased the percent of arable land used for wheat cultivation, most of the world's human population still lives in water-stressed environments. Furthermore, competition for water among agriculture, industry, environment, and human consumption may increase political friction and economic dislocations. Meeting urban water demands by siphoning off water that is needed for agriculture will, in the long run, jeopardize food security and rural economic sustainability (MacDonald, 2010). The recent drought in California pitted urban city dwellers against rural farmers – an example of just one of the conflicts that will begin to bubble up as the first signs of limiting water availability begins to manifest. It is critical to find ways to make agriculture more water use efficient, including the development of more water efficient crops. The development of water stress tolerant cultivars will constitute important

tools in a plant breeder's arsenal to meet the growing demands for food in the face of an uncertain future (Nezhadahmadi, Prodhan, & Faruq, 2013). Drought stress is among the most severe of the abiotic stresses which limit crop productivity, mainly due to deleterious and/or adaptive responses of the plants itself to water limiting conditions (Chaves et al., 2002; de Oliveira, Alencar, & Gomes-Filho, 2013). Losses in crop yield induced by drought probably exceeds that of all other causes of yield decline (de Oliveira et al., 2013).

Wheat is often grown in areas where water availability is too low for many other types of crops to grow. Wheat is better suited to these climates because it can respond by either dehydration avoidance and/or dehydration tolerance. Dehydration avoidance results when the plant responds by slowing metabolism or increasing the rooting depth to extract more water. The inclination of plants to keep hydrated under drought is called dehydration avoidance (Blum, 2005). Drought tolerance involves the ability of plants to dehydrate partially and then revert back to growth when water becomes available. Tolerance inclines the plant to survive water inadequacy (Blum, 2005). Factors that influence wheat's response to water stress include the underlying genetic composition, the developmental stage when water stress is experienced, the duration and severity of the stress, and the plasticity of the plant genome in response to stress (Nezhadahmadi et al., 2013).

Drought tolerance in wheat is controlled by many genes (Nezhadahmadi et al., 2013). Limited knowledge is available on drought-responsive genes and the roles they play during drought-stress. Most of the studies focus on the wheat seedling and vegetative stage of development. While important, during this time, important hardening and physical traits develop, which prepare the seedling for life under droughty condition which can

permanently determine morphological and structural traits which carry over into maturity. In terms of vegetative development, recent research reveals that the junction or jointing phase (between the vegetative and flowering stage) is highly susceptible to drought as well (Nezhadahmadi et al., 2013). By a large the greatest impact on wheat productivity is found during anthesis and grain filling stages of wheat development where the wheat plant is most vulnerable. Serious stress at either of these stages will result in a dramatic loss of yield.

Beyond studying the morphological and anatomical response to stress at different stages of wheat development, recent attention has been paid to the transcriptional and translational responses to periods of water stress. The use of genome-wide transcript profiling and gene expression analysis under drought stress can identify drought-responsive genes. Recently, many gene classes have been confirmed to be up- or down-regulated by drought stress (Hu & Xiong, 2014; Langridge & Reynolds, 2015). The most efficient technique for identifying differentially expressed genes uses next-generation sequencing technology of RNA. RNA-sequencing permits the quantification of expression based on sequence abundance under control and treated conditions. A major advantage of next-generation sequencing is in its increasing processing power and decreasing costs per sequence. RNA sequencing allows investigators to simultaneously monitor the expression of thousands of drought-responsive genes (e.g. dehydration-responsive element-binding gene, aquaporin, late embryogenesis abundant proteins and dehydrins) and their pathways. Here we use RNA-seq to evaluate the transcription profile of two wheat cultivars exposed to water limitations.

The importance of the flag leaf and seed head in supplying and receiving carbohydrate during, respectively, during grain filling in cereals crops such as wheat, barley, oats, triticale, etc. cannot be overstated. It is the top most leaf and as such it intercepts quite a lot of radiation during the most critical stages of development. Assimilation translocation from the flag leaf (source) to the panicle (reproductive sink) is enhanced by the proximity of the flag leaf to the sink. After anthesis, it is generally accepted that the flag leaf provides a significant percentage of assimilates to the developing grain. When the flag leaf of a small grain cereal crop is lost or destroyed, grain yield is significantly reduced (Surya, 2015). In winter barley (*Hordeum vulgare L.*) and at the time of maximum stem mass, high molecular weight carbohydrates comprise approximately 30% (dry weight) of the stem (Bonnett & Incoll, 1993). In wheat and during filling, carbon labelling (^{14}C) studies showed that pre-anthesis carbon is remobilized from the flag leaf into the stem and subsequently remobilized again into the grain (Yang et al., 2004). Understanding the relationship between the source (flag leaves) and developing seed head during grain filling will provide significant insight into the transcriptomic associations of this source sink relationship. Currently there are no studies to date that examine simultaneously these two essential organs. Accordingly, our objectives are exploratory in nature in order to characterize the transcriptomic response in flag leaves and seed heads in response to decreasing water availability in two contrasting cultivars of soft white spring wheat.

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CHAPTER II

REVIEW OF LITERATURE

Wheat, one of the first domesticated food crops, has been the basic staple food of the major civilizations of Europe, West Asia, and North Africa for 8,000 years (Curtis, 2002). Currently, its production leads all crops in terms of land area and is one of the most important crops for human nutrition (Bruinsma et al., 2003). Wheat is grown globally in a wide range of environments under irrigated and rain-fed conditions. About 20% of 1.5 billion hectares worldwide are irrigated and provide nearly 40% of the world's wheat production with the remaining 60% is from rain-fed agriculture (Molden, 2007). With climate change, drought is considered one of the major limitations (among other abiotic stresses) to growth, development, and productivity of global wheat in many countries. The impact of climate change on wheat yields is predicted to be negative in the coming years (Cheng et al., 2016; Li, Ye, Wang, & Yan, 2009; Mwadzingeni et al., 2016).

According to its growing season, wheat can be classified into two categories: winter wheat which can be planted in the fall and harvested in the spring (about 75% of wheat in U.S), and spring wheat which can be planted in the spring and harvested in early fall or late summer. Moreover, wheat can also be categorized by its hardness (hard and soft) and its color (red and white). In general, there are six types of wheat grown in the US: hard red spring, hard red winter, soft red winter, durum, soft white wheat, hard white wheat.

Common bread wheat (*Triticum aestivum* L.) is the most widely grown type of wheat used for producing flour for bread, pancakes, muffins, and cakes to name a few. On the other hand, durum wheat (*Triticum turgidum ssp. durum*) is usually used in pasta and couscous. Soft white wheats are generally for pastries and cakes.

Drought is the main limiting factor for crop production. Periodic droughts worldwide have participated in the reduction of wheat productivity throughout the ages. Historically, Australia one of the four largest wheat exporting countries in the world suffered a devastating drought between and 25% reduction in overall yield from 2002-2008 (Long & Ort, 2010). In the US, drought caused extreme crop damage in 29 states in 2012, (Gilbert, 2012). This was ranked as one of the most expensive natural disaster in U.S. history exceeded $17 billion from Federal crop indemnity payments alone (USDA, 2013).

Drought resistance is the ability of plants to grow and develop under limited water availability. For the study of drought resistance, wheat is an excellent system to study mechanisms associated with drought resistance due in large part to its large complex polygenic genome. The completion of the wheat genome sequence for hexaploid bread wheat was a major step in facilitating the identification of key genes that control complex traits like drought (Edmeades, 2008). Drought resistance can be divided up into three areas including: avoidance, tolerance, and escape. Avoidance of drought is the ability of plants to maintain plant water content by reducing transpiration and by increasing water uptake once the water is available (Agbicodo et al., 2009; Izanloo et al., 2008; Khan & Iqbal, 2011). Furthermore, drought tolerance means that plants are capable of tolerating water deficiency conditions via biochemical and physiological mechanisms and subsequently

avoiding drought effects (Khan & Iqbal, 2011; Mitra, 2001). Drought escape from drought is the plant ability to complete the plant life annual cycle before starting of the drought season. Drought resistance mechanisms vary for each genotype requiring extensive analysis to identify the principle mechanism associated with a given cultivar.

Tolerance to drought stress is a complex quantitative trait with a complicated physiological and biochemical phenotype that is controlled by many genes. Under molecular genetic analysis some of these genes are recognized as quantitative trait loci (QTL). The quantitative trait loci (QTL) exhibit effects when many genes combine into additive and non-additive effects. Tolerance to drought has low heritability because of polygenic nature of inheritance subject to environment interaction (Fleury et al., 2010; Khan & Iqbal, 2011). Despite these challenges, the genomic diversity and constituency within varieties still form the basis for improving these quantitative traits (such as, drought tolerance). Moreover, genetic variability in wheat can be explored by studying germplasm from centers of origin and diversity which typically contain plants that form the basis of our modern wheat cultivar pedigrees (Mwadzingeni et al., 2016; Nezhadahmadi et al., 2013).

Biotechnology and Drought Tolerance in Wheat:

Genomics-assisted selection has not yet contributed much to wheat drought resistance improvement (Berkman, Lai, Lorenc, & Edwards, 2012). Most improvement is based on conventional breeding approaches. Private companies and public entities continue to seek to increase drought tolerance through their breeding programs or by using transgenic approaches. For example, the Monsanto Company is considered one of the

leading companies in transgenic research for drought tolerance in maize. They recently started commercial sales of a transgenic and drought tolerant maize product in 2012. These lines were produced by inserting a cold shock protein from a bacterum into corn resulting in increased drought stress tolerance and an average of 15% yield improvement under drought conditions. Investigations into mechanisms of this commercial transgenic corn indicated an increased sensitivity to abscisic acid associated with more rapid stomatal closure. The stomatal response was shown to reduce water loss via transpiration. However, Monsanto has since conceded that improvements in water use efficiencies varies based on other environmental factors indicating a substantial gene by environment interaction. Initial results from Bayer have resulted in other genes that decrease oxidant load that lead to tissue damage (Edmeades, 2008). Other genes that are being actively promoted to enhance transgenic tolerance to drought include genes from the DREB and CBF transcription factor families. Thus the transgenic approach holds some promise, but requires extensive characterization and adjustments for it to be practicable (Edmeades, 2008).

Breeding for Drought Tolerance

Many efforts have been undertaken world-wide through the use of conventional or molecular breeding approaches for improving drought stress in wheat (Mwadzingeni et al., 2016; Saleem et al., 2016). Tolerance to drought is a complex trait and its expression is typically affected by the environment. In wheat, the greater variability in genes associated with drought can be better explored from selections obtained from the germplasm centers of origin and diversity (Dvorak, Luo, & Akhunov, 2011), in the case of wheat the fertile

crescent area of the Middle East (Nevo, 1998). To improve drought tolerance, the diversity within breeding populations needs to be enhanced and used for genetic selection or modification of adaptive mechanisms (i.e. drought escape, dehydration tolerance, and dehydration avoidance) (Blum, 2010). In plant breeding systems, most traditional marker techniques cannot detect specific genes associated with transposable elements, non-coding regions, low-copy genomic regions, and less prolific repeats that could play critical roles in phenotypic traits (Edwards, Batley, & Snowdon, 2013; Elshire, 2011). Most breeding approaches examines genetic selection based on yield components to make progress in obtaining drought responsive cultivars. Yield components such as overall grain weight per plant, numbers of grains per plant, and individual grain weight have the greatest impact on overall yield. Additional factors that best explain yield response are under drought conditions include harvest index, water use efficiency and minimizing genotype by environment interaction (Eskridge, 1990; Gauch & Zobel, 1997). All these can be used in breeding programs to under water stress management to identify superior selections for wheat improvement purposes.

Metatranscriptomics

Whole transcriptome analysis enables scientists and researchers to better understand changes in gene expression-level responses to environmental stress. Transcriptomes of non-model organisms have been reported for many plants including wheat (Davidson et al., 2012; S. Singh, Parihar, P. Singh, R. Singh, & Prasad, 2016; Wan et al., 2008). Furthermore, study of drought resistance and drought tolerance by transcriptomic analysis is becoming more widespread within the scientific community

(Zhou et al., 2015). The advent of next-generation sequencing technologies has greatly expanded our capabilities by allowing for massively parallel sequencing efforts at a greatly reduced cost. With regard to NGS technology and its advances, RNA sequencing (RNA-seq) has been widely used in plant breeding, especially in those plants that lack complete genomic information. In parallel, efforts to sequence the proteome in wheat treated with salinity and drought, salt (Guo et al., 2012; Peng et al., 2009) have provided translational insights into drought responsive mechanisms. Moreover, results coming from RNA-seq may facilitate the identification of new and interesting biochemical traits (Wang, Gerstein, & Snyder, 2009). The RNA-seq technologies generate large amounts of transcriptomic data in real time, so this data requires investments and expertise in bioinformatics for data management. Furthermore, genes involved in drought tolerance can be functionally characterized by transgenic incorporation and analysis. This is most easily performed in model species such as Arabidopsis, rice or brachypodium where functional information is much more available. Hexaploid wheat has one of the largest genomes of any crop species (17 gigabases in size) encoding for more than 124,000 gene loci. So far only 76% total genome has been sequenced (International Wheat Genome Sequencing Consortium [IWGSC], 2014). With the near sequencing of the wheat genome functional annotation by homology is becoming increasingly useful, but is a long way from complete in comparison to model organisms. The size and complexity of the wheat genome make transcriptomics especially challenging. In addition, RNA-seq and proteomic analysis may lead to the development of markers for a variety of traits to more efficiently advance cultivar development in breeding programs.

Instrumentation

There are many approaches for RNA-seq. The Illumina company has created a wide range of kits and instrumentation to identify differentially expressed genes, targeted DNA sequencing, whole-genome sequencing, targeted RNA-seq, and whole-transcriptome sequencing. Other platforms have been used for RNA-seq analysis including Roche/454 pyrosequencing (the first commercial platforms for NGS), SOLiD (developed by Life Technologies), MinION (Oxford Nanopore Technologies**),** and PacBio (Pacific Biosciences of California, Inc.). The latter two are currently referred to as third generation sequencing technologies and are much more capable of sequencing much larger fragments than the Illumina sequencers achieving of wider *de novo* genome sequencing and gene expression analysis (Berkman et al., 2012; Elshire et al., 2011; Mwadzingeni et al., 2016; Poland et al., 2012). The choice of platform to use often depends on the funds available, the difficulty in dealing with error rates, and requirement of transcript size. The earlier platforms provide more but small sequences while the more recent platforms provide much larger and fewer sequences. Matching the platform to the questions is one of the most critical steps in the process. At the time of initiation of this research the Illumina platform made the most sense for our purposes in that it provided greater read depth with sufficient sequence information for identification purposes and a relatively low error rate.

Wheat Development and Drought Stress

In most crops the most sensitive stage to yield loss due to stress is often during anthesis and grain filling. This is certainly true for corn and soybeans where large crop yield reductions were realized under short periods of water stress for several days (Hunt et al.,

2014; Kebede, Fisher, & Young, 2012; Prasad, Pisipati, Momčilović, & Ristic, 2011). The Heading (pre and post anthesis) is also critical period in the development for the wheat plant. Heading begins with the emergence of the inflorescence from the sheath of the flag leaf. Early heading in certain cultivars may represent a drought avoidance mechanism that allows wheat cultivars to bypass drier and hotter temperatures during seasonal growth. (Kamran, Iqbal, & Spaner, 2014a). At heading the plant is being transformed from vegetative growth to reproductive development (i.e. redirecting assimilates to developing the spike). In winter wheats reproductive development is controlled by the vernalization gene system. Early spring growth can be controlled by one or more alleles involving vernalization: Vrn-1 (Vrn-A1, Vrn-B1, Vrn-D1) or Vrn-3 (Vrn-A3, Vrn-B3, Vrn-D3) (Kamran, Iqbal, & Spaner, 2014b). Vernalization occurs in winter wheats when wheat is exposed to near freezing conditions for 6 weeks or more. Once vernalized then vegetative to reproductive transition is completed. Without sufficient vernalization this will not occur.

Drought stress during anthesis will result in reproductive failure due to a failure of the wheat to self-pollenate, possibly leading to spikelet sterility. Stress during anthesis results in a lower percentage of floret fertilization and a decrease in numbers of developing grains in the spike (Ji et al., 2010). The vulnerability of wheat plants to water stress is especially strong during to the grain-filling stage (Saeedipour & Moradi, 2011). Under drought stress,the ultimate yield depends strongly on the severity of water deficit (Maqbool, Ali, Haq, Majeed, & Lee, 2015).

Grain Filling and Drought

Reproductive development begins prior to anthesis during the late stages of vegetative development where the number of cells that eventually will make up the growing kernel are set. A reduction in total cell numbers decreases the kernel's ability to take up carbohydrate during grain filling resulting in a lower than average test weight (Saini & Westgate, 1999). Furthermore, a dramatic reduction in grain yield can occur during the stages when the grain is being filled with carbohydrates. Maqbool, et al., 2015 concluded that water stress induced at the grain-filling stage presented a significant reduction in wheat yield in comparison with other growth stage stresses. The reduction at grain filling may either affect the ability of the grain to incorporate the carbohydrate or the ability of the leaves (mainly the flag leaf) to export to the grain the carbohydrate. In other words, water limiting conditions may act on the source or the sink and most likely both to effect a reduction in grain yield.

Flag Leaf and Seed Head Importance to Yield

The flag leaf of the wheat plant plays a crucial role in the growth and development of wheat seeds (Ledent & Renerd, 1982). The qualities or characteristics of a flag leaf are regarded by breeders as signatures for high grain yields in wheat. The upper leaves of the wheat plant shade the vast majority of the lower leaves reducing the absorption of solar radiation by a part of the canopy (Birsin, 2005). During flag leaf maturation these lower leaves and stems undergo a carefully choreographed remobilization of nitrogen and carbon compounds for export to the grain. A strong source-sink connection between the developing grains and the flag leaf and the grain, respectively is established during the

grain filling period (Li et al., 2009). First and foremost, the flag leaf is responsible for approximately 75% to 80% of photosynthetic carbon imported into the seed head during grain filling. (Ghooshchi & Omidvar, 2012).

In this manner, the flag leaf is the crucial source of assimilates for both the grain yield and grain filling at least partially because of its close physical association with the developing spike and because it likewise remains green and photosynthetically active for a longer period than the other leaves being the last leaf to senesce (Khaliq, Parveen, & Chowdhry, 2004).

Many studies have been investigated on the effects of water stress on wheat and other crop plants. Most of these studies have been conducted during the vegetative stages of wheat development, much fewer during anthesis and very few if any during grain filling stages. The fact that wheat yields during grain filling are highly susceptible to water stress indicates that this stage should also be examined in detail. The objective of this work is to identify differentially expressed genes in the flag leaf and seed head of two spring wheat cultivars in response to moderate and severe water limitations.

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CHAPTER III

CHARACTERIZE YIELD RESPONSE AND IDENTIFYING DIFFERENTIAL EXPRESSION GENES IN THE FLAG LEAF OF TWO SPRING WHEAT CULTIVARS UNDER MODERATE AND SEVERE DROUGHT STRESS

Introduction

Drought is a key environmental stress factor that can greatly impact wheat yield world-wide. Farooq et al., 2014 wrote that drought can decrease wheat yields by up to 92% depending on the time of onset, the duration and the stress intensity. Plant responses to drought stress are very complex involving plant structures, physiology and molecular and cellular responses that increases the ability of plants to survive, and reproduce under drought stress conditions (Nezhadahmadi, Prodhan, & Faruq, 2013). The impact of water stress varies depending on the developmental state, and the genetic sensitivity to and tolerance towards stress (Lal & Stewart, 2012).

Wheat can be classified into two categories: winter wheat which is planted in the fall and harvested in the spring (about 75% of wheat in U.S), and spring wheat which is planted in the spring season and harvested in early fall or late summer. Moreover, wheat can also be categorized by its hardness (hard and soft) and its color (red and white). In

general, there are six types of wheat grown in the US: hard red spring, hard red winter, soft red winter, durum, soft white wheat, hard white wheat. Common bread wheat (*Triticum aestivum* L.) is the most widely grown type of wheat used for producing flour for bread, pancakes, muffins, and cakes to name a few. On the other hand, durum wheat (*Triticum turgidum ssp. durum*) is usually used in pasta and couscous. Soft white wheats are usually used for bakery products such as cakes and pastries. Most research is performed on the hard red winter and spring wheats with much less on the soft white types.

Developmentally speaking both the reproductive and grain filling stages in wheat development are acutely vulnerable to drought stress. Grain yield in most cereals depends on what is termed yield components including: the planting density, the number of spikes per plant, the number of grains per spike and the weight of the grain (Birsin, 2005). These yield components can be severely affected by water stress. The number of grains per spike is determined prior to anthesis when the initial cells are beginning to divide and populate the immature spikelet. Stress during this period reduces total number of initial cells that can form into mature spikelets thereby reducing the overall yield. Stress after anthesis during grain filling can also significantly impact yield by reducing the translocation of carbohydrate into the growing wheat kernel thereby reducing overall weight gain. Ultimately, final grain yield depends on formation, partitioning, accumulation, and translocation of photosynthesis during the grain filling period (Fischer & Stockman, 1986; Hafsi et al., 2001).

Although, parts of the wheat plant contribute to spike development and formation, the upper three leaves in the wheat plant are exceptionally important to grain filling, and

yield (Birsin, 2005; Sen & Prasad, 1996). The flag leaf is last of the three upper leaves to appear; this leave begins it development and expansion after booting and prior to anthesis, and is clearly recognized by the collar ringing the stalk. The flag leaf of wheat was found to contribute more than 50% of the carbon associated with grain filling (El Wazziki, El Yousfi, & Serghat, 2015) indicating a critical importance to overall yield. High yield varieties prolong the grain filling activities of the flag leaf. An extension of this green phase allows more time for the production and translocation of organic carbon into the developing grain. Across 463 wheat lines from the International Maize and Wheat Improvement Centre (CIMMYT), a correlation between yield and a prolonged period of flag leaf photosynthesis was observed under drought and/or heat stress only. Wheat plants engineered with NAM RNAi gene which delays senescence carried out 40% more flag leaf photosynthesis than control plants, but the same plants had the same duration and rate in starch accumulation during grain filling and the same grain weight (Borrill, Fahy, Smith, & Uauy, 2015) indicating that flag leaf duration is only one component of a multicomponent pipeline. Focusing research on the molecular, physiological and photosynthetic activities of the flag leaf is particularly important in understanding the mechanisms associated with wheat productivity (Loss & Siddique, 1994; Turner, 1996).

With age, the photosynthetic activity of flag leaves decreases and their grain filling function are taken over by photosynthetic tissue associated with the maturing seed head and their chlorophyll laden glumes. Glume photosynthesis during and after flowering also has a strong impact on grain yield (Olszewski, Makowska, Pszczółkowska, Okorski, & Bieniaszewski, 2014). The grain ears contribution to grain yield range from 10% -76% of total yield (Abbad, El Jaafari, Bort, & Araus, 2004). Unfortunately, few studies have

focused on the molecular aspects in drought stress in the flag leaf or seed head with most studies being conducted on young vegetative plants. Thus understanding the mechanisms associated with drought response and stress tolerance in these significant tissues may be more key to uncover drought response or drought tolerance mechanisms compared to vegetative wheat tissues. Furthermore, few studies have worked with soft white spring wheats with most studies focused on bread wheat both of the winter and spring cultivars.

Whole transcriptome analysis enables researchers to better understand changes in gene expression responses to environmental stress. Furthermore, study of drought tolerance by transcriptomic analysis is becoming more widespread within the agricultural scientific community (Zhou et al., 2015). Transcriptomes of non-model organisms has been reported for many plants including wheat (Singh et al., 2016; Davidson et al., 2012; Wan et al., 2008). The advent of next-generation sequencing technologies focused on transcriptomics has greatly expanded our capabilities of identifying differentially expressed genes by allowing for massively parallel sequencing efforts at a greatly reduced cost. Promising differentially expressed genes can be cloned and evaluated functionally for their impact on drought tolerance.

There are many obstacles to understanding the wheat response to drought. The size and complexity of the wheat genome make transcriptomics especially challenging. Hexaploid wheat has one of the largest genomes of any crop species (17 gigabases in size) encoding for more than 124,000 genes making it more challenging to screen and identify candidate genes. Furthermore, only 76% total genome has been sequenced to date and many of the genes have yet to be functionally annotated with precision, (International

Wheat Genome Sequencing Consortium, 2014). The fact that the genome has only limited functional annotation makes interpreting gene expression data of limited value to wheat breeders and physiologists. Nevertheless, efforts must be made to move the work forward.

Identifying genes that enable wheat plants to adapt to drought stress is a major goal of this project. Screening for differentially expressed genes is the first step in this process. Identifying key genes that are overexpressed during stress may provide markers for breeding programs to more efficiently develop drought tolerance cultivars (Inoue, Inanaga, Sugimoto, An, & Eneji, 2004). Using RNA-seq technology is likely the best way to probe the wheat genome for differentially expressed genes and to infer gene function efficiently. Therefore, the objective in this work was analyzed changes in the transcriptome associated with two levels of water limitation in two wheat cultivars that differ in terms of tolerance to water stress, in both the flag leaf during grain filling.

Material and Methods

The experiments were performed at the #315 USDA greenhouse facility of USDA, in Stillwater, Oklahoma, during the spring season of 2016. The average of temperature was between 15- 25 °C, with 16 hours light. Two soft white spring wheat cultivars (Alpowa and Idaho) were planted in 10.2x10.2x30.5 cm. CP412CH TreePots (Stuwe and Sons Inc) to a depth of 5 cm. Pots were sanitized with 70% ethanol and then filled with 1.9 Kg of a sandy clay loam soil fertilized with ammonium nitrate to 70 kg/ha in a way that reduces pot to pot variation. Pots were planted with three seeds and upon emergence trimmed to one plant per pot. All pots were regularly hand weeded and checked for insects and were sprayed with Immunox (Spectracide, INC. OR, USA) in case of powdery mildew, and neem oil (Certis, LLC, MD, USA) in case of aphid infestation. Three tensiometers were installed in control pots to 15 cm depth, to measured water potential. Water was provided to all pots when the tensiometer reached (40 centibars) based on the recommendations of the manufacturer for wheat grown in silt loam soil (Irrometer Co. Inc., Riverside, CA). Five days after 50% of the plants showed anthers (Feekes 11.2, post-anthesis and early grain filling), wheat plants were watered with either 220 ml (100% well-watered), 110 ml (50% well-watered), and 55 ml (25% well-watered) and then water withheld for 5 and 8 day at which time plants were harvested. There were a total of three stress intensity levels (WW, MS, SS), two cultivars, two stress durations and 5 replicate plants for a total of 60 plants.

At harvest, flag leaves were removed and weighed along with the remaining tissues. and wrapped in aluminum foil and frozen in liquid nitrogen and then stored at -80 °C for later use. Tissue was fixed within 2 to 3 minutes of harvest. Weight data were transferred

to an Excel spread sheet and analyzed statistically using in JMP® (SAS institute) software using multi-factor analysis of variance (ANOVA) based on the least-square fit significant differences among the effects, including: cultivar, duration, and stress intensity with interactions. Effects were cultivar, duration, and stress intensity. Tukey's multiple comparison procedure was used to find differences within stress intensities along with interactions based on a significance p-value of 0.05.

RNA extraction for flag leaves was performed using the TRIzol® Reagent protocol (Life Technologies Inc., Carlsbad, CA). The first step involved the manual homogenizing of flag leaf tissue samples in a mortar and pestle with liquid nitrogen. Frozen tissue weighing 0.25 g was then placed in a ground glass homogenizer with 1 ml of TRIzol reagent and homogenized until all tissue was solubilized (1 min). Extracts were placed on ice, analyzed for quantity and quality, and then frozen at -21 °C. RNA was further purified using the Qiagen RNAeasy kit (Qiagen, Valencia, USA) according to manufacturer's instructions. Extracted RNA was analyzed on a NanoDrop spectrophotometer (ND-1000, ThermoFisher, MA), with the goal of obtaining extracts exhibiting 260 nm/280 nm absorption ratios greater than 1.8. (Barbas, Burton, Scott, & Silverman, 2007) and quantities greater than 33 μg/ml based on the 260 nm absorbance.

Frozen and purified RNA was 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. According to Oklahoma Medical Research Foundation prior to RNA-seq analysis quality control measures was implemented.

Concentration of RNA was ascertained via fluorometric analysis on a Thermo Fisher Qubit fluorometer. Overall quality of RNA was verified using an Agilent Tapestation instrument. Following initial QC steps sequencing libraries was generated using the Illumina Truseq Stranded mRNA with library prep kit according to the manufacturer's protocol. Briefly, mature mRNA was enriched for via pull down with 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).

Bioinformatics analysis was performed for eight duration samples, using the OSU High-Performance Computing Center (HPCC). The sequence files were downloaded in Fastq format from the OMRF database. Quality control was attained using FastQC as a stand-alone program providing a swift analysis of the dependability of the sequence reads (Andrews,2010). The Fastq files were then screened to the level of Q30. Hisat2 was utilized

to align the genes to the wheat genome (Kim, Langmead, & Salzberg, 2015) using the International Wheat Genome Sequencing Consortium (IWGSC). The reference genome in Gene transfer format (GTF annotation) was downloaded and prepared for quantification according to the Hisat2 procedure (Pertea, Kim, Pertea, Leek, & Salzberg, 2016). SAM alignment (Li et al., 2009) file conversion, sorting, and preparation was performed using the Samtools program (Li, 2011). Quantitative predictions of the transcript were given by FPKM (fragments per kilobase per million sequenced reads) levels were produced using the RNA-seq software Stringtie. Statistical comparison of all transcripts was reached using the R package Ballgown (Pertea et al., 2016). Transcripts which revealed significant differential expression were annotated utilizing the UniProt database (UniProt, 2017). The level of expression was determined based on the number of sequences for each unigene. The degree of differential expression was determined based on the numbers of sequences for a given unigene in treated (MS, and SS) compared to WW. Ballgown was used to determine significant differential expression based on a p value < 0.05 and adjusted for false discovery rate (FDR). The fold up and down-regulation was calculated and those 20 differentially expressed genes with the highest fold change were identified. Differentially expressed genes were further functionally assigned GO: Terms based on the wheat genome annotation. The top 20 GO terms with the most differentially expressed gene members were determined using an Excel spreadsheet for each cultivar and stress intensity.

RESULTS AND DISSCUSION

Flag Leaf Weight (FL):

Here we examine the response of two soft white spring wheat cultivars to drought stress under controlled greenhouse conditions, focusing our attention on the flag leaf during grain filling. Alpowa and Idaho, two white spring wheat cultivars are believed to be resistant to drought based on higher biomass levels (Alotaibi, 2018) and greater yield (Li, 2011) under water limiting conditions. The mechanisms of resistance need to be investigated, whither from the perspective of avoidance, escaping or tolerating water limitations to be of use to plant breeders.

Here we determined that flag leaf weights were significantly affected by wheat genotypes, drought stress intensity and their interactions at both durations (Figure 1). Flag leaf weight has been correlated with grain yield (Johnson, Bruckner, & Morey, 1990) as is flag leaf length and area (Yang et al., 2016) and under well-watered and drought conditions (Qian, Jing, Wang, & Chang, 2009). Surprisingly, flag leaves from susceptible Idaho contained significantly more mass than those of tolerant Alpowa under well-watered conditions after 5 and 8 days under stress intensity. This indicates that flag leaf mass is not a likely factor in the resistance differential between the two cultivars. In contrast to flag leaves shoot mass was greater in Alpowa than in Idaho (Alotaibi, 2018).

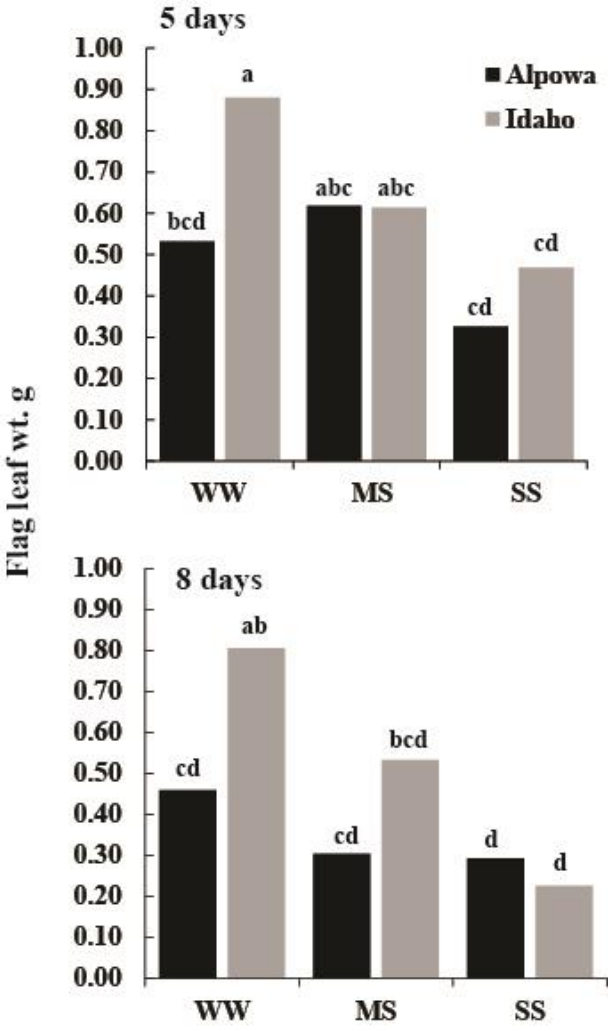
There were cultivar differences associated with increased stress intensification going from WW to MS to SS. With increasing stress intensity there is an ever decreasing trend in flag leaf weight in Idaho at 5 and 8 day durations, while in Alpowa the trend is

much less pronounced, and probably non-linear. Alpowa flag leaf weights decrease less as a percentage of well-watered than Idaho at both 5 and 8 days. Alpowa flag leaf weights averaged across durations were 91% and 62% of well-watered under MS and SS, respectively, while Idaho was 68% and 41% under MS and SS, respectively. Guendouz et al., (2016) studied the effect of irrigation on specific leaf weight of durum wheat found that water stress reduced the specific leaf weight to 42% of well-watered. This decrease in leaf weight is most likely due to senescence induced dehydration which is much greater in Idaho than in Alpowa. Consequently, the Alpowa response showing a shallower decline in senescence induced dehydration may actually be associated with the resistance mechanism. Less senescence induced dehydration with stress intensification may actually permit greater level of photosynthesis which ultimately leads to greater yield. Inoue et al., (2004) who reported that the photosynthetic rate of; ear and flag leaf were significantly higher and less affected by drought in drought resistant wheat cultivars than in drought sensitive ones. It would be of interest to monitor flag leaf water potential throughout time and stress intensification to verify this suggestion.

The grain filling process is complex and dynamic in nature beyond the limited nature of the treatment structures reported here. It also must be noted that the stress imposition here began 5 days after 50% anthesis which is likely to be early in the grain filling stage of development. It has been proposed that flag leaf photosynthesis contributes more during the early phases of grain filling compared to later stages. There are reports that later stages of grain filling are completed by stem fructan conversion and remobilization of carbohydrate into the developing grain (Borrill et al., 2015). Fructans storage in stem tissue may be a mechanism to store excess carbon produced

photosynthetically in flag leaves and other tissues while grain filling is under way and where grain import of carbohydrates is sink limited (Borrill et al., 2015). Thus speculating, Alpowa may have smaller but more photosynthetically active flag leaves and may be more capable of storing photosynthetic reserves than Idaho for later grain filling activities.

Fig 1. Flag leaf weights for two soft white spring wheat cultivars (Alpowa and Idaho treated under WW conditions to Feekes 11.2 and then treated under WW (well-watered, 100%) MS (moderate stress, 50% well-watered), SS (severe stress, 25% well-watered) for 5 and 8 days thereafter. Different letters on top of columns represent significant differences (HSD p-value < 0.05).



Flag Leaf Transcriptomic Changing

The sources sink balance associated with high yield are likely the result of metabolic activities associated with the flag leaf and seed heads. Here we concentrate on the transcriptomic response of flag leaf from two cultivars of wheat to water limitations exposed to MS and SS, and differentially expressed genes associated with comparisons between WW and MS and WW and SS stress. The numbers of up and down-regulated transcripts in Alpowa and Idaho under the two stress intensities are listed in Table 1. Overall there were 2.32 and 3.9 fold more up and down-regulated genes, respectively, indicating a much higher level of overall transcriptional activity in Alpowa compared to Idaho. There was a 10.1-fold greater transcriptional response in MS-Alpowa compared to SS-Alpowa. However, on the surface this imbalance may actually be artefactual in nature given that the sequences obtained under SS conditions were of substantially lower quality than those under WW or MS conditions making comparison across stress intensities more problematical. In MS-Idaho the imbalance is not as pronounced except for down-regulated transcripts where MS-Idaho showed 4.5 fold more down-regulated transcripts than SS-Idaho. Shared transcripts between stress intensities MS and SS constituted only 3 to 17% of the overall differentially expressed transcripts in both Alpowa and Idaho, indicating that the two stress intensities share very little in common in terms of transcriptional response.

Table 1. Transcriptome changes in Alpowa and Idaho cultivars in the flag leaf as a response to moderate and severe water stresses conditions for eight duration treatment

	Regulation	MS	MS+SS	SS	Total
Alpowa	Up	33704	8974	7962	50640
	Down	41448	1393	3619	46460
Idaho	Up	9936	1761	8361	20058
	Down	8527	1468	1880	11875

Gene Ontology

It's clear that RNA next-generation sequencing (RNA-seq) provides many minute details concerning the transcriptional landscape under considered treatment conditions. Gene ontology provides a systems biology approach that is simple and widely used to understand and highlight functional processes that are affected in response to treatment. Differentially expressed transcripts are grouped into preformatted functional categories including biological processes, cellular processes and molecular processes (Young, Wakefield, Smyth, & Oshlack, 2010; Glass & Girvan, 2014). Here we restrict our analysis to biological processes which constitute a category that gives functional information for a given set of differentially expressed transcripts. The GO analysis was achieved for the two cultivars under study (Alpowa and Idaho) in the two organs (flag leaves and seed heads) under two conditions of stress intensity (Moderate-Stress (MS) and Severe-Stress (SS) in comparison to Well-Watered (WW). This research constitutes the first analysis of transcriptional activities in the flag leaves during the critical stage of grain filling when a significant portion of grain yield is determined. The closest research associated with transcriptional activities in the flag leaf during grain filling was conducted in rice with a focus on stem remobilization of carbohydrates and not the transcriptional activities of the flag leaf itself (Wang et al., 2017), or in the flag leaf but not during grain filling (Xu et al., 2011)

The top 20 GO terms in of biological process for up and down-regulated transcripts, in response to MS and SS treatments have been selected (Table 2). In addition, the ratio of up to down-regulation is given as an indicator of the transcriptional direction. Most of the

top GO terms were predominantly down-regulated with a ratio of less than 1.0. This is especially true with Alpowa where all of the top 20 GO terms contained more down-regulated than up-regulated DE genes under both stress intensities. Idaho showed a strong tendency towards down-regulation with 16 out of 20 under MS and 12 out of 20 under SS stress conditions. Thus in terms of biological processes it appears that in both cultivars, especially Alpowa, the turning off of genes is favored over turning on of genes. This is likely given that the wheat plants at the time were at Feekes 11.3 where senescence processes are likely to be operative and normal metabolic channels are likely to be more turned off than turned on. However, this contrasts with the data observed in Table 2. where for the most part up and down-regulation of gene expression are evenly balanced, and even in favor of up-regulation under SS conditions in Idaho. It must be remembered that only a small fraction (~2 - 6%) of total numbers of differentially expressed genes are represented with GO terms due to the lack of functional annotation in the wheat genome. A more complete analysis can be obtained by resorting to a GO Terms from another closely related model species such as *Brachypodium*, *Rice*, or even *Arabidopsis* which genomes are much more functionally annotated.

In Flag leaves across all treatments, cultivars and up and down-regulation, the most abundant GO terms are related to transcriptional biological processes and to a lesser extent metabolic, transport, and translational activities. The GO term with the absolute greatest number of members was regulation in transcription (GO: 6355) and the next most abundant was transcriptional processes (GO: 6351). Of these two, down-regulation dominated across all treatments. Their child terms which include the negative regulation of transcription (GO: 45892) and the initiation of templated transcription (GO: 6352). Negative regulation

of transcription child terms was predominantly down-regulated for 28 DE transcripts in MS-Alpowa and 18 DE transcripts in MS-Idaho. This may indicate a general reduction in transcription factors that reduce transcription overall or the rate of transcription. The child term positive regulation of transcription was not observed either. Thus under water stress, it appears that the brake for transcription is down-regulated and the accelerator is not operative supporting a reduction in transcriptional regulation during grain filling. A reduction in regulation of transcription is likely to occur as a prequel to senescence induced cell death. The other predominant child term, initiation of transcription, also favored down-regulation with 62 transcripts in MS-Alpowa and 22 in SS-Idaho showing reduction in comparison to control. Thus, initiation of transcription and control of transcription are downregulated late in wheat development during the early stages of grain filling and under drought stress conditions.

Other active processes related to nucleic acid activities included DNA integration (GO:15074), DNA repair (GO: 6281), translation (GO: 6412). and RNA processing (GO: 6396). RNA processing is associated with the maturation of RNA transcripts prior to translation. Child terms for with RNA processing in this study are those associated with the organelles: chloroplast and mitochondria. In general, RNA processing term showed substantial down-regulation in MS-Alpowa and MS-Idaho under the two water limiting conditions. Down-regulation in RNA processing corresponds nicely with reduced initiation and a general down-regulation of transcriptional control alluded to above. DNA repair is the mechanisms that operates to restore damaged DNA to functional activity. These DE transcripts exhibited down-regulation in both cultivars. The only child term was for mismatch repair (GO: 6298). Mismatch repair was predominantly down-regulated in

Alpowa across all treatments, but up-regulated in Idaho indicating a cultivar difference. Mismatch repair has been shown to be more active in response to environmental stress (Kunkel & Erie, 2015). Mismatch repair genes have been shown to be down-regulated under stress conditions in Arabidopsis (Kiselev, Ogneva, Dubrovina, Suprun, & Tyunin, 2018). Greater need for mismatch repair in Idaho may reflect a greater level of oxidative stress leading to DNA damage. Translation (GO: 6412) is the cellular metabolic process that produces nascent proteins. Translational activities were for the most part down-regulated under MS and SS Alpowa but strongly upregulated under MS and SS conditions in Idaho in both cultivars indicating a strong cultivar bias. Translational responses to drought stress are known to change dramatically under water limiting conditions compared to well-watered treatments (Lei et al., 2015). DNA integration (GO: 15074) which means the incorporation of small DNA fragments into larger DNA molecules such as a chromosome, were down-regulation in all treatments. While no child terms were found for integration activities, typical integration events in wheat may involve transposition events, given that 68% of the wheat genome has its origin with transposition (Li, Zhang, Fellers, Friebe, & Gill, 2004). Furthermore, transposition activities appear to be up-regulated under drought stress (Alzohairy et al., 2014). However, here, it appears that transposition is predominately down-regulated during the grain filling stages of wheat development under stress whereas in the vegetative stages it was up-regulated (Alotaibi, 2018) indicating that transposition activities are likely associated with pre compared to post-anthesis processes.

Gene Ontology terms related to metabolic process (GO: 8152) were substantially down-regulated under stress in both cultivars. Metabolic processes are a general term that

includes anabolism and catabolism whereby macromolecular molecules are synthesized or degraded. Child terms of significance in this project included protein polyubiquitination (GO: 209) nitrogen compound metabolic process (GO: 6807), the general term biosynthetic processes (GO: 9058) and cellular metabolic processes (GO: 44237). Protein polyubiquitination involves the addition of multiple ubiquitin groups to protein destined for degradation or for endocytic trafficking. For the most part this process was down-regulated in both MS-Alpowa and MS-Idaho but upregulated in SS-Alpowa. Nitrogen compound metabolic processes involve metabolism of nitrogen containing compounds including proteins and amino acids. This process was up-regulated in MS-Alpowa and down-regulated in the other treatments. Presumably nitrogen metabolism is critical to the recycling of nitrogen containing compounds for export to the developing grain. In previous work, nitrogen compound metabolic process were predominantly up-regulated under crowding (Choe, Drnevich, & Williams, 2016). The other two child terms associated with metabolism includes biosynthesis and cellular metabolism, both of which were strongly down-regulated in Alpowa indicative of the general relaxation of biosynthesis as opposed to catabolism during grain filling in the flag leaves. Additional GO terms related to metabolism included carbohydrate metabolic process (GO: 5975), lipid metabolic process (GO: 6629), and ubiquitin-dependent protein catabolic process (GO: 6511). Carbohydrates metabolic process (GO: 5975) are processes involving the synthesis, degradation of carbohydrates, or the attachment of carbohydrates to another molecule. For the most part these biological processes were well represented with on average of over 152 DE transcripts across cultivars and stress intensities. Down-regulation was especially evident in SS-Alpowa where only 18 genes were up and 107 genes down-regulated. Child terms of

carbohydrate metabolic processes were not well represented within this biological process. The down-regulation of carbohydrate metabolism during grain filling in the flag leaf is somewhat puzzling given carbohydrate synthesis is a major function of the flag leaf during grain filling. It may be that some synthesis processes associated with export are maintained but other carbohydrate metabolic activities associated with growth are curtailed. Lipid metabolic process (GO: 6629) were not as prevalent compared carbohydrate metabolisms in the flag leaf, and were down-regulated in flag leaf tissues under stress. The only major child term represented was for lipid catabolic processes. Lipid catabolism associated with oxidative stress in the flag leaves is known to function during senescence and drought stress (Simova-Stoilova, 2009). Along with the predominant down-regulation of Lipid and carbohydrate catabolism includes the GO term Ubiquitin-dependent protein catabolic process (GO: 6511) which involves the ubiquitin mediated degradation of proteins coinciding with the down-regulation of polyubiquitinization referred to above. This GO term was substantially down-regulated and the major child term associated with these activities involve the proteasome, a protein complex associated with protein degradation. Along with protein degradation activities in the flag leaf, protein folding (GO: 6457) was also down-regulated except under severe stress in the flag leaves of SS-Idaho where it was strongly up-regulated. The down-regulation of protein folding is likely co-regulated with the overall protein degradation activities except in the cultivar Idaho. Thus under stress in the flag leaf both carbohydrate, lipids and proteins catabolism appears to be substantially down-regulated in both cultivars.

During drought stress conditions cellular transport processes were substantially altered. Biological processes including the general GO term Transport (GO: 6810) which involves the movement of molecules between cellular components or between cells. Child terms for the general transport GO term included transmembrane transport (GO: 55085), the transportation of solute through the cell membrane, intracellular protein transport (GO: 6886) which is the movement of protein between particular cellular compartments, and vesicle-mediated transport (GO: 16192) a cellular transport process that involves membrane-bounded vesicles. This latter process is a major contributor to protein integration into membrane structures. All of the above mentioned transport processes are down-regulated in all cases except for SS-Idaho where there is a slight up-regulation in transport associated DE transcripts. Interesting child terms associated with these include: vacuolar transport, protein targeting, exocytosis, and Golgi vesicle transport. Thus it appears under stress conditions there is significant transport of proteins to the vacuole and/or likely the exterior to the plasma membrane. Vacuolar transport may be associated with detoxification activities while exocytosis may be associated with senescence associated export of proteins for cell wall degradation.

A variety of other GO Terms were influenced by drought stress in the flag leaves. These include cell wall organization (GO: 71444), response to oxidative stress (GO: 6979), biosynthetic processes (GO: 9058), recognition of pollen (GO: 48544) and embryo development and seed dormancy (GO: 9793). With few exceptions these reflect substantial down-regulation. Changes in cell wall organization (GO: 71555) includes child terms such as cell wall organization, and modifications all down-regulated functions and involved in

maintaining cell shape, protection them from osmotic lysis, processes of assembly or disassembly in both cultivars and stress intensities. The response to oxidative stress was primarily down-regulated in both cultivars and stress intensities and particularly in SS-Alpowa. This GO Term is associated with the cells reaction to oxidative stress as a result of exposure to high levels of reactive oxygen species, e.g. superoxide anions, hydrogen peroxide, and hydroxyl radicals. Oxidative stress under abiotic stresses or senescence processes creates an imbalance in the redox status of plant cells (Das, Nutan, Singla-Pareek, & Pareek, 2015) leading to cell death. Oxidative stress response is indicated to be significantly connected with water limitations in wheat (Devi, Kaur, & Gupta, 2012) and other plants (Sharma, Jha, Dubey, & Pessarakli, 2012). The last two GO terms are recognition of pollen (GO: 48544) embryo development ending in seed dormancy (GO: 9793). This is unexpected in that we are well beyond pollination. However, it is possible that these transcripts may have alternative functions unrelated to pollination. Embryo development ending in seed dormancy (GO: 9793) which constitutes a cascade of events that initiates in embryo development and terminates in seed dormancy. This function was slightly down-regulated in MS-Alpowa, SS-Alpowa and MS-Idaho but was up-regulated in SS-Idaho.

Table 2. Flag leaf gene ontology terms for Alpowa and Idaho cultivars after 8 days of water limiting treatments and under two conditions of stress intensity, moderate and severe stress. The number and ratio of up and down-regulated transcripts is given under each of the top twenty terms.

		Moderate Stress (50% WW)						Severe Stress (25% WW)					
		Alpowa			Idaho			Alpowa			Idaho		
Gene Ontology Terms	GO#	Up	Down	Ratio	Up	Down	Ratio	Up	Down	Ratio	Up	Down	Ratio
Transcription, DNA-templated	6351	408	702	0.58	176	299	0.59	38	157	0.24	95	153	0.62
Regulation of DNA transcription	6355	587	1023	0.57	231	493	0.47	75	250	0.30	168	267	0.63
DNA integration	15074	68	138	0.49	44	89	0.49	8	44	0.18	14	61	0.23
DNA repair	6281	81	143	0.57	51	71	0.72	12	26	0.46	25	35	0.71
Translation	6412	166	183	0.91	270	76	3.55	14	33	0.42	89	34	2.62
RNA processing	6396	65	81	0.80	46	39	1.18	6	22	0.27	26	21	1.24
Metabolic process	8152	241	408	0.59	119	164	0.73	20	94	0.21	65	90	0.72
Carbohydrate metabolic process	5975	222	390	0.57	77	227	0.34	18	107	0.17	76	102	0.75
Lipid metabolic process	6629	94	131	0.72	22	61	0.36	10	40	0.25	27	32	0.84
Ubiquitin protein catabolism	6511	81	148	0.55	23	72	0.32	8	24	0.33	18	29	0.62
Protein folding	6457	93	108	0.86	29	53	0.55	11	20	0.55	51	35	1.46
Transport	6810	106	129	0.82	31	61	0.51	7	35	0.20	31	28	1.11
Transmembrane transport	55085	118	178	0.66	50	95	0.53	14	46	0.30	42	36	1.17
Intracellular protein transport	6886	157	186	0.84	23	79	0.29	15	30	0.50	47	43	1.09
Vesicle-mediated transport	16192	93	115	0.81	4	43	0.09	10	16	0.63	25	26	0.96
Cell wall organization	71555	59	110	0.54	29	54	0.54	11	25	0.44	16	29	0.55
Response to oxidative stress	6979	79	116	0.68	33	52	0.63	6	32	0.19	18	25	0.72
Biosynthetic process	9058	72	98	0.73	20	49	0.41	12	33	0.36	26	25	1.04
Recognition of pollen	48544	59	113	0.52	52	48	1.08	7	18	0.39	16	34	0.47
Embryo development seed dormancy	9793	71	87	0.82	37	39	0.95	8	25	0.32	29	21	1.38

Gene Expression

The difference in the gene expression under the two stresses (MS and SS) in comparison to control (WW) was characterized, and the top 20 up and down-regulated genes were presented in Tables 3 and 4, respectively, depending on UniProt classifications and definitions (UniProt, 2017). The most striking aspect of the transcriptional response is the up and down-regulation of the top twenty differentially expressed genes associated with MS-Alpowa. MS-Alpowa exhibited 7.2 and 5.1 times the numbers of top 20 transcripts for up and down-regulated genes as the average across the other three treatments. This large differential expression in MS-Alpowa is matched by the overall imbalance exhibited in Table 1 for the same treatments. Thus MS in the cultivar Alpowa is transcriptionally very active with respect to the top twenty most abundant transcripts.

The top twenty up-regulated transcripts were grouped into five functional categories (Photosynthesis, Photorespiration, methyl transferase, amino transferase and unknown) as presented in Table 3. Genes involved in photosynthesis and in particular the carboxylation reactions were most prominent including over half of the most active transcripts. These include: Rubisco small subunit, Rubisco activase, Carbonic anhydrase and a protein associated with photosystem II. This is not surprising given the known activity of photosynthesis in the flag leaf during grain filling. Clearly rubisco small subunit transcripts were highly upregulated in flag leaves to support their CO_2 photoassimilation functionality. The small subunit of Rubisco is part of a multicopy nuclear encoded portion of the holoenzyme that apparently has substantial role in improving Rubisco catalytic efficiency and CO_2/O_2 specificity (Spreitzer, 2003; Genkov & Spreitzer, 2009). Here three small subunits showed very large and concurrent transcript expression in MS-Alpowa and

to a lesser extent in SS-Idaho, SS-Alpowa, and MS Idaho. The small subunit along with Rubisco Enzyme Rubisco activase both A and B were highly upregulated except in MS-Idaho. Rubisco activases appear to function in adjusting the conformation of the enzyme regulating it carboxylation efficiency (Portis, 2003) and is necessary to activate Rubisco function. Furthermore, Rubisco activase is known to assist Rubisco in its adaptation to abiotic stresses including heat, salt and osmotic stresses (Chen et al., 2015; Portis, 2003), conditions that are likely operative prior to wheat harvest. Carbonic anhydrase functions inter-converts CO_2 to bicarbonate ions in aqueous solutions. Bicarbonate serves as substrate for amino acid and lipid synthesis. The role of carbonic anhydrase in CO_2 concentration mechanisms is well established in C4, but its precise role in C3 photosynthesis is much less clear. Proposals for multiple roles in stomatal movement, recycling of respiratory CO_2 into photoassimaltes, amino acid and lipid synthesis and seedling assimilation of CO_2 have all been proposed (DiMario, Layton, Mukherjee, Ludwig, & Moroney, 2017). The fact that flag leaves are highly active in terms of photosynthesis may suggest that at this stage of development carbonic anhydrase has a role in photoassimilation of CO_2. Furthermore, evidence suggests that carbonic anhydrase may be necessary to maintain photorepiratory fluxes (Hodges et al., 2016). This is particularly intriguing because photorepiratory enzymes are also highly upregulated. The last transcript associated with photosynthesis is an unnamed Photosystem II polypeptide of unknown function. This is likely a structural protein supporting the photosystem II function of which there are many.

Beyond photosynthesis there are four transcripts associated with photorespiration. A significant portion of the activity of Rubisco uses oxygen instead of carbon dioxide to

produce 2 phosphoglycolate. There exists a complex metabolic process commonly called photorespiration that is used to convert the toxic 2 phosphoglycolate into a usable form that can be metabolized by the Calvin Cycle, but this occurs at a considerable cost in terms of energy and carbon product. Thus photorespiration was considered to be a wasteful process. Some have considered this process to be like a safety valve to get rid of excess reductive power provided by the light reaction of photosynthesis avoiding free radical activities, especially under stress conditions (Stuhlfauth, Scheuermann, & Fock, 1990). However, this safety valve concept is probably too simplistic give that the photorespiratory pathway interacts with compounds associated with a range of metabolic pathways including nitrate assimilation, amino acid metabolism, C1-metabolism, and basic metabolic pathways. Furthermore, photorespiration is likely important in both biotic and abiotic stress response (Hodges et al., 2016). Moreover, photorespiration metabolic activities have been associated with decreasing productivity in terms of biomass and limiting nitrogen assimilation to maintain a constant C/N ratio (Dellero, Lamothe-Sibold, Jossier, & Hodges, 2015). Furthermore, activities of photorespiration appear to be co-regulated with carbon fixation itself (Timm et al., 2012).

Three of the enzymes of the photorespiratory pathway are represented among the top 20 differentially expressed up-regulated genes. These include glycine decarboxylase P subunit, serine hydroxymethyltransferase, and serine-glyoxylate amino transferase. The first two are associated with the mitochondria while the last one is associated with the peroxisomes, all part of the photorespiratory pathway. Glycine decarboxylase is a pivotal enzyme in this pathway. This enzyme takes glycine and cleaves off ammonium and CO_2 producing 5, 10 methylenetetrahydrofolate to produce serine with the assistance of serine

hydroxymethyltransferase. The ammonia produced is recycled as is the CO_2 through the Calvin cycle and the 5, 10 methylenetetrahydrofolate is used as a methyl donor for purine and methionine metabolism (Hodges et al., 2016). Glycine decarboxylase activities were shown to correlate with net photosynthetic activities indicating a connection between photorespiration and photosynthesis (Timm et al., 2012). The activities of serine hydroxymethyltransferase are downstream of glycine decarboxylase using a methyl from 5,10 methylenetetrahydrofolate and glycine itself to form serine. All of these activities exist in the mitochondria. Subsequent events of the photorespiratory pathway occur in the peroxisomes where r serine and the enzyme serine gloyoxylate amino transferase takes glyoxylate to produce hydroxypyruvate which is two enzymatic steps away from reentry into the Calvin cycle. The fourth enzyme associated with photorespiration is the peroxisomal (S)-2-hydroxy-acid oxidase which functions in the production of H_2O_2. The H_2O_2 is often noted as a signaling molecule in biotic defense reactions. The activities of the photorespiratory enzymes mentioned above were all greatly induced in MS-Alpowa, moderately induced in SS- Idaho and very lightly induced or not induced in SS-Alpowa and MS-Idaho. It is interesting that there was a cultivar difference in that induction occurred in MS conditions in Alpowa and SS conditions in Idaho.

The last five transcripts that were strongly upregulated in MS-Alpowa included 2 methyl transferases, one amino transferase and two unknowns. All of these were up-regulated in MS- Alpowa and moderately upregulated in SS- Idaho like all the others referred to above. S adenosyl methionine decarboxylase is an enzyme in the pathway of synthesis of polyamines which are nitrogen based compounds that show some regulatory features of stress responses (Minocha, Majumdar, & Minocha, 2014). End products of the

pathway include spermidine, tetramine spermine and damine putrescine. Polyamines may act in a dual manner in promoting resistance to stress through reactive oxygen scavenging or by promoting active oxygen. Furthermore, these compounds may serve as stress memory compounds or may be the active ingredient in regulating stress priming (Minocha et al., 2014). Here there were two out of the twenty top up-regulated genes associated with the transcript of an enzyme connected to polyamine synthesis. Alanine amino transferase is well known for its participation in the Dicarboxylic acid pathway of C4 photosynthesis where CO_2 is pumped into the bundle sheath cells to be captured by Rubisco. This enzyme is located on the recovery side of the cycle of products of that pathway. In addition, the enzyme is prominent in the catabolism of the amino acid alanine to pyruvate. The last two top twenty transcripts involve two unknowns whose activities are highly upregulated in MS-Alpowa in a similar manner as those above.

Overall the top transcriptional activities associated with the flag leaf involve photosynthesis activities associated with carbon fixation, photorespiration and metabolic pathways associated with polyamine synthesis and amino acid metabolism. All of these show the primary pattern of extremely high up-regulation in MS-Alpowa and moderate upregulation in SS-Idaho with significant up-regulation in the remaining treatments.

Table 3. The top 20 up-regulated genes in the flag leaf with the greatest fold change under moderate stress (MS) and severe stress (SS) conditions for 8 days in comparison with well-watered control (WW) for Alpowa and Idaho cultivars. The genes are sorted based on functional categorization. ND: not differentially expressed.

Down-regulated Differentially Expressed Genes or Proteins	Functional categories	Alpowa MS	Alpowa SS	Idaho MS	Idaho SS
		Fold change			
Rubisco small subunit	Photosynthesis	309	17.3	11.5	105
Rubisco small subunit	Photosynthesis	304	18.0	13.5	80
Rubisco small subunit	Photosynthesis	299	23.8	11.7	108
Rubisco small subunit	Photosynthesis	285	13.1	12.3	89
Rubisco small subunit	Photosynthesis	285	14.6	7.3	80
Rubisco activase A	Photosynthesis	369	3.2	ND	122
Rubisco activase B	Photosynthesis	351	3.4	ND	128
Rubisco activase	Photosynthesis	346	3.7	1.1	80
Carbonic anhydrase	Photosynthesis	310	9.4	1.7	77
Carbonic anhydrase	Photosynthesis	315	8.4	2.0	89
Photosystem II polypeptide	Photosynthesis	294	ND	1.0	80
Serine hydroxymethyltransferase	Photorespiratian	311	2.3	1.1	97
glycine decarboxylase P subunit	Photorespiration	296	ND	2.8	89
Serine-glyoxylate aminotransferase	Photorespiration	284	ND	3.1	73
Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	Photorespiration	286	3.4	ND	89
S-adenosyl methionine decarboxylase	Methyl transfer	310	ND	28.4	80
S-adenosyl methionine decarboxylase	Methyl transfer	300	ND	2.3	98
Alanine aminotransferase 2	Amino transferase	298	ND	ND	80
Unknown	Unknown	288	2.2	ND	97
Unknown	Unknown	285	11.1	8.7	105

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

The top 20 down-regulated genes under the two drought stress treatments and two wheat cultivars are presented in Table 4. The differentially expressed genes in a down-regulated way can be functionally categorized into 12 distinct categories. As with up-regulation MS-Alpowa showed very strong down-regulation compared to the other three treatments. The strong down-regulation in MS-Alpowa for the top 20 genes reflect the overall down-regulation for the same treatment cultivar combination for all differentially expressed genes. Half of the top 20 transcripts were functionally unknown reflecting the lack of knowledge within the wheat genome of genes that are predominantly down-regulated in response to water stress. The other 10 genes included a disparate range of functional attributes.

In the DNA integration category, the gag polyprotein expression was 352 fold reduced in MS-Alpowa. This gene is considered to be part of the basic retroviruses infrastructure and essential for virion assembly and binding to the plasma membrane, creating spherical particles through protein-protein interactions (Jalalirad & Laughrea, 2010). GAG polyproteins have been studied in terms of HIV infections (Ganser-Pornillos, Yeager, & Sundquist, 2008), but little is known concerning their presence in transcriptomic studies in plants. It has become clear that transposable elements (TEs) are considered a significant source of evolutionary innovation in eukaryotic genomes by providing non coding DNA which may provide novel regulatory sites to modulate gene and protein expression. The wheat genome is one of the largest genomes associated with crop production, much of which is non-coding sequence, of which a large majority is associated with transposable elements. The reason why these retroviral elements are significantly reduced in response to water limiting stress is a puzzle. Could it mean that under stress

conditions during grain filling, a critical stage in the survival of the wheat plant, that retroviral interactions are minimized by some unknown mechanism. Further research in this area should be conducted concerning the association of viral resistance to water limitation during grain filling.

The VAN3-binding protein-like in Arabidopsis is responsible for formation the leaf veins via a canalization mechanism associated with the plant hormone auxin to generate a highly reproducible branched and reticulate pattern. Furthermore, VAN3 ARF-GAP may play an important role in the auxin signaling associated with vesicle transport that is required for differentiation of vascular (Koizumi, Sugiyama, & Fukuda, 2000; Koizumi, 2005). Clearly both functions are associated with growth responses which under the current circumstances are likely down-regulated.

Serine/arginine repetitive matrix protein 1 networked is a part of pre/post-transcript splicing multiprotein complexes of the small nuclear ribonucleoprotein particles (snRNPs), involved in pre-mRNA processing, formation of an exon junction complex that stimulates mRNA 3'-end cleavage, binding both RNA and DNA with low sequence specificity (Blencowe, Issner, Nickerson, & Sharp, 1998; Szymczyna et al., 2003). The down-regulation of this gene is likely part of a more pronounced down-regulation of RNA processing as indicated in the GO: Term analysis and transcription in general.

Protein networked 2A was down-regulated and may be part of membrane-cytoskeletal adapter complex associated with F actin at the plasma membrane in growing pollen tubes (Deeks et al., 2012). Actin cytoskeletal networks are major actors in terms of cell trafficking and transport. The third transcript produce B3 domain-containing proteins

that represent a large transcription factor superfamily. The B3 superfamily specific to plants are composed of three family members and plays a central role in embryogenesis to seed maturation and dormancy in plant life (Wang et al., 2012). Family members include auxin response factors, abscisic acid insensitive factors, and the RAV family of transcription factors. In *Arabidopsis thaliana*, the B3 domain of At1g16640 structure composes of a seven-stranded β-sheet and two short α-helices (Deeks et al., 2012; Waltner, Peterson, Lytle, & Volkman, 2005). All three of the B3 domain transcription factors are associated with drought response (Fu et al., 2014; Mittal et al., 2014; Wang et al., 2010). Here their expression appears to be substantially down-regulated. Lastly, translation initiation factor that is required for initiation of translation was dramatically down-regulated in the drought resistance MS-Alpowa in accordance with the general down-regulation observed under the GO: TERM analysis. A gene involved in protein catabolism containing a U-box domain-was highly downregulated in MS-Alpowa. The plant U-box (PUB) proteins contain five distinct subclasses that suggest diverse roles. The only PUB gene functionally characterized is the ARC1 gene from Brassica which is required for self-incompatibility. In yeast, the prototype U-box protein (Ufd2) was identified that involved in catalyzing ubiquitin chain formation on artificial substrates with ubiquitin-protein ligase, ubiquitin-conjugating enzyme and ubiquitin-activating enzyme (Azevedo, Santos-Rosa, & Shirasu, 2001; Hatakeyama, Yada, Matsumoto, Ishida, & Nakayama, 2001). The non-specific lipid-transfer gene in plant is a basic small protein that is typically involved in abiotic stress response, pathogen defense, reproductive development and transfer of phospholipids across membranes. Its major function is associated with cuticular lipid export to create the cuticular wax layer (Kim et al., 2012). In maize, about 63 genes of non-

specific lipid transfer proteins (nsLTPs), have been identified divided into five types (Wei & Zhong, 2014).

Bowles (1990) reported that by changing in physiological conditions of defensin-protein, the higher plants protect themselves from various stresses such as harsh growing conditions (i.e. drought, heavy metals), pathogen attacks, application of chemicals including phytohormone, wounding, air pollutants like ozone, ultraviolet rays. This process of protective reactions in higher plants called "defense responses" and the actively synthesized proteins in this process called "defense-related proteins" or defensing like protein. This defense transcript was not differentially expressed in MS-Idaho, but a significant change in it expression was observed in Alpowa cultivar. Finally, a retrotransposon protein was substantially down-regulated in response to water limitations. Retrotransposition is responsible for the bulk of the wheat genome as indicated earlier.

Table 4. The top 20 down-regulated genes in the flag leaf with the greatest fold change under moderate stress (MS) and severe stress (SS) conditions for 8 days in comparison with well-watered control (WW) for Alpowa and Idaho cultivars. The genes are sorted based on functional categorization. ND: not differentially expressed, NP: not present.

Down-regulated Differentially Expressed Genes or Proteins	Functional categories	Alpowa		Idaho	
		MS	SS	MS	SS
		fold change			
Unknown	Unknown	366	92	44	77
Unknown	Unknown	340	51	45	NP
Unknown	Unknown	333	69	6	119
Unknown	Unknown	320	59	42	107
Unknown	Unknown	309	62	2	89
Unknown	Unknown	306	65	38	80
Unknown	Unknown	303	53	3	117
Unknown	Unknown	301	59	ND	103
Unknown	Unknown	299	63	ND	99
Unknown	Unkown	310	72	107	94
Gag polyprotein	DNA integration	352	51	4	132
VAN3-binding protein	Vascular patterning	351	63	43	93
Serine/arginine repetitive matrix protein 1	Transcript splicing	344	51	1	75
Protein networked 2A	Cytoskeleton	337	51	29	NP
B3 domain-containing protein	Senescence	319	74	ND	82
Translation initiation factor IF-2	Translation	316	51	28	NP
Putative U-box domain-containing protein 42	Protein degradation	302	59	ND	79
Non-specific lipid-transfer protein 2	Lipid transport	308	65	54	111
Defensin-like protein	Disease resistance	303	51	ND	76
Retrotransposon protein	Retrotransposition	308	57	5	78

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

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CHAPTER IV

CHARACTERIZE YIELD RESPONSE AND IDENTIFYING DIFFERENTIAL EXPRESSION GENES IN THE SEED HEAD OF TWO SPRING WHEAT CULTIVARS IN RESPONSE TO MODERATE AND SEVERE WATER LIMITATIONS

Introduction

In seed bearing plants it has been reported that drought stress affects the relationship between the content of carbon in photosynthetic organs (leaves) and the content of carbon in heterotrophic organs (seeds and roots, sink) indicating that carbon partitioning processes are sensitive to drought stress (Cuellar-Ortiz et al., 2008). An essential part of the plant life cycle is senescence processes where nutrient remobilization occurs from vegetative parts to storage organs, such as: seeds, tubers fruits, roots, and stems at the end of plant life cycle (Gregersen & Holm, 2007). Success for plants depends on the reproductive process leading to a high number and mass of the seeds. Significantly lower seed yield due to drought can occur during anthesis where seed number is predominantly affected by lower fertility (Salter & Goode, 1967). Seed development is also strongly affected by the availability of water during the grain filling stage of development.

Water deficits results in a significant decrease in seed yield for most crops. These decreases can occur due to a reduction in source effects such and photosynthesis or sink effects such as carbohydrate assimilation into developing seeds. Severe stress can cause reproductive structures to abort causing a drastic reduction in yield (Boyer & Westgate, 2004; Marcelis et al., 2004; Paul et al., 2016). Grain yield can be divided into components including the weight and number of individual seeds, spiklets, seed heads and tillers. These easily accessible morphological traits are often used on breeding programs to identify good yielding varieties (Villegas et al., 2007). In wheat, it has been reported that grain yield is more closely correlated to grain number than to grain mass, so that, selecting for a high number of spikes per area unit and a high grain number per spike will lead to improved yield. Estimating the head numbers in the late spring will be more accurate than that made in the fall (Beharav, Cahaner, & Pinthus, 1998; Zamski & Grunberger, 1995).

Photosynthesis throughout plant development ultimately provides carbon that ends up in the grain. Maintaining functional green upper leaves, sheaths, and seed heads during grain filling are important for high yields. Most of the carbon destined for the grain comes from one of two sources: photosynthetic carbon produced during grain filling and carbon remobilized from vegetative tissues and translocated into the developing kernel. The flag leaf and seed head usually contribute most of the photosynthetic carbon to the developing grain, (Simmons, Oelke, & Anderson, 1985). 70 to 90 % of the final grain yield is derived from products of photosynthesis that are produced by the plant during grain filling. Depending on seasonal conditions, photosynthetic carbon produced by the flag leaf may contribute up to 50 percent of the grain yield while seed head, penultimate leaf, and other leaves can also contribute significantly. However, sink limiting conditions often apply

during grain filling which means that there is a limitation to the rate at which the developing kernel can absorb carbon. The excess carbon produced during this time, but not assimilated, can be translocated to the stem for temporary storage in the form of fructans. Once photosynthesis in vegetative tissues slows down then grain filling can continue by a remobilization of stem reserves. This usually accounts for later stage filling of the developing kernals (Borrell et al., 1989).

Grain filling begins after pollination. Within 1 to 2 weeks after pollination, carbon is rapidly transferred from leafy tissues to the developing grain where it is transformed into starch and protein. During this early grain filling time the kernel consistency is milky and soft, the soft dough stage of grain development. Three weeks into grain filling and after the soft dough stage the developing kernel continues to fill and then begins to rapidly desiccate and solidify, termed the hard dough stage. At this point the grain approaches what is termed physiological maturity. Throughout this grain filling period, seed head photosynthesis through glumes and awns contribute to the carbon flow to the developing grain. Awns due to their close proximity to the developing kernel and photosynthetic capacity contribute significantly to the final mass of the developing grain. In awned and awnless genotype barley, Bort, Febrero, Amaro, Jarius (1004), suggested that awns improve net CO_2 fixation and water use efficiency of ears throughout the grain filling. Based on shading experiments, the photosynthetic assimilation by the seed heads, from one week after anthesis to maturity, accounted for 40% of total grain filling, in the awned genotype, but only 15% in awnless cultivars. Thus the awns as photosynthetic organs contributors significantly to the carbon flow to the developing kernals. Furthermore, the awns and glumes, which are modified leaves, appear to be less sensitive to water stress conditions. During water stress, seed head

photosynthesis may in fact compensate for the reduction in photosynthesis experienced by flag leaves and other vegetative tissues (Weyhrich, Carver, & Martin, 1995). In durum wheat, photosynthesis was much greater in the ear than in the flag leaf under well-watered conditions. Whole ear photosynthesis correlated better than flag leaf photosynthesis with final grain yield (Abbad, El Jaafari, Bort, & Araus, 2004).)

For molecular studies on wheat grain development, transcriptome data will provide a valuable resource to better understand the molecular mechanisms and regulators functioning during grain filling. Unfortunately, there is little work performed on identifying the transcriptome during this important stage of wheat development. Wan et al., (2008) determined the transcriptome of developing seed heads from hexaploid wheat (Triticum aestivum, cv.Hereward) in the period between 6 and 42 days after anthesis (DAA). Transcript abundance was analyzed into several stages including post-anthesis differentiation into grain filling stage (6–10 DAA), grain fill stage (12–21 DAA), desiccation and maturation stage (28–42 DAA), and found wide changes in transcript abundance which can be related to fundamental processes leading to wheat development under environmental stress. Using Illumina paired-end RNA-sequencing, transcriptome changes during barley grain development were investigated. In both genotypes, 38 differentially expressed genes (DEGs) were found co-expressed during the barley grain development. The proteins encoded by most of those DEGs, such as alpha-amylase-related proteins, lipid-transfer protein, MYB transcription factors, Nuclear factor-Y and subunit B (NF-YBs), were detected, (Tang et al. 2017).

Our objective from this study was to identify the DEGs during the seed head stage in the two spring wheat cultivars under study (Alpowa and Idaho) under moderate and severe water limitation.

Material and Methods

The experiments were performed at the #315 USDA greenhouse facility of USDA, in Stillwater, Oklahoma, during the spring season of 2016. Two soft white spring wheat cultivars, Alpowa and Idaho were planted in 10.2x10.2x30.5 cm. CP412CH TreePots (Stuwe and Sons Inc) to a depth of 5 cm. Pots were sanitized with 70% ethanol and then filled with 1.9 Kg of a sandy clay loam soil fertilized with ammonium nitrate to 70 kg/ha in a way that reduces pot to pot variation. Pots were planted with three seeds and upon emergence trimmed to one plant per pot. All pots were regularly hand weeded and checked for insects and were sprayed with Immunox (Spectracide, INC. OR, USA) in case of powdery mildew, and neem oil (Certis, LLC, MD, USA) in case of aphid infestation. Three tensiometers were installed in control pot to 15 cm depth, to measured water potential. Water was provided to all pots when the tensiometer reached (40 centibars) based on the recommendations of the manufacturer for wheat grown in silt loam soil (Irrometer Co. Inc., Riverside, CA). After five days when plants 50% of the plants showed anthers (Feekes 11.2, post-anthesis and early grain filling), wheat plants were watered with either 220 ml (100% well-watered), 110 ml (50% well-watered), and 55 ml (25% well-watered) and then water withheld for 5 and 8 day at which time plants were harvested. There were a total of three stress intensity levels (WW, MS, SS), two cultivars, two stress durations and 5 replicate plants for a total of 60 plants. Plants were harvested at 5 and 8 days after watering. Seed heads were removed from the main stem for each plant individually, and weights recorded. Seed heads were wrapped in aluminum foil and frozen in liquid nitrogen and then stored at -80 °C for later use. Tissue was fixed within 2 to 3 minutes of harvest. Weight data were transferred to an Excel spread sheet and analyzed statistically using in JMP®

(SAS institute) software using multi-factor analysis of variance (ANOVA) based on the least-square fit significant differences among the effects, including: cultivar, duration, and stress intensity with interactions. Effects were cultivar, duration, and stress intensity. Tukey's multiple comparison procedure was used to find differences within stress intensities along with interactions based on a significance p-value of 0.05.

RNA extraction for seed head was based on Furtado et al., 2014, protocol using TRIzol® Reagent (Life Technologies Inc., Carlsbad, CA) and RNeasy Plant Mini Kit ((Qiagen, Valencia, USA)). The first step involved homogenizing tissue samples in liquid nitrogen in a mortar and pestle to a fine powder. To 250 mg of fine powder was added 1.5 ml of TRIzol in a 2 ml bead beating tube. Tissues were lysed in Mini-Beadbeater (BioSpec Products Inc., Bartlesville, OK) with sterile glass beads 2.5 mm-diameter at 5000 rpm for 1 min. The tubes were centrifuged at 14,000 rpm for 10 min at 4 °C. The upper phase (350 µl) was transferred to fresh tube and the RNA extraction was analyzed based on NanoDrop spectrophotometric measurements with the goal of obtaining extracts exhibiting 260/280 nm ratios greater than 1.8. (Barbas, Burton, Scott, & Silverman, 2007) and quantity greater than 33 µg/ml based on the 260 nm absorbance. Frozen and purified RNA was 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. According to Oklahoma Medical Research Foundation prior to RNA-seq analysis quality control measures was implemented. Concentration of RNA was ascertained via fluorometric analysis on a Thermo Fisher Qubit fluorometer. Overall quality of RNA was verified using

an Agilent Tapestation instrument. Following initial QC steps sequencing libraries was generated using the Illumina Truseq Stranded mRNA with library prep kit according to the manufacturer's protocol. Briefly, mature mRNA was enriched for via pull down with 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).

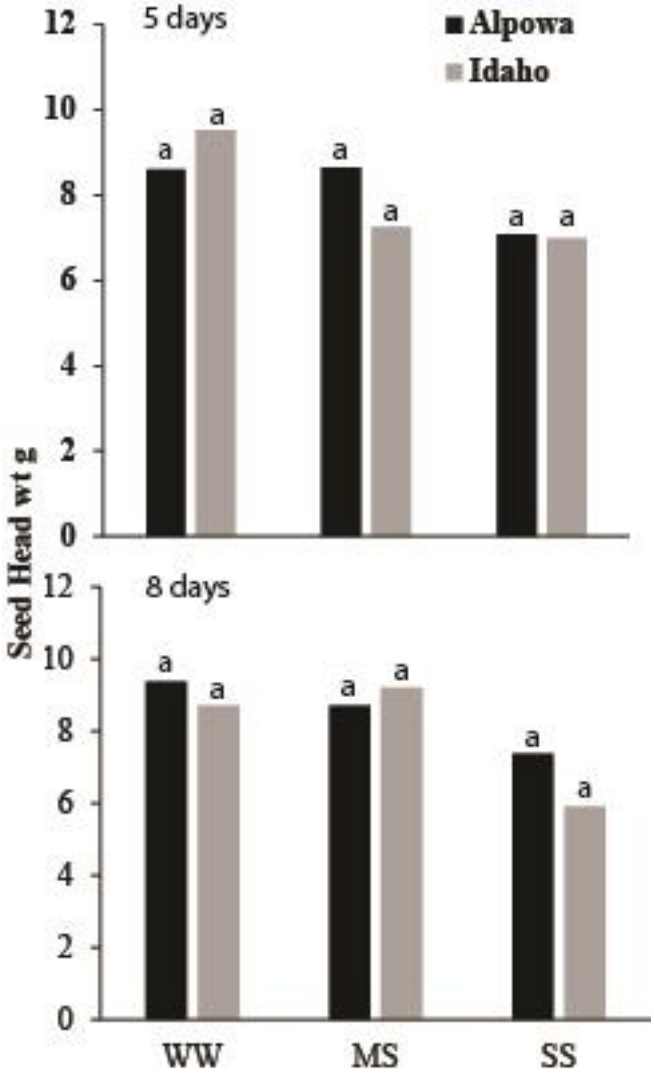
RNA-Seq data was analyzed by Brian Couger, Bioinformatics Specialists of the OSU High Performance Computing Center. The sequence data was downloaded from the OMRF where they were checked using FastQC application to insure the quality at Q30 level. Sequencing data were downloaded to the Cowboy super computer where they were further analyzed for eight durations samples based on the Bioconductor packages under the R software environment. All Fastq reads produced by sequencing were aligned to the wheat genome using the read alignment program Hisat2 (Kim, Langmead, & Salzberg, 2015). The reference genome and GTF annotation for wheat were downloaded and prepared for read quantification according to the Hisat2 protocol (Pertea, Kim, Pertea, Leek, & Salzberg, 2016). SAM alignment (Li et al., 2009) file conversion, sorting, and preparation were achieved using the Samtools program (Li, 2011). Quantitative prediction of transcript FPKM levels was 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. The functional categorization according to UniProt classifications and definitions database, (UniProt Consortium, 2017).

RESULTS AND DISSCUSION

Seed Head Weight (SH):

Here we treated wheat to three levels of water availability during the grain filling stages of wheat development. Water was withheld from wheat plants producing three levels of stress intensity and two different stress duration during the early stages of grain filling. At the end of stress imposition, transcriptional responses were analyzed using RNA sequencing procedures. Seed head weights did not differ statistically among any of the water limitations treatments, cultivars, or durations (Fig. 2). While there was no statistical difference among treatments there was a relatively large numerical reduction in overall seed weight in SS-Alpowa, MS-Idaho, and SS-Idaho and these reductions were numerically greater in Idaho than Alpowa. Grain yield weights ranged from 68 to 105% (SS-Idaho) of WW. The lack of statistical sensitivity to water limitation can be most likely be alleviated by increasing overall replication. Thus water limitation did lower overall grain weight in either cultivar, and differences between cultivars were too slight to be statistically noticeable, if at all. It may be possible that yield differences between Alpowa and Idaho are not associated with the grain filling stages of wheat development. Khan and Naqvi (2011) found significant differences in spike length as affected by water stress. Also, Abdel-Moneam et al., (2014) noticed a 21 to 26% reduction in spike length as affected by water stress.

Fig 2. Seed head weights for two cultivars, Alpowa and Idaho at Feekes 11.2 treated under WW (well-watered, 100%) MS (moderate stress, 50% well-watered), SS (severe stress, 25% well-watered) conditions for 5 and 8 days. Different letters on top of columns represent significant differences (HSD p-value < 0.05).



Seed Head Transcriptomic Changing

Transcriptional analysis was performed on RNA-seq data from seed heads treated under water limiting conditions for eight days duration. Differential transcriptional response under water limiting conditions compared with WW were based on whether transcripts were DE based on a p value of < 0.05 adjusted for false discovery. Comparing the total numbers of DE transcripts between cultivars and across stress intensities: Idaho had 5.5 time the number of DE transcripts compared to Alpowa (338 in Alpowa, and 1843 in Idaho) with only 53 transcripts shared between the two cultivars in their response to water stress (data not shown). Thus the data suggests limited similarity in transcriptional response between the two cultivars. However, the lack of similarity could reflect subtle differences in homologous sequences between the two cultivars rather than functional responses. Furthermore, resistance to drought in these spring wheats appears to be associated with a limited transcriptional response. Limited transcriptional activity in Alpowa may actually reflect a more stable metabolism under water limited conditions. In contrast the Idaho response may be more transcriptionally responsive to cope with damaging effect of water stress.

The total numbers of differentially expressed genes under MS, and SS treatments only, and under both MS and SS conditions for both cultivars are presented in Table 5. Comparing among stress intensities Alpowa showed greater up and less down regulation in response to increasing stress intensity (MS to SS). Alpowa up regulation was 20% greater in MS compared to SS conditions and down regulation 70% greater in SS than MS. Thus in Alpowa up regulation is favored under MS compared SS conditions and down regulation SS is favored compared to MS conditions. The exact opposite is true in Idaho

and to even a greater extent compared to Alpowa. Idaho exposed to SS conditions up regulated 99% more transcripts than under MS conditions while under down regulated conditions 87% more transcripts were DE under MS compared to SS conditions. Thus Idaho up-regulation favored SS stress conditions compared to SS and down regulation MS in favor of SS conditions. This pattern of transcriptional regulation may also be associated with the water limited resistant phenotype in these soft white spring wheats.

When comparing DE transcriptional responses between pre-anthesis flag leaves and post-anthesis seed heads on average over 56,308 transcripts were associated strictly with flag leaves, while only 776 transcripts with seed heads and 492 transcripts common between the two organs. The imbalance between seed head and flag leaf expression may be partially due to the difficulty in extracting and analyzing seed heads via Triazol extraction and RNA-seq technique, respectively.

Table 5. Transcriptomic changes in Alpowa and Idaho cultivars in the seed head in response to moderate and severe stress for eight days duration.

	Direction	MS	MS and SS	SS	Total
Alpowa	Up	135	0	112	247
	Down	80	0	136	216
Idaho	Up	364	0	725	1089
	Down	676	0	361	1037

Gene Ontology

The Gene Ontology analysis was performed for the two cultivars under study in seed heads under two treatment of water limitation (Moderate-Stress (MS) and Severe-Stress (SS) in comparison to Well-Watered (WW). This research constitutes one of the first analysis of transcriptional activities in the seed head during the critical stage of grain filling when a significant portion of grain yield is determined. In seed heads, the top 20 GO terms for biological process are indicated in Table 6. The trend in GO terms was predominantly down regulated especially in Idaho under both conditions (MS and SS) while in Alpowa a moderate tendency towards down regulation was detected. Not all of the top 20 GO Terms were represented in all treatments. Out of the top 20 GO Terms MS-Idaho included all 20 GO terms while all other treatments include anywhere from 6 to 10 of the top 20 GO terms. MS-Idaho GO terms were predominantly down regulated, a total of 159, while the rest of the stress intensity treatments ranged from a low of 9 to 44 in both cultivars. Thus the most represented top 20 GO terms were mostly defined by the MS Idaho down regulated treatments.

All GO Terms in Table 6 were categorized and sorted according to numbers of DE transcripts into generalized areas including transcription/translation, metabolic processes, cell wall, stress response along with two un-generalized GO terms. The numbers of DE transcripts summed across treatments associated with each GO Term ranged from a high of 53 to a low of 5. These numbers are considerably lower than those found in the flag leaves during the same developmental stages which ranged from a high of 387 to a low of 38. The much lower level of categorized transcriptional response in seed heads may be due

to difficulties in extracting sequenceable RNA from seed materials and/or to the genuinely lower level of transcription associated with seed heads.

GO terms containing the greatest numbers of DE transcripts across all treatments included: DE transcription (53) with carbohydrate metabolic processes (31), metabolic processes (23) and regulation of transcription (21) indicating that transcriptional processes and metabolic processes especially those involving carbohydrates predominate in seed tissues. This was also true for the flag leaves, where metabolic and nucleic acid associated processes dominated the list. The GO terms involving nucleic acid associated processes or contained the greatest numbers of DE transcripts including regulation of transcription (GO: 6355), DNA transcription (GO: 6351), DNA repair (GO: 6281), DNA integration (GO: 15074) and signal transduction processes (GO: 7165). Fracasso et al., (2016) reported that GO terms related to regulation of DNA replication (GO: 6275), the controlling of cell development by the extracellular stimulus (GO: 1560) were the most enriched GO terms in sorghum at vegetative stage. Regulation of transcription (GO: 6355) and transcription (GO: 6351) showed significant numbers of DE transcripts across all treatments and especially in MS Idaho where for the most part transcription was down regulated. Thus one of the most active processes in the seed head appears to be the down regulation of transcriptional processes. This is not too surprising given that seed head tissues during grain filling are undergoing senescence. In contrast, the same processes in the flag leaves were predominantly upregulated. The other DNA related processes contained a limited number of DE transcripts, and especially in MS-Idaho.

The next most abundant category in seed heads are the GO terms for metabolic process (GO: 8152) and biosynthetic process (GO: 9058), and their related child GO terms, carbohydrate metabolic process (GO: 5975), pectin catabolic process (GO: 45490), and cellulose catabolic process (GO: 30245). Metabolic processes include both biosynthetic and catabolic processes with the main purposes of conversion of food to energy to run cellular processes. This GO term showed down regulation in all cases except MS-Alpowa. One of the major child terms under metabolic processes is carbohydrate metabolic process (GO: 5975) which involve chemical reactions and pathways involving carbohydrates and formation of carbohydrate derivatives by the addition of a carbohydrate residue to another molecule. This GO term had nearly the same trend as in metabolic process GO term and shows a relatively large number of DE transcripts compared to other metabolic processes. Metabolic processes refer to both catabolic and anabolic reactions so it is not surprising that in seed heads where sugar import is transformed into starch that carbohydrate metabolic processes would be prevalent. Hübner et al., (2015) found that at least four out of 12 GO term categories in the tolerant accessions were associated with carbon metabolism. Thus carbon metabolism appears to be highly responsive to water limitation. Pectin catabolic process (GO: 45490), and cellulose catabolic process (GO: 30245) contained a surprising number of down regulated DE transcripts with 5 and 9 transcripts under MS-Idaho as indicator of plant reducing pectin and cellulose catabolism in seed heads during live grain filling. Cheng et al., 2016 identified proteins involved in protein translation/processing/degradation, metabolism, photosynthesis, and defense in two wheat cultivars (*Triticum aestivum* L.) under dehydration and rehydration conditions.

Furthermore, proteins involved in transcription, redox homeostasis, energy, cellular structure, signaling and transport were also characterized in the same study.

Seed head cell wall processes appear to be affected including: cell wall organization (GO: 71555) which is a process that results in the assembly, arrangement of constituent parts or disassembly of the cell wall; cell wall modification (GO: 42545) which leads to chemical and structural alterations of an existing cell wall that can result in loosening and increased extensibility or disassembly, and cell wall biogenesis (GO: 42546) that results in the biosynthesis of constituent macromolecules, assembly, and arrangement of constituent parts of a cell wall. Cell wall associated GO Terms were almost exclusively included down regulated DE transcripts in MS-Idaho. Down regulation was especially prominent in cell wall organization. Down regulation of cell wall functions during senescence in stressed vs well-watered conditions appears to be a significant factor in grain filling seed heads. Borrill et al., (2015) carried out GO term enrichment for differentially expressed genes in hexaploid wheat (*Triticum aestivum* L.) finding that cell wall organization was affected.

Concerning the stress response GO terms in seed head, response to oxidative stress (GO: 6979), defense response (GO: 6952), hydrogen peroxide catabolic process (GO: 42744), response to stress (GO: 6950) and response to water (GO: 9415) were all represented by DE transcripts. Hydrogen peroxide catabolic process (GO: 42744) includes metabolic process that results in hydrogen peroxide degradation and removal. This process was highly associated with MS Idaho with 8 DE down regulated transcripts. In SS-Idaho both hydrogen peroxide degradation and response to oxidative stress (GO: 6979) contained 3 up regulated DE transcripts compared to only 1 down regulated transcript. Thus in seed

heads during senescence conditions, oxidative stress response may be substantially up regulated. Oxidative stress results from the presence of high levels of reactive oxygen species, e.g. hydroxyl radicals and singlet oxygen and hydrogen peroxide. This may reflect the plant response to water limitation under severe stress in both cultivars in the face of water shortage. Defense response (GO: 6952), response to stress (GO: 6950) and response to water (GO: 9415) are related to processes that help plants survive extensive water limitation. However, for the most part these processes exhibited down regulation especially under MS-Idaho.

Finally, and during drought stress conditions the cellular transport processes were somewhat affected including a predominant down regulation of DE transcripts associated with transmembrane transport (GO: 55085) in MS Alpowa but an up regulation in SS Idaho. Transmembrane transport is defined as the transportation of solute through the cell membrane. The last GO:Term was sexual reproduction (GO: 19953) which showed exclusive down regulation in MS Idaho. This is not surprising given that the seed head tissues were harvested post-anthesis and during grain filling. Apparently stress reactions accelerate the down regulatory trend.

Unfortunately, the limited numbers of GO terms and the high numbers in one treatment (MS-Idaho) provides very limited information to develop overall conclusions concerning metabolic processes in seed heads in comparison to flag leaves. Very few child terms were present to allow us to go beyond broad generalizations. The fact that most of the GO Terms were down regulated is consistent with a senescing tissues where metabolic,

transcriptional functions are likely to be down regulated under stress conditions compared to WW conditions.

Table 6. Seed head gene ontology terms for Alpowa and Idaho cultivars after 8 days of water limiting treatments under two conditions of stress intensity, moderate and severe stress. The number and ratio of up and down regulated transcripts is given under each of the top twenty terms.

		Moderate Stress (50% WW)				Severe Stress (25% WW)			
		Alpowa		Idaho		Alpowa		Idaho	
Gene Ontology Terms	GO#	Up	Down	Up	Down	Up	Down	Up	Down
Reg. of transcription, DNA-templated	6355	2	2	6	16	1	4	3	19
Transcription, DNA-templated	6351	0	2	5	11	1	0	2	0
DNA repair	6281	0	0	0	3	0	0	0	3
DNA integration	15074	0	2	0	2	1	1	4	6
Signal transduction	7165	0	0	0	5	1	0	0	1
Metabolic process	8152	2	1	3	11	0	1	1	4
Biosynthetic process	9058	0	0	0	4	1	0	1	2
Carbohydrate metabolic process	5975	2	0	3	20	1	1	0	4
Pectin catabolic process	45490	0	0	0	9	0	0	0	0
Cellulose catabolic process	30245	0	0	0	5	0	0	0	0
Cell wall organization	71555	0	1	1	15	0	0	0	0
Cell wall modification	42545	0	0	0	9	0	0	0	0
Cell wall biogenesis	42546	1	0	0	3	0	1	0	0
Hydrogen peroxide catabolic process	42744	0	2	2	8	1	0	3	1
Response to oxidative stress	6979	0	2	2	8	1	0	3	0
Defense response	6952	1	0	2	6	1	1	0	2
Response to stress	6950	1	0	0	11	0	0	0	0
Response to water	9415	0	0	0	6	0	0	0	0
Transmembrane transport	55085	0	3	0	1	1	0	3	2
Sexual reproduction	19953	0	0	0	6	0	0	0	0

Gene Expression

While GO: Terms are useful for determining effects on broad overall functions related to biological processes they tend to provide generalized less specific information. More focused attention on specific functions may be obtained by examining those transcripts with the most elevated function. In seed heads, the top 20 up-regulated genes in the two cultivars under study and under the two treatments in comparison to WW control, are presented in Table 7. It is interesting that of the top 20 DE transcripts the highest level of up regulation is found almost exclusively in Idaho compared with Alpowa and that the intensity of expression is often correlated with increasing stress intensity. This dramatic up-regulation in Idaho was shown by the 5.5 fold greater number of differentially expressed transcripts overall as shown in Table 5. Thus overall differential expression and top 20 DE show the same pattern of response. The most abundant up regulated DE transcript is alpha-amylase/trypsin inhibitor with (228-fold MS-Idaho), followed by the drought-responsive factor, (167-fold, MS-Alpowa,), gamma prolamin (162-fold, SS-Alpowa), 18S ribosomal RNA (158-fold, MS-Idaho) and peroxidase (157-fold in SS-Idaho). The alpha amylase trypsin inhibitor is an albumin seed protein that is typically found expressed during seed development (Finnie, Melchior, Roepstorff, & Svensson, 2002; Zhou et al., 2017) and is complicit in sensitivity to wheat digestive allergies distinct from the gluten celiac disease complexes (Reig-Otero, Manes, & Manyes, 2018). This protein biological function has yet to be determined but there is evidence of its function to improve grain yield in rice (Zhou et al., 2017).

Related to drought stress, the drought-responsive factor gene up-regulated surprisingly in MS-Alpowa and approximately half as much was observed with MS-Idaho. The exact biological function of this apparent transcription factor is not known beyond its role in drought response. Through binding to their promoters, these proteins can modulate expression of a specific set of genes as regulatory proteins converting the stress-induced signals to cellular responses. Under drought and other stresses, plants activate many regulons to optimize plant growth as determined in Arabidopsis (Singh & Laxmi, 2015).

The next most DE transcripts associated with seed heads is a gamma gliadin transcript which codes for a major class of storage monomeric proteins found in wheat seeds (Wieser, 2007). Here we find strong up regulation in SS-Alpowa and MS-Idaho. The up regulation of seed storage protein production is consistent with its occurrence during seed development where storage proteins are being synthesized. The small subunit 18S ribosomal transcript was found to be highly upregulated in Idaho only. This is peculiar in that this particular gene is usually thought to be constitutively expressed in tissues, but here there is a distinct cultivar differentiation in response to stress.

Plants exposure to harsh environmental conditions results in an increase in reactive oxygen species (ROS) production of species as: hydrogen peroxide (H_2O_2), singlet oxygen and hydroxyl radical (OH). To cope with the high level of ROS, antioxidant defenses transcripts are typically differentially expressed in plants (Caverzan et al., 2012). Here peroxidase transcripts representing a response to oxidative stress was dramatically up-regulated in SS-Idaho by 71 fold and even more dramatically up regulated in SS-Alpowa (76 fold) and SS-Idaho (157-fold). As an electron acceptor to catalyze a number of

oxidative reactions, peroxidases are haem-containing enzymes that degrade hydrogen peroxide to less reactive products. Peroxidases are known to function in lignin synthesis and degradation, auxin catabolism and response to wounding oxidative stress (UniProt) and drought stress in wheat (Zhang & Kirkham, 1994). A specific 16.9 kDa heat shock protein was shown to be up regulated in Idaho under both stress conditions but not in Alpowa showing another cultivar specific response. Heat shock protein also can be expressed when plants are exposed to persistently changing stress factors such as drought, cold and hot temperatures, chemicals and salinity. These stress factors can cause plant cell damage and lead to osmotic and oxidative stresses as secondary stress. This specific heat shock protein is known to show increased expression during heat stress (Young, Yeh, Chen, & Lin, 1999). Gog- pol polyproteins were found to be up regulated in Idaho only. This gene is considered to be part of the basic retroviruses infrastructure and essential for virion assembly for binding to the plasma membrane, creating spherical particles through the protein-protein interactions (Jalalirad & Laughrea, 2010). GAG polyproteins have been studied in terms of HIV infections (Ganser-Pornillos, Yeager, & Sundquist, 2008), but little is known concerning their presence in transcriptomic studies in plants. GDP-mannose transporters were found to be strongly up-regulated in MS-Alpowa, MS-Idaho and SS-Idaho. This enzyme may be included in the import of GDP-mannose from the cytoplasm to Golgi lumen for extracellular usage in either cell wall synthesis or protein modifications in *Arabidopsis thaliana* (Baldwin, Handford, Yuseff, Orellana, & Dupree, 2001; Handford, Sicilia, Brandizzi, Chung, & Dupree, 2004). Dehydrins play a fundamental role in plant adaptation and response to abiotic stresses and especially to drought. Dehydrins are known accumulate in vegetative tissues following salinity, cold, freezing and dehydration stress,

and in seed tissue during development (Hanin et al., 2011; Wang et al., 2014; Lopez, Banowetz, Peterson, & Kronstad, 2003). Dehydrins were shown to be expressed in MS-Alpowa, MS-Idaho and SS-Idaho, more so under MS conditions than SS. The exact biological function of this important class of stress proteins has yet to be discovered but to date they are thought to be involved in protection from oxidative stress and the stabilization of membrane structure under stress conditions (Graether, & Boddington, 2014).

Other proteins involved in drought stress response include serpin 1, galactinol synthase, ervatamin-B protein and tryptophan synthases proteins. serpins are a group of proteins that inhibit chymotrypsin-like serine proteases (serine protease inhibitors) (Khan et al., 2011). Their exact role in seed tissue of wheat during grain filling is currently not known. However, serpin has been implicated in wheat allergies, reduced grain quality, and insect pest resistance (Ram, 2012; Mameri et al., 2012). Serpin protein showed a strong up regulation in Idaho across stresses compared to a very moderate up regulation in Alpowa. As osmoprotectants function, galactinol synthase included in the biosynthesis of raffinose family oligosaccharides (RFOs) and subsequently induce the plant stress tolerance to salinity, chilling, heat, a superoxide radical generating via accumulation the osmoprotective substance raffinose (Panikulangara et al., 2004). This enzyme introduced transgenically was shown to function in protecting rice grain yield from drought stress through the enhancement of several physiological functions, (Selvaraj et al., 2017). Moreover, the Ervatamin-B which functions as a cysteine protease enzyme, showed a considerable differentiation in its expression compared to the serpin protein indicating a different functional arrangement. It is interesting the dehydrins, ervatamin B protein, and galactinol synthase transcripts are very closely associated in terms of transcript expression

patterns, a possible indicator of co regulation. Tryptophan synthase was shown to be up-regulated only in Idaho indicating a cultivar differentiation. This enzyme is an important step in the tryptophan synthesis pathway which also leads to the synthesis of the growth hormone auxin (UniProt). Possible stress roles may include bacterial defense or wounding responses (Niyogi & Fink, 1992).

The final top 20 differentially expressed genes include an egg apparatus, sucrose phosphate synthase II, premnaspirodiene oxygenase, and helicase transcripts. All four of these proteins show remarkably similar expression patterns in that they are up-regulated almost exclusively in Idaho, thus showing a strong cultivar differentiation. This pattern is also matched by tryptophan synthase, gag-pol polyprotein, heat shock 16.9 kDa protein and two unknowns possibly indicative of coregulation. The egg apparatus protein is a secretory protein that is known to be associated with pollen tube attraction to female gametophytes (UniProt) (Gray-Mitsumune, & Matton, 2006). However, given the post-anthesis stage of development it may be possible that this protein has additional functional activities that are associated with stress and likely with cytoskeleton actin filaments. The premnaspirodiene oxygenase enzyme is involved in the biosynthesis of solavetivone (potent antifungal phytoalexin). This enzyme promotes the hydroxylations of premnaspirodiene and solavetivol (Takahashi et al., 2007). Thus antifungal activities are likely to be important to protect the wheat seed heads during its last stages of its vegetative life cycle from fungal infection. The last top 20 DE transcript codes for helicase enzyme. This enzyme use ATP to remodel or bind RNA or ribonucleoprotein complexes (RNPs). RNA helicases are found in all kingdoms of life and participate in eukaryotes in nearly all aspects of RNA metabolism (Jankowsky 2011).

Table 7. The top 20 up-regulated genes in seed head with the greatest fold change under moderate stress (MS) and severe stress (SS) conditions for 8 days duration in comparison with well-watered control (WW) for Alpowa and Idaho cultivars. The genes are sorted based on functional categorization. ND: not differentially expressed

Up-regulated Differentially Expressed Genes or Proteins	Functional categories	Alpowa		Idaho	
		MS	SS	MS	SS
		Fold change			
Alpha-amylase/trypsin inhibitor	Alpha-amylase endopeptidase inhibitor	20	ND	228	1
Drought-responsive transcription factor	Drought response transcription factor	167	ND	74	ND
Gamma gliadins	Seed protein storage	ND	162	74	ND
18S ribosomal RNA gene	Translation	ND	ND	158	ND
Peroxidase	Oxidative stress	ND	76	71	157
No significant similarity	Unknown	ND	2	64	149
Heat shock protein 16.9 kda	Protein homooligomerization	ND	ND	69	142
Putative gag-pol polyprotein?	DNA integration	ND	ND	67	139
GDP-mannose transporter	Transmembrane carbohydrate transport.	41	ND	64	138
Dehydrin	Response to drought stress	136	ND	76	13
Serpin 1	Serine-type endopeptidase inhibitor activity	3	7	105	133
Hypothetical protein TRIUR3_27653	ABA inducible protein	ND	ND	72	129
Galactinol synthase	Osmoprotection, drought stress	127	ND	64	2
Ervatamin-B protein	Cysteine-type peptidase activity	124	ND	64	4
Tryptophan synthase	Tryptophan biosynthesis	ND	ND	74	123
Hypothetical protein TRIUR3	ABA inducible protein	ND	123	65	ND
Egg apparatus	Pollen tube attraction to ovary	5	ND	89	123
Sucrose phosphate synthase II	Sucrose biosynthetic process	ND	ND	88	123
Premnaspirodiene oxygenase	Sesquiterpanoid sysnthesis	ND	ND	73	122
Helicase	RNA processing	ND	ND	99	120

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

Down regulation provides information on biological processes that are substantially reduced in activity and importance in seed heads exposed to water limiting conditions. The top 20 down regulated transcripts in seed heads exposed to MS and SS in comparison to control are found in Table 8. The overall pattern of expression differs from that of up-regulated DE transcripts in that the differences in degree of expression is more uniform across stress intensities and cultivars with MS Idaho having a slightly higher degree of expression than the other stress intensities. The overall average for the top 20 down regulated transcripts was 92 fold with a range from 230 fold for a maximum and 26 for a minimum. MS-Idaho had an overall average of 106 somewhat higher than the overall average. Of the top 20 down regulated transcripts five were also found in the up-regulated list, including: alpha-amylase/trypsin inhibitors, 18S rRNA gene, drought responsive factor, two egg apparatus proteins and a putative gag pol polyprotein. These were discussed extensively above and will not be discussed here. These proteins likely belong to multigenic families which exhibit complex regulation where a portion of the genes are up-regulated or down regulated depending on the conditions. In addition to the functionally known DE transcripts, discussed above, there were several transcripts with basically unknown functions. While functionally unknown, they appear to be important transcriptional responders to changes inherent in water limiting conditions. These may serve as important target for further research into the mechanisms of drought stress.

Seed head transcripts that are not found on the top 20 up-regulated genes include myrosinase-binding protein (MyroBP), two plasma membrane H+ ATPases, a RPM1 interacting protein and a SEC1 transport protein. The Myrosinase-binding proteins (MyroBP) have been studied in *Arabidopsis thaliana*. This gene was found in plants as a

complex with the glucosinolate-degrading enzyme that plays a role in defense against pathogens (Takeda et al., 2008; Rask et al., 2000). The two plasma membrane H+ATPase are plants plasma membrane proton-pumping ATPase (H+-ATPase) that activate most of the ion and metabolite transport via proton motive force across the plasma membrane (Morsomme & Boutry, 2000). The two distinct transcripts show very similar down regulated expression patterns except for SS-Alpowa where expression was changed 183 fold in comparison to WW treatment. With the application of water limitation, the RPM1 interacting protein was substantially down regulated. This protein is an essential signal transduction regulator of plant defense in the case of pathogen infection and is targeted to recognize selected bacterial avirulence genes triggering the defensive hypersensitive response that helps in limiting the spread of disease (Mackey et al., 2003). The SEC1 family transport protein SLY1 is utilized in vesicular transport between the Golgi and the endoplasmic reticulum for exocytosis (Halachmi & Lev 1996). These proteins are highly conserved among species and are most known to be involved in synaptic transmission in mammalian systems (Halachmi & Lev, 1996). Their association with plants is not known.

The next set of down regulated proteins induced by water limitation include a serine/threonine protein kinase PBS1, a retrotransposon protein, a pectinesterase, a tetratricopeptide repeat protein and a period clock protein. The serine/threonine protein kinase PBS1 has been shown in Arabidopsis to be a signal transduction regulator of pathogen defense mediated by plant resistance proteins (Swiderski & Innes, 2001). The expression patter of this transcript is also similar with the RPM1 interacting proteins suggesting a co-functional unit. Retrotransposon proteins are involved in DNA integration activities associated with “jumping genes”. These transcripts which facilitate this activity

are substantially down regulated in seed heads possibly indicating a protection of the seed tissues from transposition. This process is likely to be very active especially in germline tissues during stress to induce evolutionary genomic changes. The down regulation in vegetative seed tissue indicates a deactivation of a process which in vegetative tissue will have no evolutionary effects. The pectin esterase is a cell wall-associated enzyme that facilitates plant cell wall modification and breakdown. Pectin is one of the central components of the plant cell wall. This enzyme catalyzes the de-esterification of pectin into pectate and methanol. The tetratricopeptide repeat (as interaction modules and multiprotein complex mediators) is highly conserved across all kingdoms of live. Proteins containing this repeat are typically associated with diverse biological processes, such as biomineralization, protein import, organelle targeting, and vesicle fusion to name a few (Zeytuni & Zarivach, 2012). A clock protein plays a central role as a transcription factor in regulating the circadian rhythms in mice. In mice this protein is associated with the regulation of the renal epithelial sodium channel and control of sodium balance (Gumz et al., 2009). While studied in mice and other mammals, this protein has yet to be identified in plants. The remaining 4 proteins all have unknown functions which presents an opportunity for further research in to novel drought responsive mechanisms.

Table 8. The top 20 down-regulated genes in the seed head with the greatest fold change under moderate stress (MS) and severe stress (SS) conditions for 8 days in comparison with well-watered control (WW) for Alpowa and Idaho cultivars. The genes are sorted based on functional categorization. ND: not differentially expressed.

		Alpowa		Idaho	
Down-regulated		**MS**	**SS**	**MS**	**SS**
Differentially Expressed Genes or Proteins	**Functional categories**	**Fold change**			
Alpha-amylase/trypsin inhibitor	Protein degradation	72	89	ND	230
18S rRNA gene	Protein synthesis	78	183	28	77
Drought-responsive factor	Stress	166	70	ND	63
Egg apparatus	Pollen tube attraction to ovary	76	90	153	73
Egg apparatus	Pollen tube attraction to ovary	76	90	132	73
Putative gag-pol polyprotein	DNA integration	84	122	141	66
Myrosinase-binding protein	Flower development, defense	83	72	133	62
Plasma membrane H+-ATPase	ATP biosynthesis, salt stress	76	71	129	84
Plasma membrane H+-ATPase	ATP biosynthesis, salt stress	78	183	125	62
RPM1-interacting protein	Disease resistance	83	103	127	62
SEC1 family transport protein SLY1	Transport	81	76	ND	124
Serine/threonine-protein kinase PBS1	Disease resistance	74	71	124	86
Retrotransposon protein	Retrotransposition	87	86	120	75
Pectinesterase	Pectin catabolic process	87	75	120	62
Tetratricopeptide repeat protein	RNA editing	119	78	61	100
Period clock protein	Circadian rythmns	84	122	26	62
Unknown	Unknown	71	79	122	64
Unknown	Unknown	76	121	26	70
Unknown	Unknown	74	78	ND	150
Shematrin-like protein	Unknown	110	71	121	64

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

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