Physiological and molecular responses to water-stress in local Saudi wheat cultivars

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by

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Abstract

The kingdom of Saudi Arabia (KSA) is among the top countries in terms of agriculture in desert areas, the country uses highly developed agricultural technologies to grow different crops under challenging environmental conditions. The global climate change and the consequent increase in temperature and drought especially in the arid and semi-arid regions made the situation even more challenging. This work aimed at determining the physiological and molecular mechanisms underpinning drought-tolerance in wheat using six local Saudi wheat cultivars. Understanding the phycological responses and gene regulations under water stress could contribute to improving wheat cultivation in Saudi Arabia. My thesis was divided into four main experimental chapters, each chapter describes one experiment. **The first experiment** was to assess drought tolerance in a collection of six known wheat Cultivar grown in different regions of the KSA by comparing their growth and yield under well-watered conditions and water-stress conditions. Shoot weight and length, Root weight and length, Root: shoot ratio, RWC, proline content, soluble sugar content and protein content in addition to yield were used as selection criteria for drought resistance. The results allowed to group the studied wheats into two groups, drought resistant (193 Najran (Cv2) and 357 Sama (Cv3)) and drought sensitive (181 Jizan (Cv1), 377 Rafha (Cv4), 562 Ma'ayah (Cv5) and 981 Najd (Cv6)). In **the second experiment,** RNA sequencing was performed in leaf samples harvested from water control and PEG-treated plants of one drought resistant 193 Najran (Cv2) and one drought sensitive cultivar (377 Rafha (Cv4)) at the vegetative, flowering and grain filling stage. Transcriptomic analysis aimed at finding differentially expressed genes and associated metabolic pathways in the two Saudi wheat cultivars under water stress at the three growth stages. This yielded 24.2 GB of sequence data. At least 40 million reads of 85 bp each were obtained per sample, Although genes from different pathways changed expression under water-stress, the increase in expression of genes associated with Photosynthesis, Amino acid metabolism and Secondary metabolism such as phenylpropanoid biosynthesis seemed to be the most important. **The third experiment** investigated the drought induced changes in the proteome in drought resistant 193 Najran (Cv2) and drought sensitive cultivar (377 Rafha (Cv4)) at the vegetative, flowering and grain filling stage, Proteomics analysis showed tangible changes in protein levels indicated a general regulation trend of plant defence under water stress, such as Stress/defence/detoxification proteins, Photosynthesis proteins, Carbohydrate metabolism proteins and Amino acid metabolism proteins. In **a fourth experiment,** based on the results of the transcriptomics and proteomics results together with the literature, the expression of four drought

related genes The four genes included Dehydrin gene (DHn3), Bidirectional sugar transporter (Sweet), Phenylalanine ammonia-lyase (Pal5) and Serine hydroxy methyl transferase (Shmt) found to be various regulation in the six wheat cultivars based on their response to water stress. using qRT-PCR. The qRT-PCR analysis confirmed increased expression levels of these genes known to be up-regulated under water stress.

From this work, I could conclude the following:

1. There is a big difference in the speed of response to water-stress between wheat Cultivars, this difference is associated with variation in transcript and protein expression levels at three growth stages.
2. The vulnerability of wheat plants to water stress is higher at the flowering stage compared to the vegetative and grain filling stages. Attempts to improve drought tolerance in wheat should be targeted to this growth stage.
3. The phenylpropanoid metabolic pathway plays a key role in resistance to water-stress in wheat and might be a target for improving drought resistance in this crop.

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List of abbreviations

DEGs	Differential Expression Gene
DEPs	differentially expression proteins
FAO	Food and Agriculture Organization of the UN
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
NGS	next generation sequencing
PAL	Phenylalanine ammonia-lyase
PEG6000	Polyethylene glycol
QC	Quality control
QRT-PCR	Quantitative real-time polymerase chain reaction
QRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNA-seq	high throughput cDNA sequencing
ROS	Reactive oxygen species
RWC	Relative water content
sc-PDSI	The Palmer Drought Severity Index
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
SHMT	serine hydroxymethyltransferase
Sweet	Bidirectional sugar transporter
UniProtKB	UniProt KnowledgeBase
YSK2 dehydrin 3	Salt-induced YSK2 dehydrin 3

List of equation

equation 1 $\mathbf{RWC}\ (\%) = [(\mathbf{FW} - \mathbf{DW}) / (\mathbf{SW} - \mathbf{DW})]\ \mathbf{x\ 100}$ 33

equation 2 $\mathbf{WST} = \mathbf{Ps/Pc}$ 35

equation 3 $e = 100 * (-1 + 10 - 1/\text{slope})$ 134

equation 4 ratio = efficiency amplification ^ − (ct target gene (control − treatment)) /
efficiency amplification^(ct reference gene (control − treatment) 134

Chapter 1 Introduction

1.1.General Introduction

Plant diversity is a section of the biological diversity of living organisms, as the United Nations Environment Program (UNEP,1992) has defined biodiversity as the variation between different organisms in the ecological habitats. Biological diversity divided into three main aspects, Ecological diversity, which is the variation between species of living organisms at the level of ecosystems, Genetic diversity, which is the variation between the different species in terms of patterns of a genetic structure at the level of taxonomic units, and Species diversity, which is the species variation of a community. Rapid changes of physiological and developmental traits occurred due to the high impact of climate change on events, such as phenotypic plasticity, epigenetic modifications, and genetic adaptation. Molecular analysis, primarily through omics approaches, of the abiotic impact has revealed the underlying biochemical and physiological mechanisms, thus characterizing the links between phenotypic plasticity and climate change responses (Bigot et al., 2018; Anderson and Song, 2020).

1.1.1. The economic impact of drought.

Water constitutes approximately 90% of the non-woody plant biomass, it is the major medium for transporting metabolites and nutrients for the plant's physiological processes. Fully understanding the importance of water to life and whole creatures are essential "We made every living thing from water" (Quran 21.30-31). Drought is generally defined as a deficiency of average precipitation, in addition to soil moisture deficit, which results in plant stress and results in yield loss, and crop failure. The severity of the drought depends on the occurrence, intensity, and frequency (Boken et al., 2005, Hayes et al., 2012, IPCC, 2014, Walz et al., 2018). Drought could have several definitions depending on the context, but the main issue is always water deficit; some of these different definitions of drought are given in figure 1.1 below.

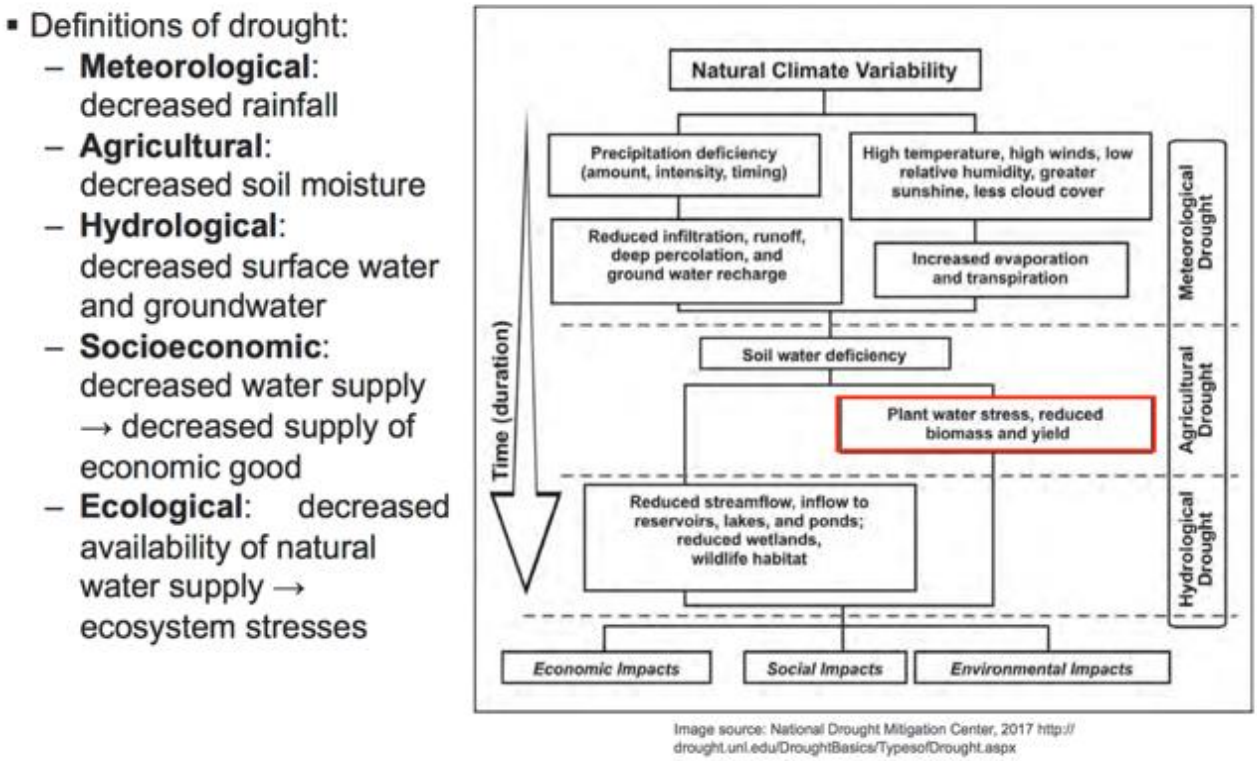


Figure 1. 1 Definition of drought, the red highlighted definition applies to the current study investigation (National Drought Mitigation Center, 2017).

Climate change mainly increasing the frequency of drought in dry regions such as Africa, central and southern North America, Southern Europe, and the Mediterranean region (Dai, 2011) (figure 1.2). Ethiopia is an example of the largest wheat-producing countries in Sub Sahara Africa. Dehydration occurs in the rain season (June) was affect the good wheat harvest in November or December of Ethiopia wheat production (White et al., 2001). Even though climate events such as earthquakes, extreme temperatures, flood and storms may lead to chronic economic loss, the drought-caused financial loss is a persistent issue more than all climate events. For example, the typical time scale associated with a heatwave is on the order of a week, while drought may continue for months or even years (Mishra and Singh, 2010). Australia is one of the major wheat export countries in the world. In 2019/20, Australian wheat production was reduced by more than 11% because of flowed three years drought event, which causes decreasing in wheat exports and damages Australia's economy (Packham, 2019).

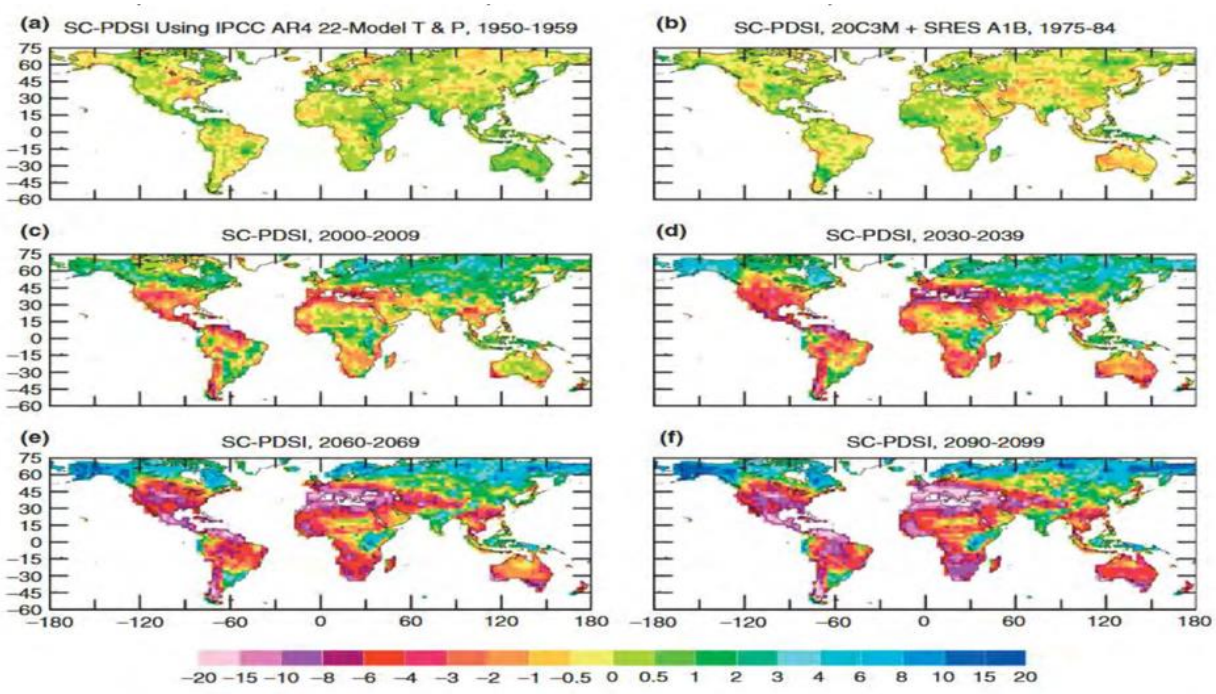


Figure 1. 2 Drought under global warming. Showing the dry patterns for years (a) 1950–1959, (b) 1975–1984, (c) 2000–2009, (d) 2030–2039, (e) 2060–2069, and(f) 2090–2099. Red to pink areas are extremely dry (severe drought) conditions. Blue colors are wet areas. *x*, latitude degrees. *y*, longitude degrees. sc-PDSI (The Palmer Drought Severity Index). Image source: (Dai, 2011).

Drought affects many life aspects, not only in the agriculture sector but also in non-agriculture sectors. For example, drought can cause a substantial decrease in the water level of rivers, which would affect the transportation of goods. Drought impacts the economy at three levels: **Individual**, increase in food prices, **Businesses**, negative impact on goods transportation and **National**, A one-per cent increase in the area affected by drought can slow a country's gross domestic product (GDP) growth by 2.7 per cent per year (McDermott et al., 2013; Dai, 2011; Brown et al., 2013; Shiferaw et al., 2014; EPA, 2015).

Drought affected 1.5 billion people in the 1998-2017 period (Wallemacq, 2018). It is the most relevant hazard in the world in terms of economic losses. In 1998-2017 approximately 124 billion US$ economic losses were recorded due to drought (Wallemacq, 2018). In 2003 a severe drought event was recorded in Italy; it caused a 29.6% decrease in crop production with a 9.9% decrease in cereals (Mishra and Singh, 2010). Also, in Texas (USA), drought caused significant damage to the cropping area and crop yield between 2013 and 2016 (Ray et al., 2018). Table 1.1 and figure 1.4 show the crop decrease in different countries affected by drought. However, some countries such as Russia have a good wheat growing strategy to overcome drought events and increase wheat production by combining spring and winter wheat cultivars in the same year. Therefore, Russia's

wheat production increased by 30% in 2013/14 compared to 2012 when severe drought sharply reduced spring wheat yield in the country (USDA, 2013).

Saudi Arabia is another example of countries successfully overcoming or controlling the impact of drought on their economy by constructing desalination plants as early as the 1970s and by investing in other countries where it grows its food to be exported back to the Kingdom (DeNicola et al., 2015).

Countries	Drought events	Crop production (%)	Recourse
Italy	2003	-29.6	Mishra and Singh,2010
Portugal	2004-2006	-17.1	Mishra and Singh,2010
East Texas	2013-2016	-125.3	Ray etal.,2018
Kansas (USA)	2016-2018	-16.8	USDA,2018
Australia	2017-2018	-27.6	Bond and Liefert,2018

Table 1. 1 Decreased wheat production in different countries affected by drought.

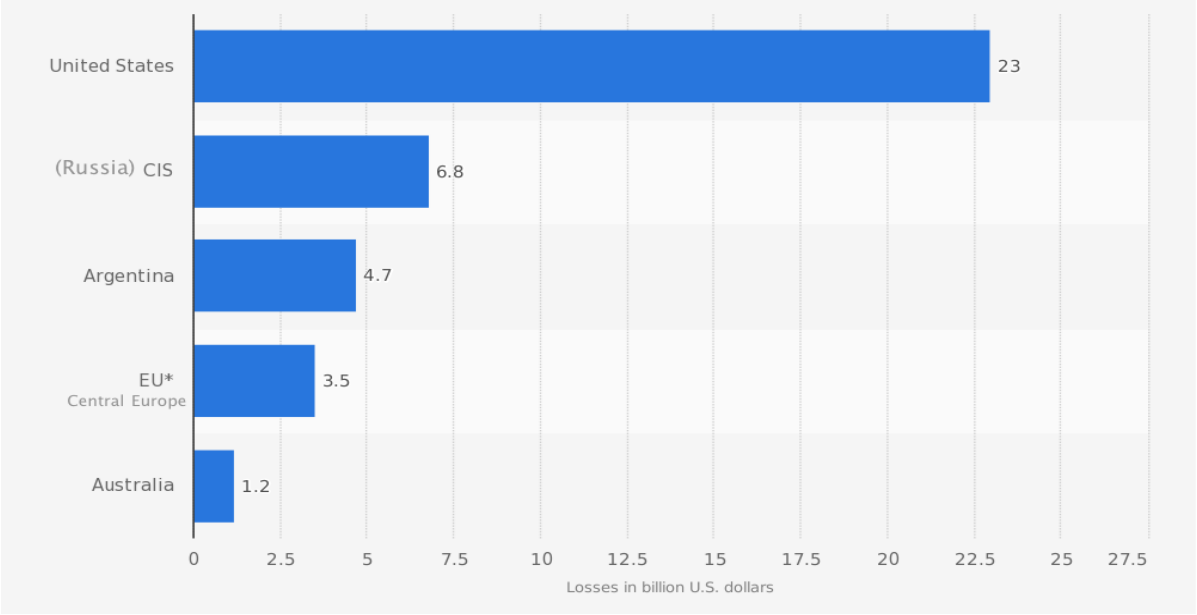


Figure 1. 3 Major global crop losses due to drought in 2012, by region (in billion U.S. dollars). Image source: Syngenta,2019.

Many countries have active hazard monitoring systems that measure the drought level based on hydrological droughts such as low water levels in rivers or reservoirs and meteorological drought such as lack of rainfall (Mishra and Singh, 2010; Walz et al., 2018). Many computer software such as InfoCrop v.21, Cropsytv 4.19, DSSATv4.5, and WOFOSTv1.5 were used to simulate the crop growth, soil productivity, and sustainability of agriculture production. These simulations aim to avoid the effect of climate change via the input of specific information about plant growth, soil, and weather (Eitzinger et al., 2004; Shekhar et al., 2008; Hadiya, 2016; Hadiya et al., 2018). However, this is not enough from the agricultural point of view; using different varieties of crops can be used to cope with drought challenges under drought stress (Ray et al., 2018). Beads on this concept, the need to find new resistant crop cultivars.

1.1.2. Plant adaptation and responses to drought

Plants have the ability to adapt under severe abiotic and/or biotic stress through specific changes at physiological, morphological, molecular, and cellular levels. The high ability of plants to adapt to severe water deficit is described as tolerance to drought. However, the meaning of drought tolerance in xerophytes such as cacti is survival during a drought, whereas in crops are the quality and quantity of production (Belhassen, 1997; Obidiegwu et al., 2015) (figure 1.5). Therefore, the meaning of "drought tolerance" in this study is going to be defined in terms of yield under a limiting water supply. Plant's responses to stress might happen at different levels; for example, under long-term drought stress, plants respond by inhibiting shoot growth, gene expression, and metabolite accumulation. Under short drought, plants respond via stomatal closer and osmatic adjustment (figure 1.6) (Chaves et al., 2003). Moreover, plants reduce transpiration by shedding their old leaves under high drought stress after remobilizing hydrocarbons and minerals from leaves to roots and stems to minimize water loss and increase plant growth (Arndt et al., 2001). Plants respond to drought stress in three different ways: avoidance, escape, and tolerance (Levitt, 1972; Turner, 1986; Ahanger et al., 2014). Also, plants can combine different strategies to survive under severe drought (Ludlow, 1989). For example, watermelon adaptation strategies under drought stress are based on an increase in various defense responses such as antioxidants, protein protection, osmotic adjustment, wax accumulation, hormone signaling, and melatonin biosynthesis to decrease potential damage that would happen in the cell membrane and other cell components (Li et al., 2019a). There are many traits that explain plant adaptation to droughts, such as root size and depth, phenology, hydraulic conductivity, and storage of reserves (Chaves et al., 2003). One of the widely measured plant's adaptation traits had been the accumulation of cellular solutes such as proline and soluble sugars, Mostajeran and Rahimi-Eichi (2009) and many researchers found the increase of

proline and soluble sugars was to protect the macromolecular components from denaturation under drought stress. From gene expression insight, Ergen et al. (2009) found noticeable differential gene expression patterns in drought-tolerant and sensitive wild emmer wheat genotypes under drought. Such as differential usage of IP-dependent signal transduction pathways, ethylene, and abscisic acid (ABA) signals. Local crop cultivars are considered a useful genetic resource due to the better adaptation to environmental stress. For instance, wild barley was used as a source of genes for breeding programs (Ellis et al., 2000; Pickering and Johnston, 2005; Hübner et al., 2015).

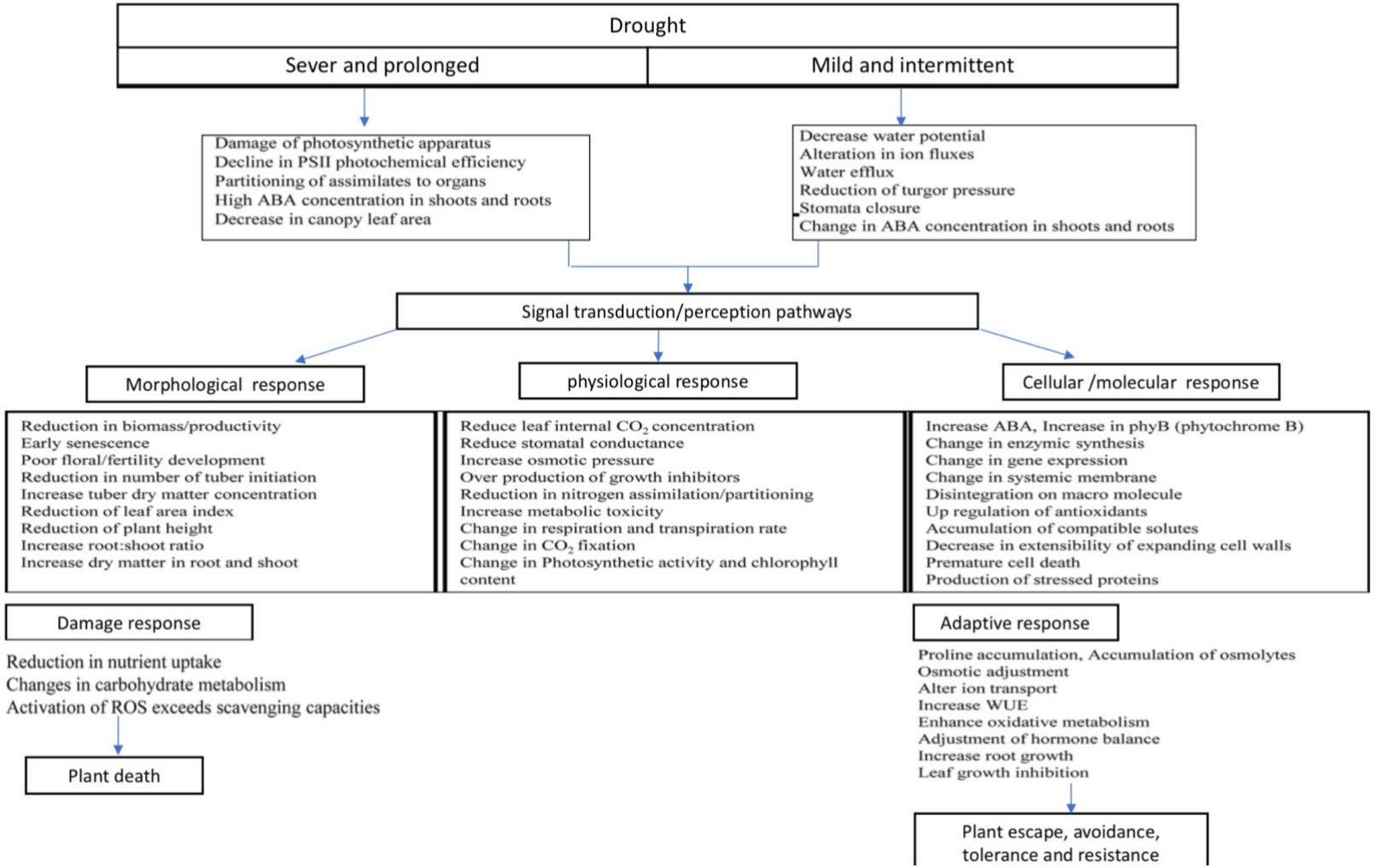


Figure 1. 4 Flow chart detailing the effect of different levels of drought and how plants respond to the water-stress stimulus at molecular, physiological, and morphological levels. (modified from Obidiegwu et al., 2015).

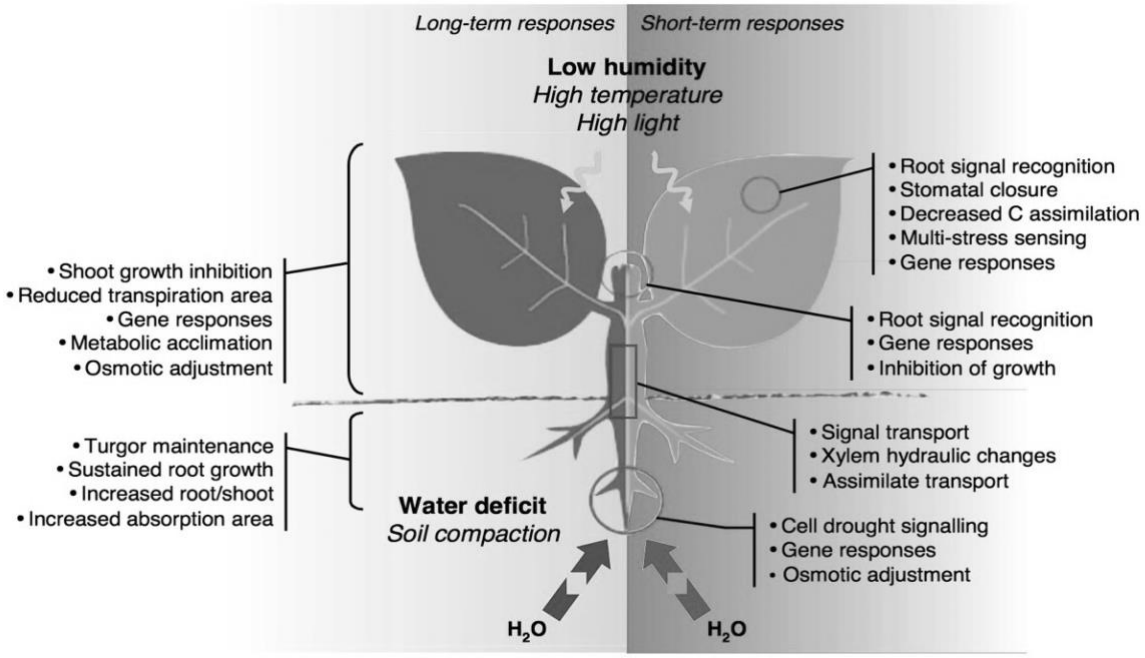
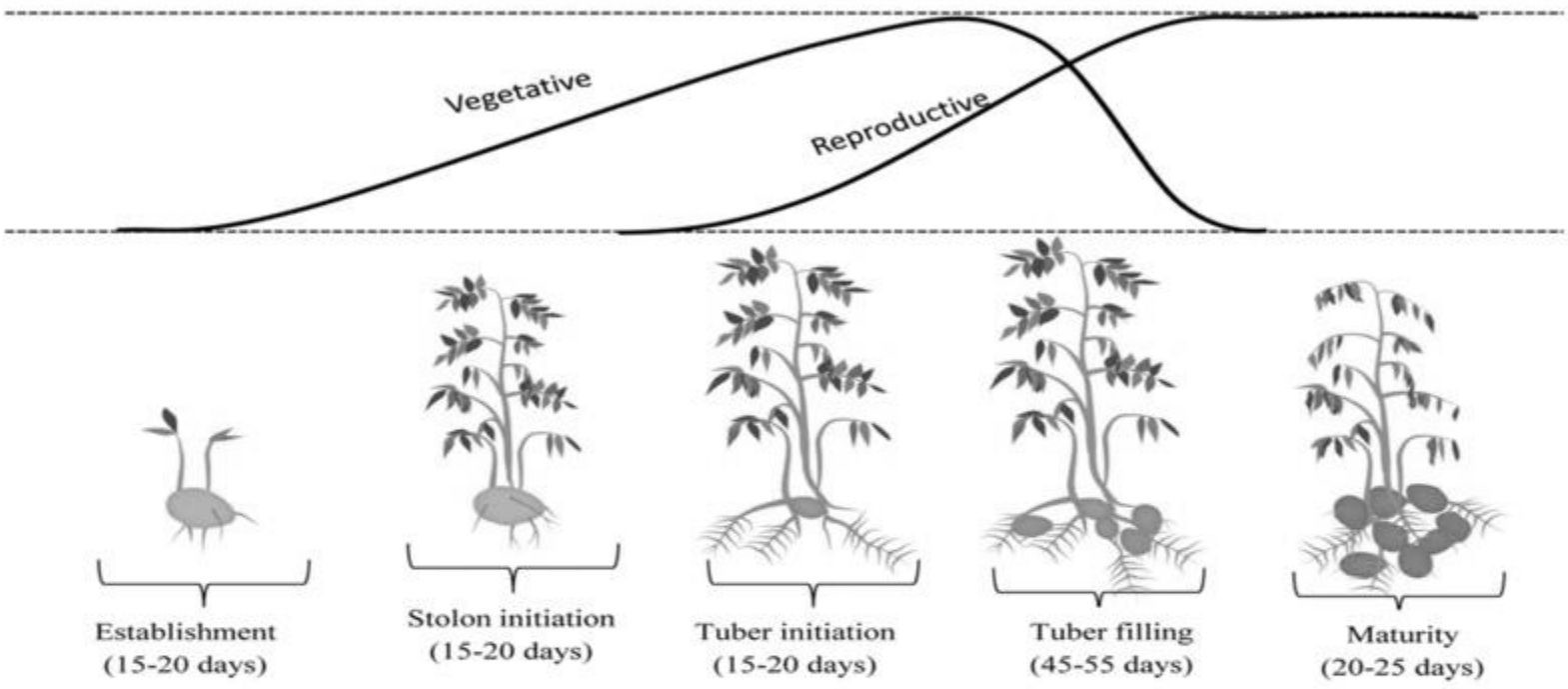


Figure 1. 5 Whole-plant response to drought stress. Left, long-term or acclimation responses; right, short-term response. Image source: (Chaves et al., 2003).

1.1.3. Age and developmental effects on drought resistance.

Plants respond variably to drought during their development. The most affected stage is seed germination; seeds' ability to germinate under a severe environment is the first step in drought resistance. It was found that seed germination significantly declined under drought in crops such as wheat (*Triticum aestivum*) (El-Nakhlawy et al., 2015, Nagy et al., 2018), rice (*Oryza sativa* L.) (Swamy et al., 2017), maize (*Zea mays* L.) (Janmohammadi et al., 2008), Barley (*Hordeum vulgare*) (Guo et al., 2009) and other herbs and tress such as sunflower (*Helianthus annuus* L) (Kaya et al., 2006); *Cupressus ari- zonica, Sophora japonica, Pinus nigra, Cupressus sempervirens,* and *Pinus brutia* (Sevik and Cetin, 2015). Some crops considered as drought-sensitive such as Potato (*Solanum tuberosum* L.) (figure 1.7) show a response to drought from establishment stage to maturity stage (Obidiegwu et al., 2015).



Impact of water deficit	Delayed emergence Restrict root establishment Restrict plant development Presence of fewer stalks/stem	Restrict plant development Limited stolon initiation Limited number of stolon Restrict root growth Restricts uptake and response to nutrients	Limited foliage and plant development Limited tuber initiation Limited number of tubers	Limited plant and tuber development Restricts tuber size Promotes distorted tuber shape Faster senescence	Limited relative tuber density Limited tuber size

Figure 1. 6 Effect of water stress at different growth stages of potatoes. Image source: (Obidiegwu, et al. 2015).

Drought tolerance of wheat leaves decreased with plant age (Blum and Ebercon, 1981), embryo abortion is a widespread phenomenon in plants if abiotic stress occurs during the reproductive stage (Setter et al., 2010; Feng et al., 2011; Hayes et al., 2012). This effect has been reported for many cereal crops, such as wheat (bread wheat) (Kakumanu et al., 2012). In terms of wheat growth stages, it was found that wheat in tillering and anthesis stages was more affected by drought stress with regard to the obviously decreased grain weight and grain yield (Sheoran et al., 2015). Tolerance to drought is increased if plants are exposed to water-stress before the anthesis stage (Wang et al., 2014a). It's obvious from the many research to consider the importance of selected crop varieties adjusted to the local climate to mitigate the huge drought impact on cereal crops production (Daryanto et al., 2017).

1.1.4. Wheat production, types, economic importance

Wheat (*Triticum aestivum*) is at the top of the worldwide food list since 8000-10000 B.C., together with rice and maize (Shewry, 2009; Okay et al., 2014; Khan, 2016). The first documented growing area of wheat was the fertile crescent (8000 to 10000 years ago) (Gill and Friebe, 2002). Current wheat resulted from hybridization between cultivated emmer wheat which is tetraploid (AABB, *Triticum dicoccoides*) and the diploid wild goatgrass (DD, *Aegilops tauschii*) (Shewry, 2009; Brenchley et al., 2012 ; Vu et al., 2017). Figure 1.8 shows the evaluation of hexaploid wheat. It has a very large (17-gigabase-pair) polyploid genome (Brenchley et al., 2012; Bierman, 2015). Wheat belongs to a huge plant family called Poaceae, which has the most economical plants such as rice and barley (figure 1.9).

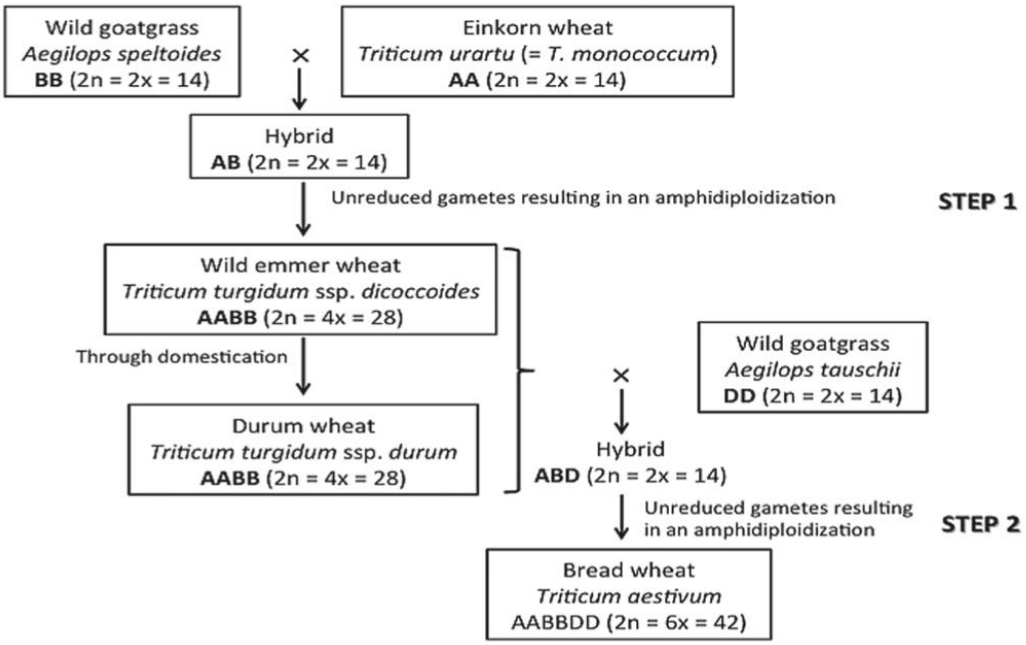


Figure 1. 7 The evolution of hexaploid wheat *Triticum aestivum* L. Source: (Jauhar et al., 2009; Harris et al., 2014).

Wheat grows successfully between the latitudes of 30° and 60° North and 27° and 40° South (Nuttonson,1955) this region has an optimum temperature of about 25°C with a minimum of 3°-4° C and a Maximum of 30°-32° C, thus wheat is being grown and harvested somewhere in the world in any giving month. Wheat harvesting in the temperate zones occurs between April and September in the Northern hemisphere and between October and January in the Southern hemisphere. Based on seasons of growing, wheat is classified into spring or winter wheat. Spring wheat is planted in spring and matures in late summer or in the autumn in south Asia and middle east. In Winter wheat, the vegetative stage would be exposed to a period of cold winter temperature (0°-5° C) (Curtis, 2002).

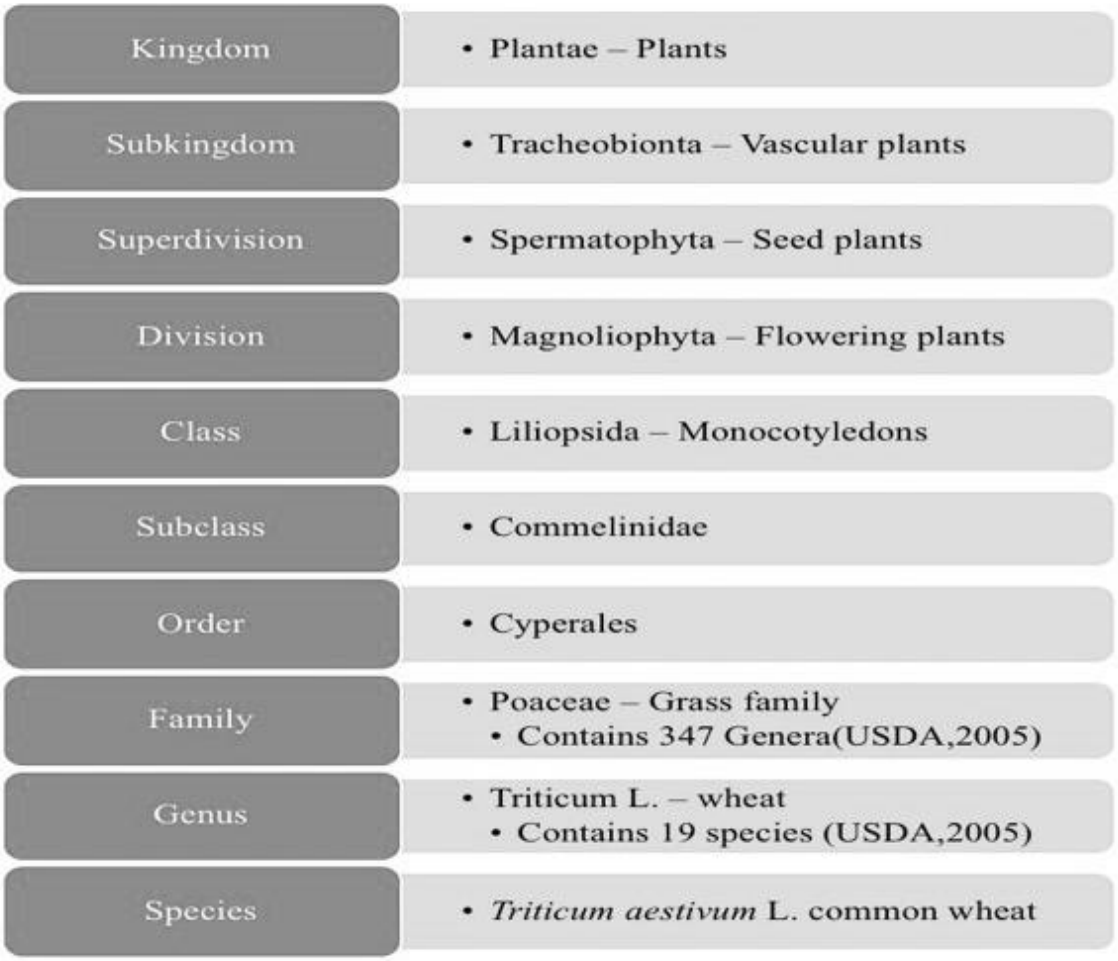


Figure 1. 8 Wheat taxonomic classification.

Wheat growth divides into several stages based on the *Feekes* scale (Large, 1954) and *Zadoks* scale (Zadoks et al., 1974). Both of the scale ratings are based on leaf development and grain filling and the difference between the two scales is that *Zadoks* scale gives more details to the vegetative stage than *Feekes* scale (figure 1.10).

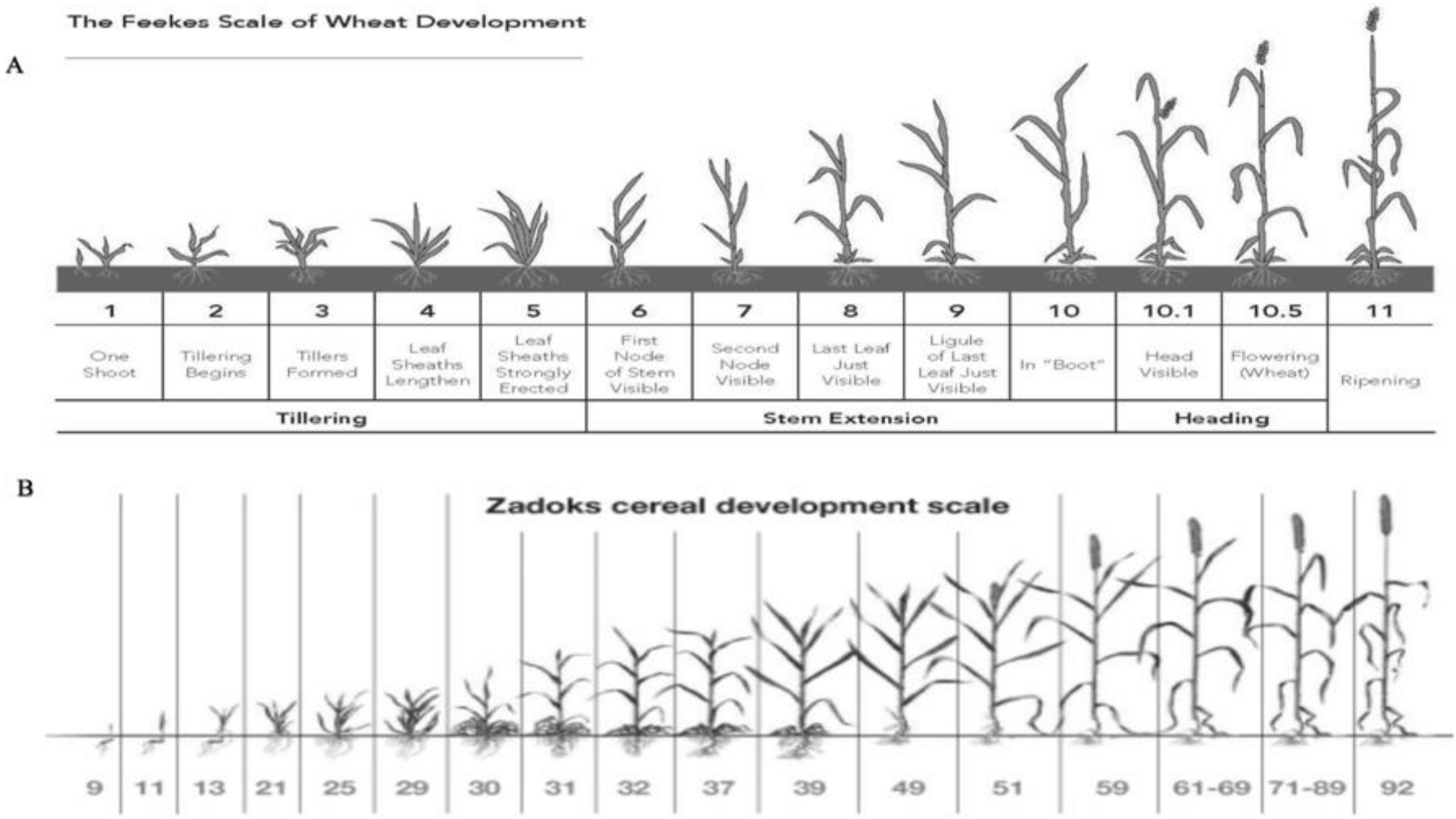


Figure 1. 9 Wheat development stages. A. Feekes scale (Large, 1954). B. Zadoks scale (Zadoks et al., 1974).

Wheat (*Triticum aestivum*) is a cereal crop of high economic and social importance across the Globe with an estimated global production of 754.1 million metric tons in 2018 (FAO). According to the Global leading wheat producing countries, 2017/2018 Statistical report, the European union (Andersen et al., 2002) was among the top 5 wheat producing countries by 137.6 million metric tons followed by China with 132.5 million metric tons in 2018/19. However, there are noticeable ups and downs in wheat production during the last three years due to weather variables (Table 1.2). Wolf (1993) indicated a reduction in E.U. winter wheat production under increasing wind speed, temperature, and solar radiation.

Due to the expected increase in the world population (9.7 billion by 2050) a steady increase in crop production of at least up to 60% is needed to fulfill the extra demand (Godfray et al., 2010). Asseng et al. (2019) assumed that wheat yield could increase by 7% if new stress-adapted wheat cultivars were introduced. Unfortunately, due to environmental stresses, particularly increased drought and salinity, wheat production has not increased as expected during the last ten years. It is expected to decline in the future if no new wheat cultivars with higher tolerance to drought and/or salinity are developed.

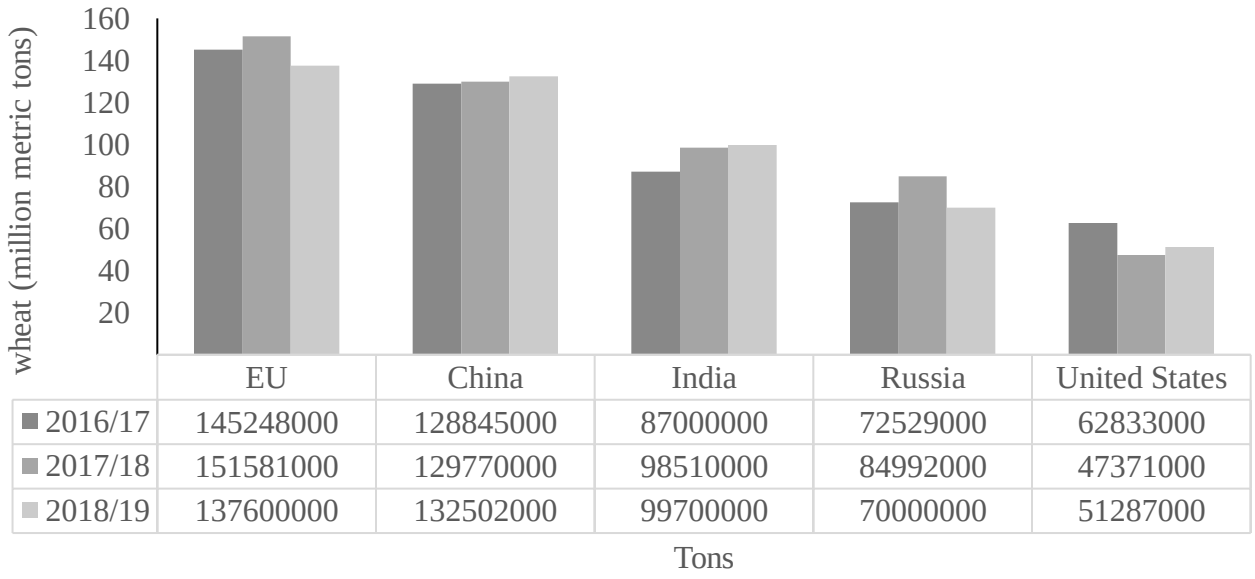


Figure 1. 10 Five leading wheat producers worldwide in 2016/17, 2017/18 and 2018/19 (data as million metric tons). Source: (Statista, 2019, Cook, 2019).

1.1.5. Wheat in Saudi Arabia.

Wheat is the main crop in the Kingdom of Saudi Arabia (KSA), in the 1970s, the country was considered the sixth wheat exporting country in the world (Hartmann et al., 2012). And in the early 1990s, it was considered self-sufficient in wheat (Al-Shayaa et al., 2012, Albokari et al., 2012). Wheat cultivars sown in the different regions of the country have different traits; for instance, in the middle of KSA, which has low rainfall with high average temperatures, wheat cultivars such as SAMA and Al-Lugaimi, which are considered as drought tolerant, were common (Ashraf et al., 2013). The wheat cultivar Al-Hassa which is considered salinity resistant according to the 2015 list of wheat accessions – ministry of Agriculture Saudi Arabia, was grown on the east coast of the country.

Saudi wheat has high genetic diversity (AlJuwaeid, 1989, Alghamdi et al., 2017a) with Cultivars highly adapted to arid and saline conditions; this has allowed KSA to be among the wheat exporting countries. In 1982, the proportion of wheat production to total cereal production was 85.3% (Elhadj, 2004); however, limitations in natural water resources increased in recent years with severe consequences on wheat productivity. Saudi Arabia lacks rivers and lakes, and the level of annual rainfall is now limited to around 40-144 mm in wet years with high evaporation rates (Hasanean and Almazroui, 2015). The main water resource in the country is desalination of seawater, which, if used for irrigation, would cost a lot of energy with a high environmental impact due to the consequent CO_2 emissions (Stokes and Horvath, 2009; Fiaz et al., 2018; Wakeel et al., 2016). In 2008, the Saudi government decided to decrease local wheat production by 12.5 % and gradually reduce domestic wheat production starting from 2016 to decrease water consumption in irrigation (Ahmed et al., 2013). The production of wheat in 2007-2008 was 2.35 million tons; it has, however, sharply declined to 30,000 tons in 2015-2016 (Agrochart, 2014). However, wheat research is actively encouraged in light of the fact that wheat studies could lead to wheat cultivars adapted to increasing drought and possibly other adverse environmental conditions like high temperatures and salinity.

1.1.6. Lack of resistant wheat cultivars

Enhanced wheat resistance to biotic and abiotic stress is of high economic importance. Wheat crop is affected by a number of diseases, insects, pests, or environmental changes. Due to the rapid progress of these stress factors and their aggressive impact, a Sustained effort is made to find resistant wheat cultivars such as in Bangladesh (Haque et al., 2020), Australia (Yadav et al., 2019a), Russia (Di Paola et al., 2018) and Europe (Senapati et al., 2019). However, many

countries exposed to severe environments, such as Saudi Arabia (case of the current study), need to decrease the water consumed in agriculture due to a lack of drought resistant cultivars.

The improvement of response to drought is complicated due to the complexity of the wheat genome (Nezhadahmadi et al., 2013) and the interplay between a large number of genes encoding components of cellular signal transduction pathways and proteins and enzymes responsible for the stress-tolerance response (Jiang et al., 2017a, Barnabás et al., 2008). Therefore, convenient approaches are needed to select/develop a such resistance. These approaches must be informed by a good understanding of the morphological, physiological, and molecular responses of wheat together with changes in biotic and abiotic stress genetic characteristics.

1.1.7. Response of wheat to drought (plant, organ, protein, gene)

Some wheat Cultivars have the ability to grow under severe drought through specific changes at the morphological, physiological, cellular, and molecular levels (Ashley, 1993, Chaves et al., 2003) (figure 1.5). Under abiotic /biotic stress, plant cell organelles such as plastids, mitochondria, and peroxisomes initial response is to increase toxic components such as reactive oxygen species (ROS), which are produced by the aerobic metabolism that leads to an imbalance between the production and scavenging of ROS content (Bailey-Serres and Mittler, 2006). ROS is highly toxic and can damage important components of plant cells such as proteins, lipids, carbohydrates, and DNA, which ultimately results in oxidative stress (figure 1.12) (Gill and Tuteja, 2010). Mitochondria may play an important role in the generation of ROS through energy-dissipating systems such as the plant uncoupling protein PUCP; the ATP-sensitive plant mitochondrial potassium channel PmitoKATP; and the alternative oxidase (AOX) (Pastore et al., 2007, Pastore et al., 1999).

There is strong evidence to suggest that significant changes in the accumulation of wheat contents are a way to adapt to drought stress (Gregorová et al., 2015; Bayramov, 2017). For example, it was found that under drought, wheat has reduced overall protein content with increased Rubisco and PEPC enzymes (Bayramov, 2017). In addition, accumulation of free proline in drought-tolerant wheat was higher than in wheat cultivars which were considered as sensitive to drought (Kocheva et al., 2013) and the same was found for phenolic and flavonoid contents as well as malondialdehyde (MDA) content (Al-Ghamdi, 2009; Gregorová et al., 2015). Some enzyme' activities were found to be increased under drought (Sheoran et al., 2015); among them, chitinases and glucanases increased under severe water stress (Gregorová et al., 2015).The activities of the antioxidant enzymes such as superoxide dismutase (SOD) were higher in drought tolerant wheat, whereas the higher expression level of Mn-SOD in the same wheat under drought conditions

showed that SOD might play an important role to protect wheat tissues from the effect of severe drought (Sheoran et al., 2015). In contrast, Han et al., (2015) found that water-stress could lead to reduced leaf area, dry weight and total root length, they also found that sucrose content declined in roots yet it increased in leaves of Wangshuibi wheat cultivars which are considered as sensitive to drought stress. On another hand, Marcińska et al. (2013) found significant increase in root length, plant high, relative water content (RWC) in leaves and root, proline and carbohydrate content in leaves of resistant wheat cultivars than in sensitive cultivars. These increases could be a way to regulate the osmatic pressure in wheat (Delauney and Verma, 1993). According to Hura et al. (2007) during osmotic stress imposed by PEG-treatment, drought resistant wheat showed a capability for osmoregulation, which enabled it to maintain a relatively high turgor of protoplasts and leaf-water content, also decreased activity of the photosynthetic apparatus occurred to prevent the over-reduction of electron transport components. This is in agreement with the findings of other research where a decrease in chlorophyll index and water content (RWC) was shown in different wheat sensitive to drought under drought stress (Sheoran et al., 2015, Gregorová et al., 2015). Moreover, wheat under drought stress can show degraded chloroplasts with a drastically reduced photosynthesis (Nagy et al., 2013a) .

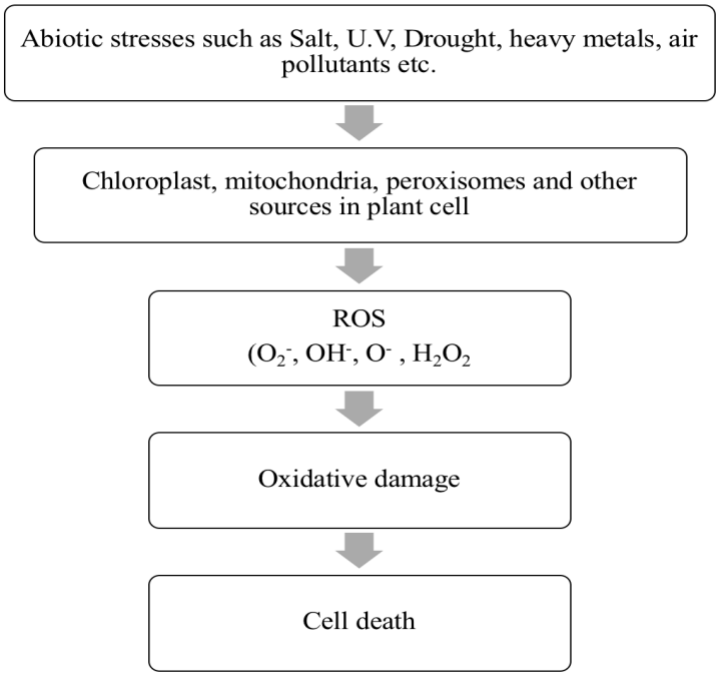


Figure 1. 11 Abiotic stress induced ROS production and cell death. (modified from Nagy et al., 2013a).

Moreover, drought stress could significantly decrease Nitrogen efficiency ratio (NER), nitrogen uptake efficiency (NUpE), nitrogen use efficiency (NUE), nitrogen fertilizer utilization efficiency (NfUE) in winter wheat. All the decrease was higher in drought-sensitive than drought-resistant wheat (Sheoran et al., 2015). This would significantly influence protein synthesis, the decrease in total protein content in plant tissues is an excellent physiological parameter to indicate the sensitivity of wheat cultivars to stress. Nagy et al. (2013a) found that total protein content was increased in drought-tolerant wheat and decreased in drought-sensitive wheat. Gregorová et al. (2015) found that drought stress would affect shoots and roots in different ways, they showed that dry weight and length of wheat shoot decreased while they increased significantly in roots. A high ability to improve root systems under insufficient soil moisture is an important feature in wheat cultivars. This can enable wheat to produce more dry matter under drought stress because wheat can uptake much water from the soil (Saidi et al., 2008). At the gene level, the elevated expression of TaMYB33 genes in wheat under osmotic-stress as a result of drought and salt stress, mediated by stimulated ABA production correlated with increased resistance in wheat tissues to severe drought and high NaCl concentrations (Qin et al., 2012). Similarly, Baloglu et al. (2014) indicated higher expression of TaMYB33 and TaWLIP19 genes in drought and salt tolerant wheat species. Also Zhou et al. (2015) found that the high accumulation of TaFBA1 transcripts in wheat could enhance the oxidative stress tolerance of wheat, which may be involved in drought tolerance. Some genes induced during drought stress are responsible for producing important metabolites and proteins to protect cells from the impact of water deficit (Shinozaki and Yamaguchi-Shinozaki, 1997). For instance, Expansins which are important cell wall proteins involved in the loosening of the cell wall during cell elongation, were affected by drought, particularly during the elongation stage during leaf development. It was shown that the transcript level for the Expansins gene was higher in drought tolerant than in drought-sensitive wheat cultivars (Zhou et al., 2015). Ergen et al. (2009) found noticeable changes in gene expression patterns in drought tolerant and sensitive wild emmer wheat genotypes, such as genes involved in IP3-dependent signal transduction pathways, ethylene and abscisic acid (ABA)–dependent signaling pathways. Products of these stress-induced genes include enzymes required for the biosynthesis of various osmoprotectants such as sugars, proline, and Glycine-betaine (Shinozaki and Yamaguchi-Shinozaki, 1997). Measurements of proteins, soluble sugars, and proline contents together with global profiling of gene transcripts could be used as good markers to investigate the regulation of metabolism under drought stress. Proteins are directly involved in metabolism and cellular development. Thus, proteomics analysis has been an essential tool for understanding the mechanisms and biological interactions involved

in plant responses to the environment (Gong and Wang, 2013). For example, glutamine synthetase isoenzymes are good markers of plastid status and nitrogen metabolism under stress conditions (Nagy et al., 2013a). Late embryogenesis abundant proteins that may protect macromolecules and membranes under water-related stress are also good markers of water-stress related metabolic changes; expression of the TaDlea3 gene which encodes LEA protein increased in wheat during developmental stages under drought stress (Chen et al., 2016a, Asseng et al., 2019). A plastid outer envelope protein (TaOEP16-2) increased under heat and drought stress in wheat (Zang et al., 2017). Differentially expressed genes in response to treatment could be identified and associated with a specific treatment (Hübner et al., 2015). Therefore, the mechanisms by which plants respond to stress conditions could be easily understood by profiling plant transcriptome (Chung et al., 2017).

1.1.8. The perspective of research on drought resistance in wheat.

Wheat products have been main course in humankind's meal since a long time. The rapid rising in population imposes the need that wheat production is increased. Unfortunately, there is no balance between the population rise and wheat production due to the significant changes in the world environment, which negatively impact the growth of the available wheat cultivars.

Therefore, the need for new wheat cultivars that are resistant to different environmental stresses. Understanding the regulation of the genes involved in drought tolerance in wheat could pave the way for improving the physiological response to drought stress in wheat cultivars.

The wheat genome is complex because of its hexaploidy, huge size (17 Gb, almost six times the human genome size ~3.2 Gb) (Kumar et al., 2015a; Poersch-Bortolon et al. 2016b; Alipour et al., 2019,) and high repetitive sequences (85%) (Jia et al., 2018). The three biochemical datasets that provide the genomic information are Transcriptomics, including the complete set of mRNA molecules that result in generating proteins, Proteomics, the complete collection of proteins, and Metabolomics, the complete series of metabolites produced in the cell (Romero et al., 2006, Horgan and Kenny, 2011). As previously mentioned in 1.1.7, the availability of genomic tools and resources allowed us to understand transcriptome and proteome changes under different environmental stresses, demonstrating how plants cope with different stresses. Regulation of several major classes of genes has been suggested to assist cellular adaptation under stressful conditions (Bray, 2004). For instance, upregulation of TaCRT transcripts in wheat response to drought stress (Islam et al., 2015b) and transcription factor dehydration-responsive element-binding protein (DREB) regulate the expression of genes response to salt stress in wheat (Jiang et al., 2017a). And TaZFP34 gene was upregulated under abiotic stress resulting in enhanced root elongation and reduced shoot growth in wheat (Chang et al., 2016). Raney (2012b) found from

RNA-seq analysis of *Chenopodium quinoa* under water stress that there was an overlap between drought stress tolerance and other abiotic stress mechanisms. In addition, water stress promoted the expression of 27 genes in *Arabidopsis thaliana*. These genes were related to six different functional categories: transport, signaling, transcription, hydrophilic proteins, metabolism and unknown functions (Bray, 2004). The most preferred technique to identify and characterize the differentially expressed genes (DEGs) with their functional annotation under different environmental stress in a plant is high throughput cDNA sequences (RNA seq) based on Next-generation sequencing (NGS) technology (Kumar et al., 2015a, Poersch-Bortolon et al., 2016b). High resolution and accuracy in genomic analyses are one of NGS's advantages (Lee et al., 2013b), NGS has many applications such as whole genome sequencing, gene expression profiling, target sequencing and small RNA sequencing (Lee et al., 2013b). To obtain all possible information, selecting suitable analysis tools for the massive amount of data generated by NGS is crucial. It depends on the biological questions or the aims of the study. For example, the most common workflow or experimental design to find differentially expressed genes (DEGs) based on RNA-seq is alignment or mapping of short sequencing reads (FASTQ file) against related reference genomes from the National Center for Biotechnology Information (NCBI), the University of California, Santa Cruz (UCSC). This allows us to infer which transcripts are expressed by using advisable tools such as Bowite, Bowite2, which is extremely fast, general purpose short read aligner with TopHat to align RNA-seq reads to the genome or against de novo assembled RNA transcripts for novel species (Trapnell et al., 2012a). These software packages include SAMtools that allow to summarise the aligned results into BAM files. Follows with the normalization tools for differential expressions such as DESeq, baySeq, edgeR or Cufflinks software. Count reads per transcript are determined with HTSeq or Cufflinks software (Lee et al., 2013b). This workflow can lead to trusted conclusions about genes or biological pathways in the plant under study. Due to the need for good knowledge and background in some programing language such as python, R language or any scripting language, many bioinformatics companies tried to decrease the programming by combining all these software packages in one commercial software such as Geneious and CLC genome workbench, so the researchers can gain the benefits without any expertise in statistical programming, which can be one of RNA-Seq technology preferred reasons (Marguerat and Bähler, 2010; Bowman et al., 2013; McGettigan, 2013; Yang et al., 2015). Khalil et al. (2009) related the high increase of RNA content in Egyptian wheat cultivar (Giza 168) under high temperature stress to the induction of genes encoding certain enzymes involved in responses to heat stress. In parallel with proteomics analyses this could be used to determine the mechanisms used by plants under

different environmental stresses (Peng et al., 2009b). The proteins are the main effectors of most cellular function (Caruso et al., 2009) and are used to adjust the growth (Ingram and Waites, 2006), physiology (Yi and Deng, 2005) and metabolism (Walker et al., 2007) as biochemical reactions in plant cells under development or environmental stress (Aryal et al., 2014b). Proteomics studies provide an important opportunity to advance the understanding of wheat response mechanisms under water stress by defining proteins that change in abundance, form or activity (Thelen and Peck, 2007). Cheng et al. (2015) identified 77 unique proteins in the drought tolerant Ningchun 47(NC47) wheat; these proteins were involved in Carbon metabolism (23.4%), photosynthesis/respiration (22.1%) and stress/defense/detoxification (18.2%), they also found that some drought related proteins in the NC47 wheat were more upregulated under drought than in Chinese spring wheat which is sensitive to drought. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) results can determine complete and comprehensive biological pathways (at the protein level) linked to plant resistance or sensitivity to different environmental stresses (Aryal et al., 2014a; Hossain et al., 2015; Nouri et al., 2015; An et al., 2016; Wang et al., 2016a). For example, levels of at least 15 proteins have been shown to be significantly regulated in the root of wheat seedlings under flooding stress (Kong et al., 2010). Similarly, important proteins associated with maize defense against viral pathogens have been identified using these techniques (Poersch-Bortolon et al. 2016b). The general experimental workflow of proteomic studies is to extract proteins from intact plant tissues by homogenization and differential centrifugation; after separation by SEC (size exclusion chromatography), proteins are identified by LC-MS/MS and MASCOT database searches (Aryal et al., 2014a).

To sum up, transcript-protein-metabolite correlation and comparison between control and stressed plants could identify the biological processes regulated by different stresses (Soda et al., 2015, De Filippis, 2017).

1.1.9. Research limitations to develop stress resistant wheat cultivars. (research gaps)

The limitations of current research can be divided into three parts: Firstly, the poor background knowledge of the local Saudi wheat genetic resources. Only some breeding programs to determine the diversity of wheat parent genotypes using molecular markers (RAPD and ISSR) have been undertaken (Motawei et al., 2007; Barakat et al., 2010). Secondly, although the whole-wheat genome assemblies have improved contiguity (Appels et al., 2018), there is still a lack of global sequence contiguity with complete genome coverage and full annotations. Thirdly, mass data collection and processing are challenging (De Filippis, 2017), the handling of mass genomics data obtained by High Throughput sequencing using Next Generation Sequencing (NGS) technology

and proteomics data obtained using liquid chromatography-tandem mass spectrometry (LC-MS/MS) requires experience and careful planning.

1.1.10. Omics approaches to understand drought stress responses

Transcriptomics, proteomics and metabolomics are analytical system approaches of choice for understanding plant functions (Duque et al., 2013) (figure 1.13).

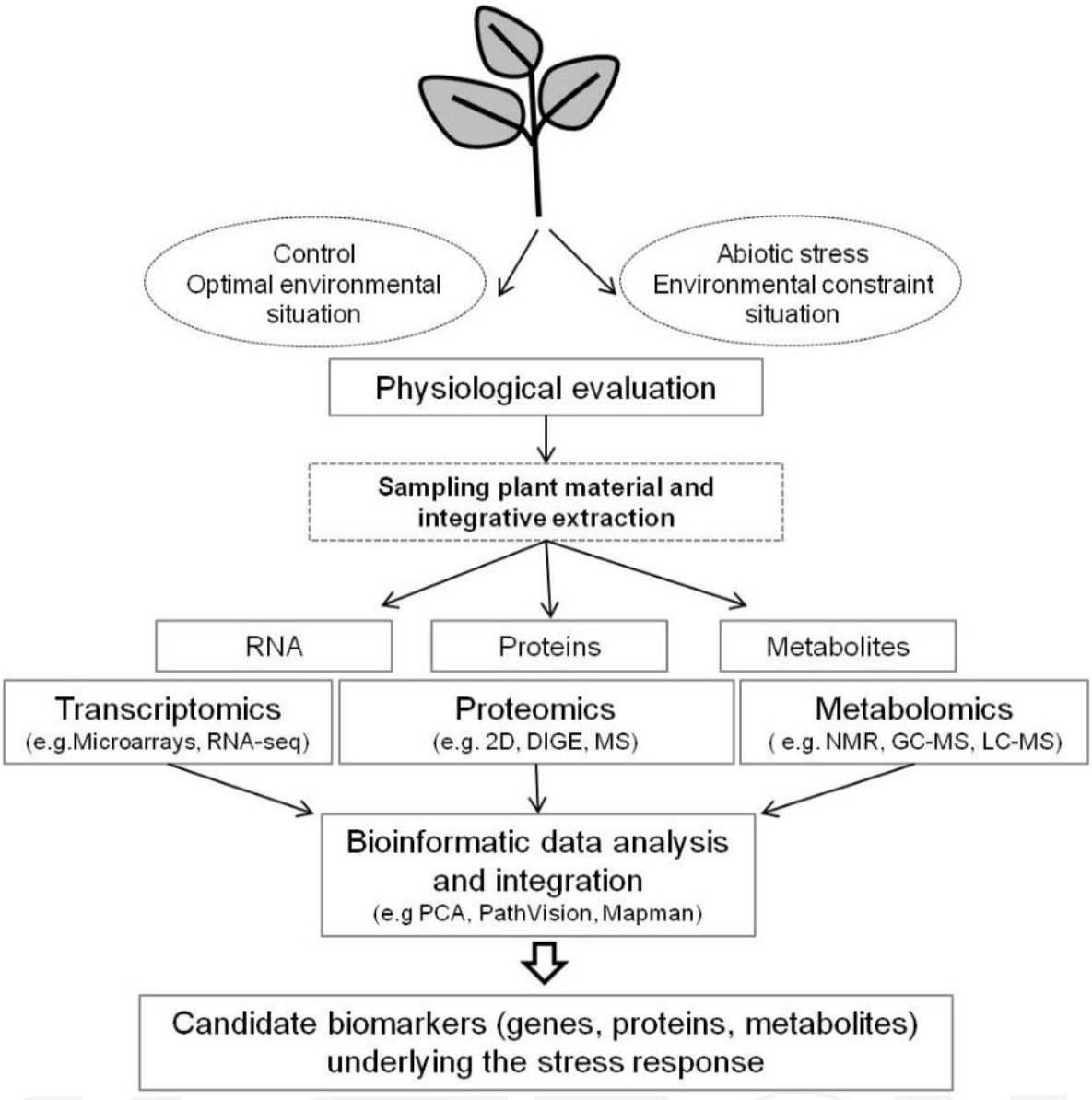


Figure 1. 12 Flowchart of common plant system biology approaches to study effect of abiotic stress. (Duque et al., 2013).

Three main different sources of information as initial starting material based on the different output applications and the sequencing technologies are shown in figure 1.14. There are different types of NGS, for example, 454 technology, Ion torrent, Proton and Illumina paired sequencing platforms.

Although they share the general workflow shown in (figure 1.15), Illumina paired sequencing platforms have some main features such as more accurate read alignment and the ability to detect insertion-deletion (indel) variants (Fuentes-Pardo and Ruzzante, 2017). NGS supersede the old sequencing technology "Sanger," which was used to sequence the human genome within a decade, while NGS can achieve this within a single day (Behjati and Tarpey, 2013).

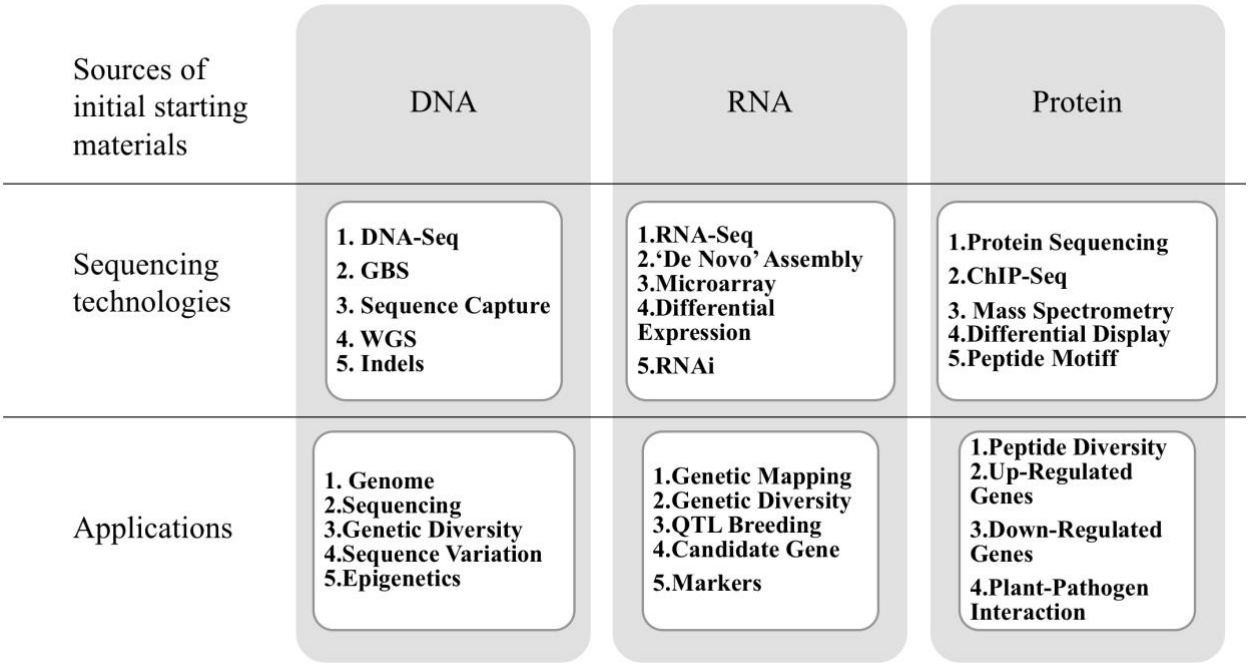


Figure 1. 13 from three different sources of initial starting plant genetic martials to the sequencing technologies and data applications (De Filippis, 2017).

The majority of the analytical tools used in NGS are based on clustering algorithms. These algorithms allow to group genes with similar expression patterns in a set of experiments (De Filippis, 2017). This can be done without transcriptome assembly when the complete genome sequence of the species under study is available. Every technology has different tools and programs that allow the exploitation of the output data depending on the aims of the project. Therefore, the development of computational resources (computationally tractable) and algorithms (statistically sound) for analysis and the resources to store the growing quantities of data is crucial (Attwood et al., 2011).

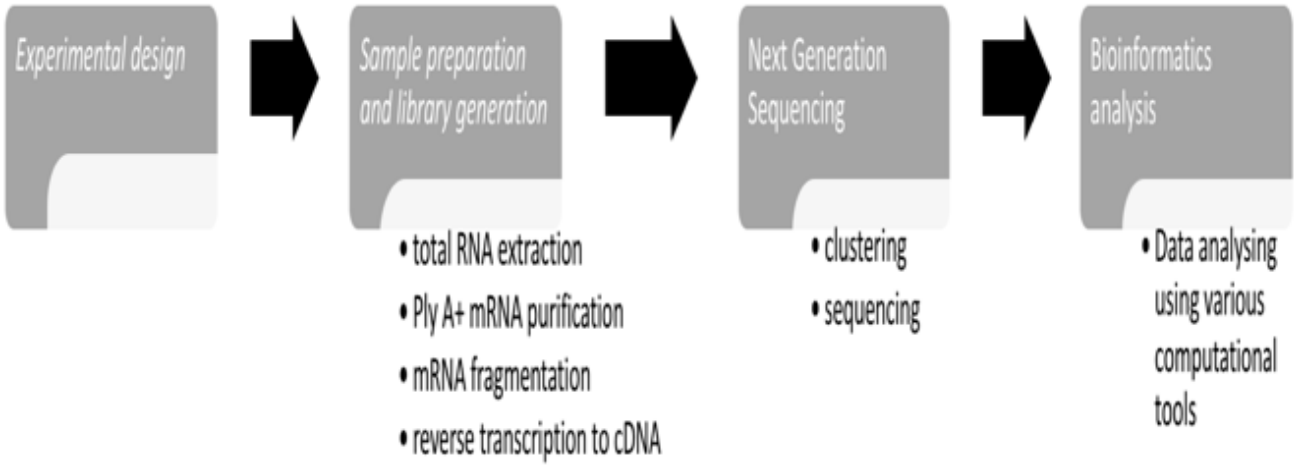


Figure 1. 14 General workflow of next generation sequencing (NGS) (Hardwick et al., 2017, Sudhagar et al., 2018).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method needs a well-annotated genome sequence and sufficient information on subcellular localization of proteins to increase the ability of peptide identification from the targeted tissues (Aryal et al., 2014b). This method is based on analyses of spectral data generated from a peptide mixture separated by L.C. and ionized by electrospray. Eluting peptides from the L.C. column at any given time will generate a full M.S. spectrum; each ion species in the spectrum represents one peptide. Peptides are identified by. They were matching the spectra to a previously established reference database of peptides (figure 1.16) (Xie et al., 2011).

Finally, the current study could be an example of combining the transcriptomics and proteomics approaches, which could lead to a mass of data that would reveal important information, including identifying the biological processes regulated in plants under different stresses (figure 1.17).

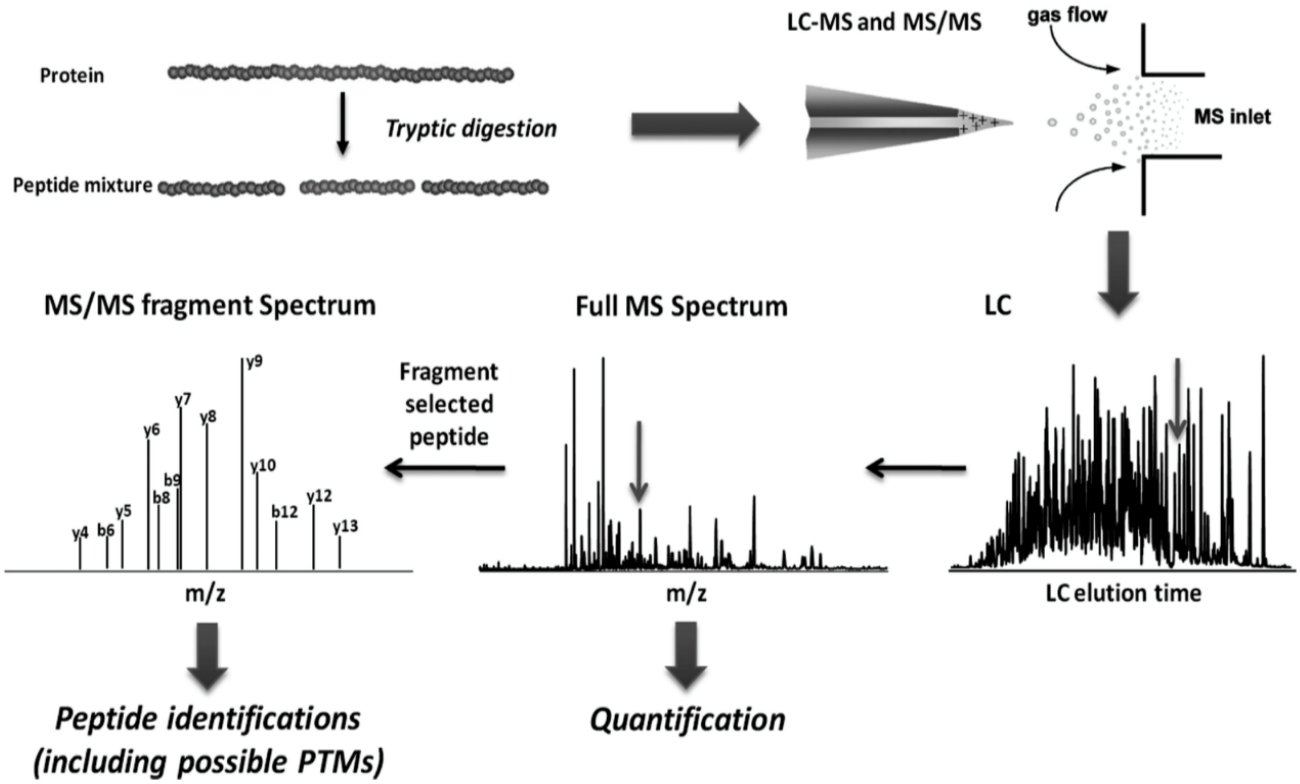


Figure 1. 15 General workflow of LC-MS/MS (Xie et al., 2011).

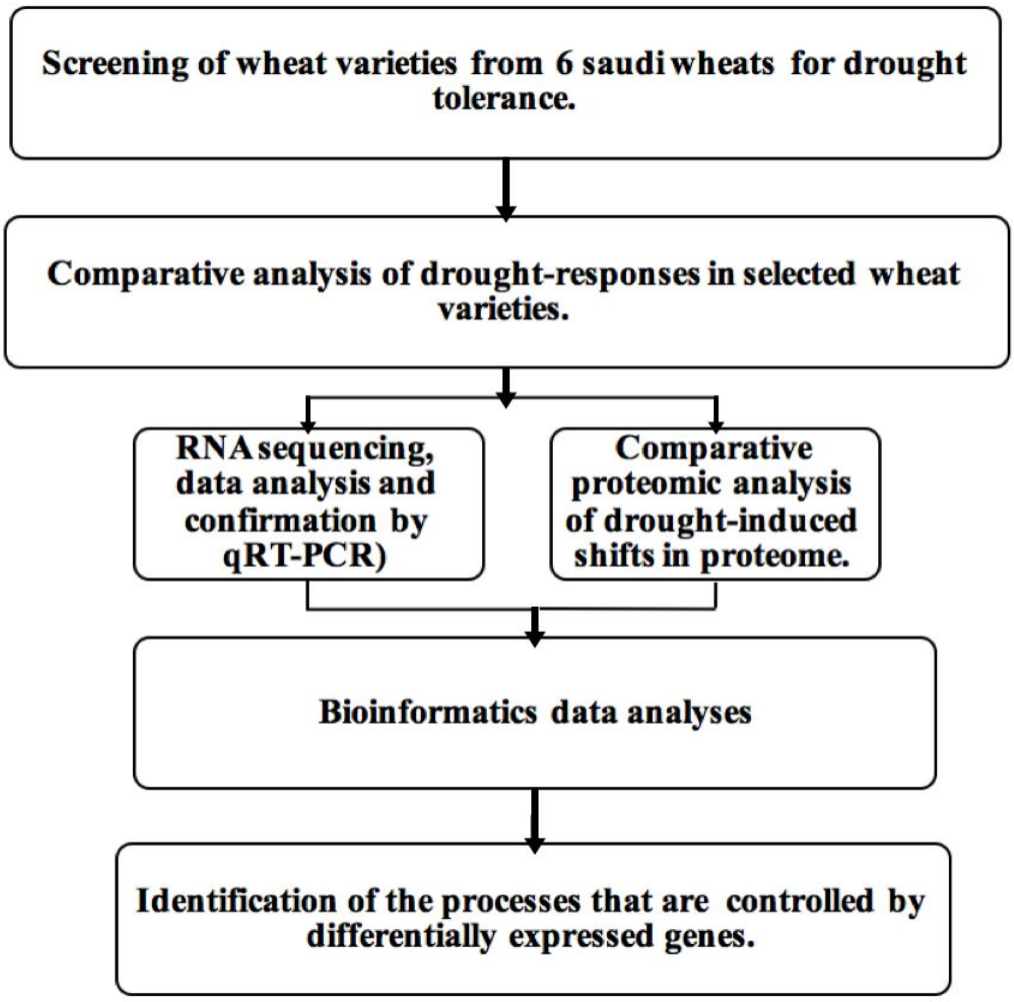


Figure 1. 16 The current study workflow showed that the combined study of transcriptomics and proteomics approaches identifies the processes controlled by differentially expressed genes

1.2.Aim of the project

This work aimed to determine the physiological and molecular mechanisms underpinning resistance to water stress in six local Saudi wheat cultivars. This could contribute to improving wheat production in Saudi Arabia by unraveling the pathways and genes regulated under water stress.

1.3.The hypothesis of the project

The shifting in mRNA and proteins in wheat cells under drought stress could be associated with drought regulated gene expression. This gene regulation might impact several biological pathways involved in drought resistance. Therefore, the transcript-protein-metabolite correlation and its comparison between control and stress plants could identify the biological processes regulated by drought and shed light on the key mechanisms of drought resistance in some wheat cultivars.

Chapter 2 Analysis of level of drought tolerance in seven known wheat cultivars (Cvs) grown in different regions of the Kingdom of Saudi Arabia (KSA).

2.1. Introduction

The kingdom of Saudi Arabia (KSA) is among the top countries in agriculture in desert areas. The country improved agriculture by implementing major irrigation projects and adopting large-scale mechanization to grow different crops under challenging environmental conditions. However, because the used technologies are high-energy consumers and have highly negative impacts on the local environment with significant negative effects on land and groundwater, this Agricultural model is unsustainable. The global climate change and the consequent increase in temperature and drought, especially in the arid and semi-arid regions, made the situation even more challenging. Traditionally the main grown crop of the country was bread Wheat (*Triticum aestivum*). However, recently the KSA government decided to stop the cultivation of this crop to avoid a water crisis in the country and because the cost of wheat production in the arid area was higher than any other wheat producing country (Al-Hamoudi et al., 1997). In KSA, Wheat was cultivated in four areas: the central region of the country (Riyadh and Qasseem, 57%), the South of the country (Jizan, Najran and Asir, 19%), the north of the country (Al Jouf, Tabouk and Hayel, 13%) together with the Eastern and Western regions approximately 11% (Agriculture, 2017).

Different wheat cultivars having different traits are grown in different regions of the country. For instance, in the central region with low rainfall and high average temperatures, wheat cultivars such as SAMA and Al-lugaimi, which are considered drought-tolerant cultivars, were common (Boutraa et al., 2010, Ashraf et al., 2013). The Al-Hassa wheat cultivar, which is considered salinity resistant according to the 2015 list of wheat accessions – ministry of Agriculture Saudi Arabia, was grown on the East coast of the country. Unfortunately, limited studies on Saudi wheat cultivars and their molecular and physiological adaptations to water stress despite some breading programs undertaken in different Saudi universities focused on developed foreign genotypes and only some local cultivars. Furthermore, most research was about agricultural aspects such as the response of wheat cultivar (cv. Yecora Rojo) to irrigation and nutrient levels in the KSA (Patil et al., 2014), the Pathogens stress in Tabuki wheat cultivar (Southern regions of KSA) (Moussa et al., 2013) as well as the yield performance and stability of SAMA local wheat Cv (Al-Otayk, 2010). Furthermore, the other research was to study foreign wheat cultivar under KSA conditions since these foreign cultivars have enough background data. Although lacking sufficient information of

Saudi wheat Cvs growing under severe conditions have high genetic diversity for morphological and yield related traits. Therefore, they could be useful in breeding programs to improve wheat production under environmental stress (Alghamdi et al., 2017b). Different wheat Cvs local to different regions of KSA were selected and seeds obtained from the Agriculture and Animals National Centre in Riyadh, KSA. (table 2.1). These different growing wheat regions had different drought levels, which could impact wheat drought resistance (figure 2.1). This section of the research aimed at assessing drought tolerance in a collection of 6 known wheat Cvs grown in different regions of the KSA by comparing their growth and yield under well-watered conditions and water-stress conditions. Shoot weight and length, Root weight and length, Root: shoot ratio, RWC, proline content, soluble sugar content and protein content as a selection criterion for drought tolerance.

2.2. Material and methods

2.2.1. Plant growth conditions and water-stress treatments

a. Greenhouse conditions

A purpose-built greenhouse that mimicked semi-natural conditions with regard to light intensities, temperature and relative Humidity was used. At mid-day the relative humidity was 50% - 60%, temperature was on average 30 °C during the day and 22 ºC during the night, the photoperiod was on average12 h light/12 h dark.

b. Plant growth

The different wheat Cvs (*Triticum aestivum* L) used in this study included 181 Jizan (Cv1), 193 Najran (Cv2), 357 Sama (Cv3), 377 Rafha (Cv4), 562 Ma'ayah (Cv5), 981 Najd (Cv6) (table.2.1) were sown directly and separately into 18 pots (height 18 cm, diameter 23 cm) containing each 800g of mixture of soil and peat moss (1:3 v/v). Plants were watered with tap water; no nutrient solution was used since the used soil mix contained enough nutrients. After 14 days of growth wheat plants were split into three groups as following: first group for vegetative stage samples, Second group for flowering stage samples and third group for grain filling stage samples. Each group has four pots (two pots for control two pots for treatment) and each pot had six plants.

c. Water-stress treatments

Plants were grown under two treatments: tap-water only (- 0.05 MPa), used as control (C) and a Polyethylene glycol (PEG6000) solution (- 0.34 MPa) 5, 10 and 15% (w/v) applied in steps to cause water-stress to the plants. Plants were watered once every other day with the PEG treatment at vegetative stage started after 14 days from first stem emerged and lasted two

weeks, and the PEG treatment at flowering and grain filling stages started from first emergence of stage signs accordingly to Feecks scales (Large, 1954) for two weeks (figure 2.1, table S1.1).

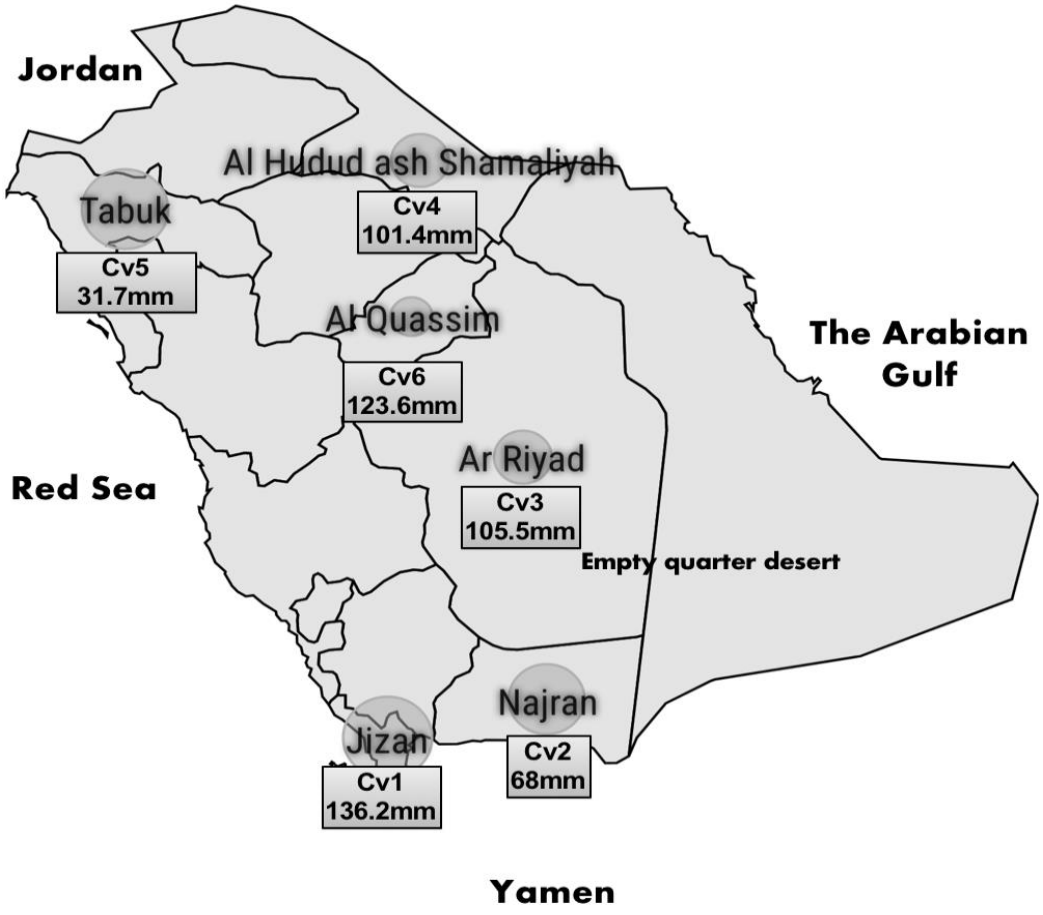


Figure 2. 1 Location map (Saudi Arabia) for the studied area of the six Wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6). The map shows Precipitations in mm and average temperatures in ^{0}C (1985-2019). **source:** National Centre of Meteorology, Saudi Arabia.

Table 2. 1 Name and origin of 6 Wheat cultivars grown in the KSA and used in the study.

Wheat cultivars	Local name	Origin	Temperature (C^0)
Cv1	181 Jizan	Jizan (south region)	35.4-26.4
Cv2	193 Najran	Najran (south region)	33.3-17.5
Cv3	357 Sama	Riyadh- Aldahna (central region)	33.4-18
Cv4	377 Rafha	Al hudud ash Shamaliyah (north region)	31-16
Cv5	562 Ma'ayah	Tabuk (north region)	30-15
Cv6	981 Najd	Al Quassim (central region)	32.2-17.6

d. Plant sampling

Plant samples (shoots + roots) were harvested at the middle of the day and divided into two groups, first group for measuring physiological parameters and the second group for performing biochemical measurements. Samples were placed in labeled foil bags (each sample separately) the first group was placed on ice and measured in the same day of harvest, and the second group was snap-frozen in liquid nitrogen and stored at -80ºC until used.

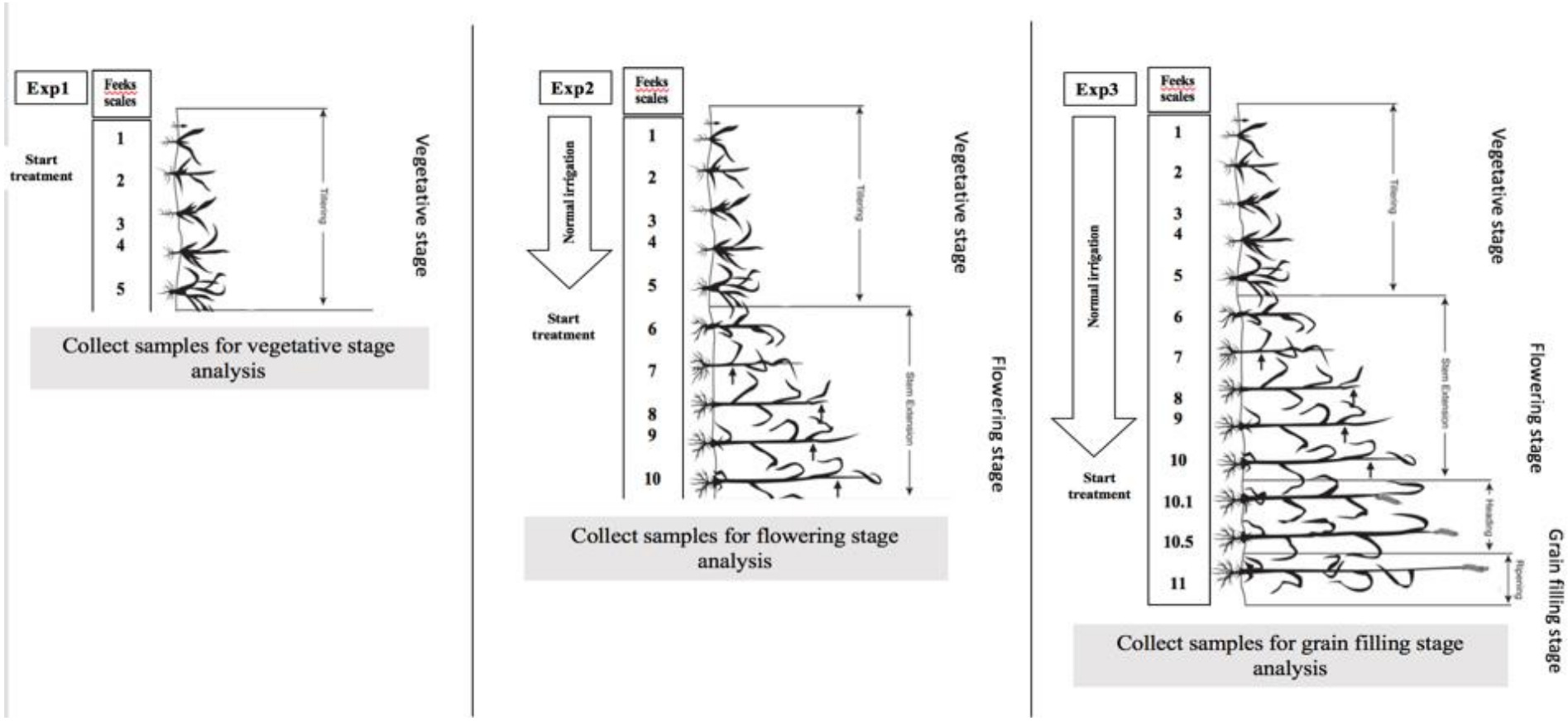


Figure 2. 2 Experimental design and wheat sample collection and watering periods at three life stages of wheat plants (vegetative, flowering and filling stages) according to Feeks scales (1:One shoot stage; 2: Tillering begins stage; 3:Tillers formed stage; 4:leaf sheaths strengthen stage; 5:leaf strongly erected stage; 6: first node of stem visible stage; 7:second node visible stage; 8:last leaf just visible stage; 9:ligule of last leaf just visible stage; 10: In boot stage; 10.1+10.5: flowering stage ; 11: grain filling stage (Large, 1954).

2.2.2. Physiological parameters

a. Plant growth measurements

The height (cm) and dry weight (g) were measured at three developmental stages (vegetative, flowering and grain filling stage) and compared between the plants subjected to water-stress and the controls, the plants height was taken one day before the plant was harvested.

b. Relative water content

At the same day of harvesting, leaves were cut into sections of about 5 cm^2, covered with foil and placed on ice. The fresh weight (FW) was recorded then each sample was placed in a pre-weighed glass Petri dish full of water to obtain full turgidity hydration approximately 3-4 h under normal room light and temperature conditions. After hydration, the samples were lightly dried with filter paper and immediately weighed. To get full starvation weight (SW) the samples were oven dried at 80°C overnight and weighed (after being cooled down in a desiccator) to determine the dry weight (DW). RWC was determined by a standard method (González and González-Vilar, 2001) and calculated using the following equation:

Equation 1 $\mathbf{RWC}\ (\%) = [(\mathbf{FW} - \mathbf{DW}) \,/\, (\mathbf{SW} - \mathbf{DW})]\ \mathbf{x\ 100}$

Where FW: fresh weight, SW: Saturated weight and DW: dry weight.

2.2.3. Biochemical measurements

a. Measurement of Proline content.

Shoots were ground under liquid nitrogen and 200 mg FWt. material were homogenized in 1ml of 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was transferred into a centrifuge tube and span at 13000 rpm for 10 min. at 4ºC. The supernatant (approximately 200 µl) was transferred to a glass tube and 1ml of glacial acetic acid and 1ml ninhydrin reagent (2.5g ninhydrin/100 ml of a solution containing glacial acetic acid, distilled water and ortho-phosphoric acid 85% at a ratio of 6:3:1) were added. The mixture was incubated in a water bath at 80 ºC for one hour. The reaction was let to cool down for 5 min. at room temperature. Spectrophotometric reading was taken immediately at a wavelength of 546nm. The proline concentration was determined from a standard calibration curve and calculated on fresh weight basis (µmol proline g FWt^{-1}) (Claussen, 2005). Proline content was expressed in this report in relation to leaf fresh weight. The Fresh/dry weight ratios were determined and the results were expressed in relation to dry weight.

b. Measurement of soluble sugar content.

Phenol/sulphuric acid method was used for determination of content of sugars and related substances by Dubois et al., (1956). Shoot material was ground under liquid nitrogen and

200mg of DWt ground material extracted in 1 ml of 80% methanol. The extract was transferred into a centrifuge tube and heated in a hot plate at 80 ºC for 40 min. and span at 13000 rpm for 10 min. In a glass tube, 0.5 ml of upper phase solution + 0.5 ml H_2O + 0.5 ml 5% phenol + 2.5 ml sulphuric acid were added and mixed with a glass rod and left to cool down to RT for 15 min.. Spectrophotometric reading was taken immediately at a wavelength of 483nm. (DuBois et al., 1956). The sugar concentration was determined from a standard calibration curve of Glucose concentration at 483nm. Sugar content was expressed in this report in relation to leaf dry weight.

c. Measurement of Protein content.

Bradford standard assay was used for determination of total protein concentration. Shoots were ground under liquid nitrogen and 100mg of DWt ground material was homogenized in 1 ml of 20-50 mM, pH 7.0 - 9.0 Tris buffer. The homogenate was span at 13000 rpm for 10 min. and 30µl from the supernatant was mixed with 970µl of Bradford reagent. Spectrophotometric reading was taken after 5min of incubation at RT at a wavelength of 595nm. The protein concentration was determined from a standard calibration curve prepared using Bovine Serum Albumin (BSA). (Stoscheck, 1990, Bradford, 1976).

2.2.4. Seed germination

After completion of the filling stages, the seeds were collected from the plants and let to dry at room temperature. Twenty seeds from each cultivar were cleaned with 5% hypochlorite for 5 min then rinsed several times with sterile water and sown in petri dishes on wet filter paper. The Petri dishes were sealed with parafilm to prevent evaporation and kept in a growth chamber at a temperature of 25±1°C in the dark with a relative humidity of 70%. Seeds were considered as germinated at 2 cm extension of shoot or root.

2.2.5. Experimental design and Statistical Data Analysis

Statistical analysis was performed using SPSS 2. The data represent means calculated from four replicates for the measured parameters. The analysis of the main effects and interaction effects of growth stages within water stress was done using ANOVA followed by F-test analysis. The analysis of the main effects and interaction effects of water stress was done using L.S.D. Statistical significance was set up to $p < 0.05$ for both analyses. The values in tables are means ±SE.

2.2.6. Ranking and scoring of cultivars for water stress tolerance

In order to rank and compare the wheat cultivars under study from tolerant to sensitive to water stress, water stress tolerance (WST) was calculated for each parameter measured for each cultivar based on the method described in (Goudarzi and Pakniyat, 2008)

Equation 2 $\mathbf{WST = Ps/Pc}$

Where Ps is the mean of the cultivar under water stress and Pc the mean of cultivar under control condition. The indices were then used to score and rank the cultivars according to the method used by (El‐Hendawy et al., 2007, Khan, 2014). Cultivars were classified into four classes according to the formula: number of classes = 1+3.3 log10 n, where n is the number of tested cultivars (El‐Hendawy et al., 2007). Scores were assigned from the highest value to the lowest value (indicated by 1 to 4) for the following growth parameters: shoot weight and length, Root weight and length, Root: shoot ratio, RWC, proline content, soluble sugar content, protein content, seed weight, number of seeds per spike and germination rate. For instance, score number 1 for shoot height means that this cultivar had the highest shoot height compared to others. (table S1.12). The level of tolerance to water stress across different wheat cultivars was improved from previously described methods in case of salinity tolerance (Munns and James, 2003; El-Hendawy et al., 2007; Genc et al., 2007; Khan, 2014,). In current study, water stress tolerance (WST) was calculated as the percentage according to previous equation 2.2.6. As a result, the 6 wheat cultivars were classified into four levels of water stress tolerance: highly tolerant maintained a high level of WST (> 100%), moderately tolerant cultivar showed moderate WST (80 - 100%) , sensitive cultivars showed a low level of WST (60-80%) and highly sensitive cultivars showed a lowest level of WST (<60%). (table S1. 13).

2.3. Results

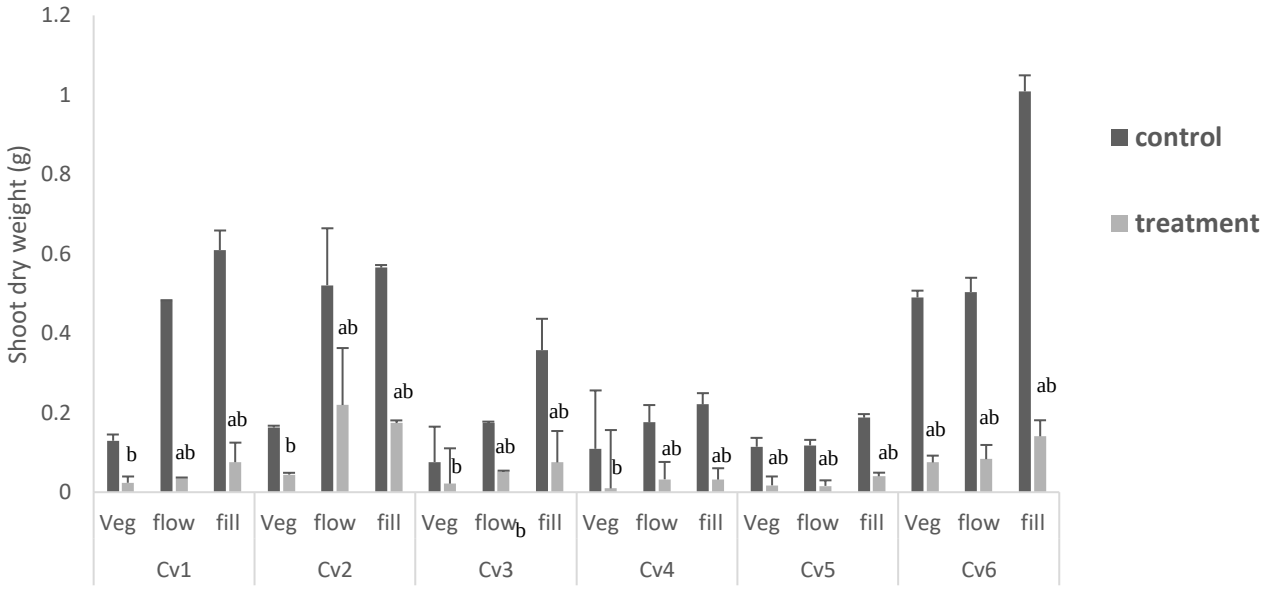
2.3.1 Plant Growth

The six cultivars germinated after 3 days from sowing. Water-stress created by irrigating wheat plants with 15% PEG6000 caused variable changes on shoot and root growth, Relative water content (R.W.C.), free proline content, soluble sugar content and protein content compared to the unstressed control plants depending on the Cv and growth stage.

Shoot measurements

The studied six wheat Cvs showed a relatively important reduction in shoot dry weight under PEG treatment at all growth stages with maximum significant ($p<0.000$) reduction shown by Cv1 at flowering stage (92.5% reduction of weight) and minimum significant ($p<0.016$) reduction 58% shown by Cv2 at the same stage. All reductions and the mean differences between water-stressed plants and control plants were significant at all growth stages (figure 2.2.a; table S1. 2). Water-stress caused also a reduction in shoot length, however the mean reduction caused by water-stess was not statistically significant. There were significant mean differences in shoot length reduction between life stages. The highest reduction was in flowering and grain filling stages in all Cvs, water-stress caused a reduction in shoot length of a maximum average of 40% in Cv1 at grain filling stage and a minimum average of 4% in Cv2 at the same growth stage (figure 2.2.b; table S1. 3).

a



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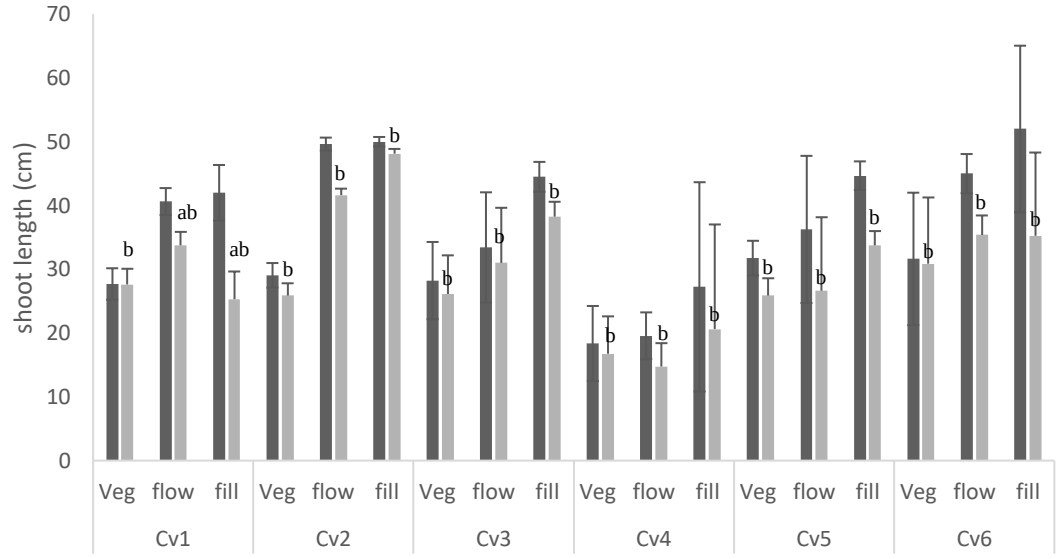


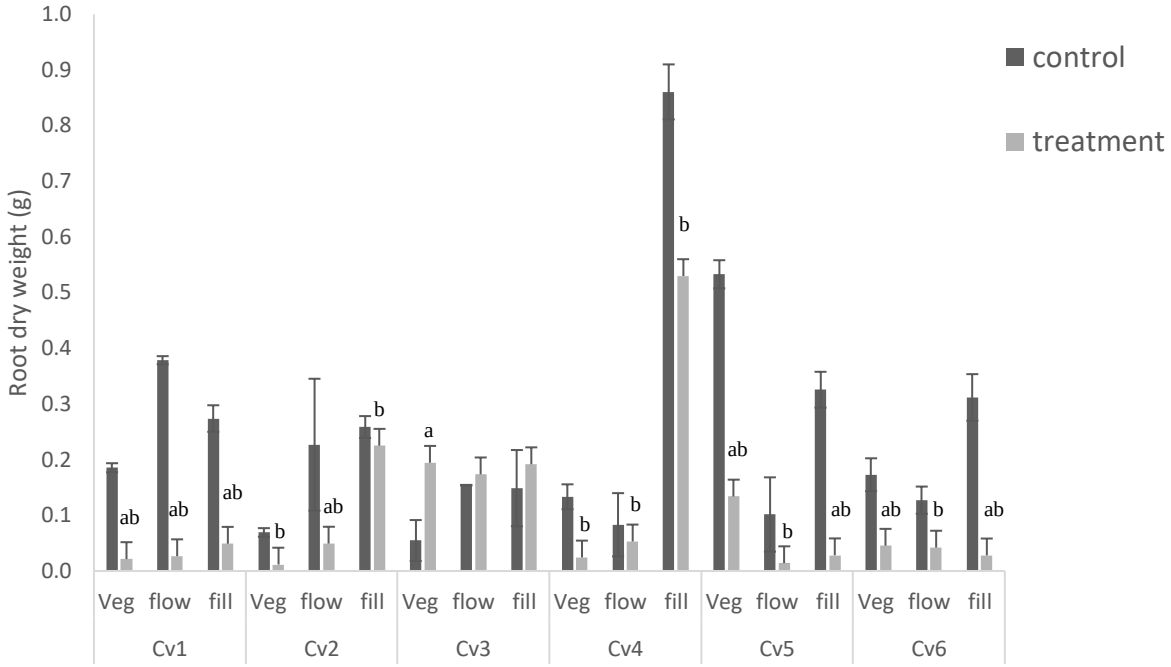
Figure 2. 3 Effect of water stress on plant growth parameters. a. Shoot dry weight (g) b. Shoot length (cm) measurements in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. n=4, bars are standard errors.

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means. [b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Root measurement

High decrease was recorded in Root dry weight under PEG treatment at all growth stages with maximum reduction shown by Cv1 at flowering stage (93%) and minimum reduction of 13% shown by Cv2 at the same stage. In contrast, Cv3 showed a non-significant increase in root dry weight between either water-stress or growth stage comparative under PEG treatment at flowering and grain filling stages, yet it showed a significant ($p<0.022$) increase at the vegetative stage by 72% (figure 2.3.a; table S1. 4). All Cvs showed an increase in root length at least at two growth stages under PEG treatment with an average increase of 40%. Cvs 3, 4 and 6 had an increase in root length at all growth stages with maximum significant increase ($p<0.000$) of 87% showed by Cv3 at vegetative stage and minimum non-significant ($p<0.456$) increase of 26% showed by Cv2. However, Cv6 root length showed notable decrease at all plant growth stages under PEG treatment with maximum significant ($p<0.002$) reduction of 55% at grain filling stage and minimum non-significant ($p<0.962$) reduction of 3.2% at vegetative stage (figure 2.3.b, table S1. 5).

a



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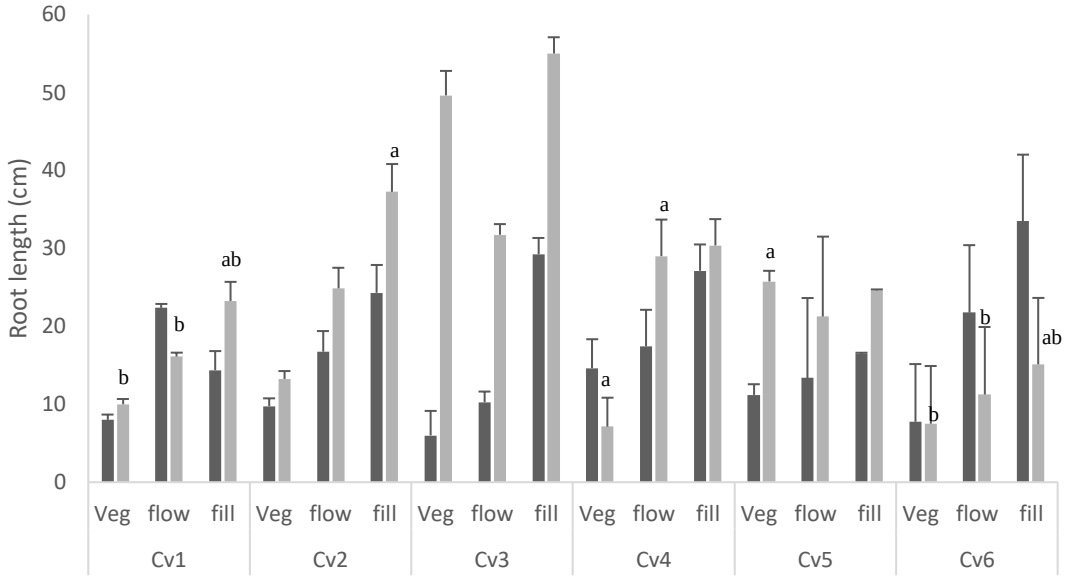


Figure 2. 4 Effect of water stress on plant growth parameters a. Root dry weight (g) b. Root length (cm) measurements in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193 Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15%PEG6000. n=9, bars are standard errors.

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means. [b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Root:shoot biomass ratio was significantly increased in Cv2 at flowering and grain filling stages, and significantly decreased at vegetative stage (F-test = $p<0.027$), compared to the control. Cv3, Cv4, Cv5 and Cv6 showed at all growth stages an increase with maximum increase of 92% by Cv3 at vegetative stage, and with minimum increase of 1.9% shown by Cv5 at flowering stage. Interestingly, root: shoot ratio has shown a decrease only in Cv1 at all growth stages, Cv2 at vegetative stage. and Cv5 and Cv6 at grain filling stage by 59% and 35% respectively. (figure. 2.4; table S1. 6).

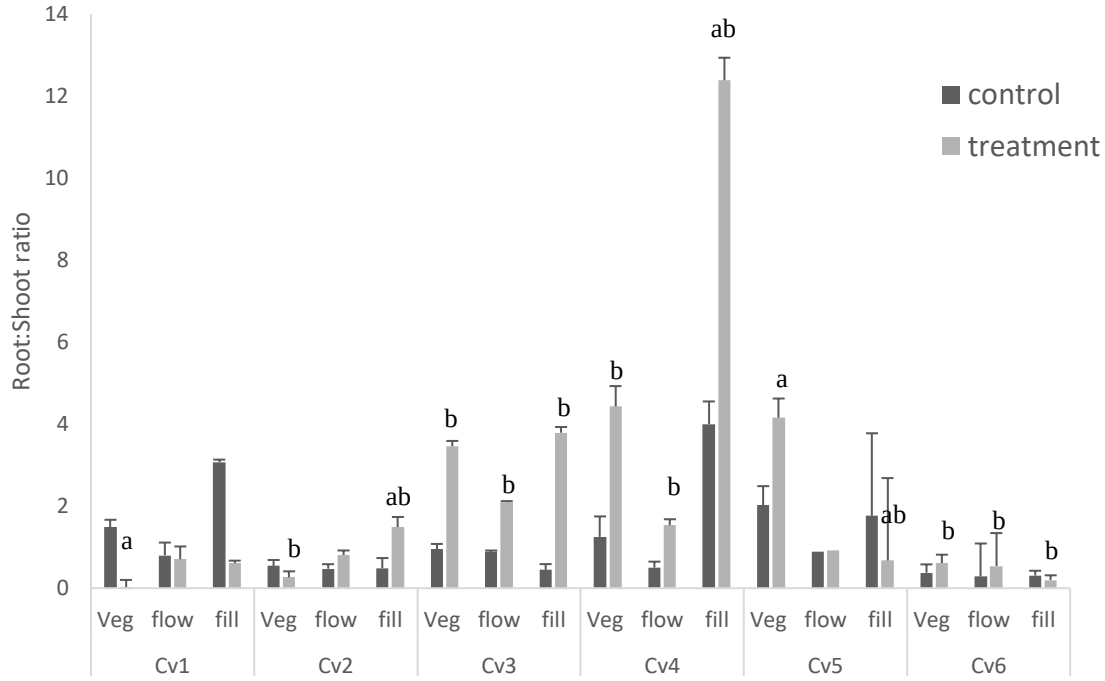


Figure 2. 5 Effect of water stress on plant growth parameters a. Root: shoot ratio biomass ratio measurements in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193 Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15%PEG6000. n=9, bars are standard errors.

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means. [b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

2.3.2 Relative water content

Relative water content (RWC) in shoot declined at filling stage in most Cvs under PEG treatment, there was a significant decrease in Cvs 3,4,5 and 6 compared to the unstressed control, the decline varied between Cvs and went from 9% in Cv1 to 37% in Cv5. In contrast, Cv2's RWC has increased at all growth stages compared to the unstressed control (figure 2.5, table S1. 7).

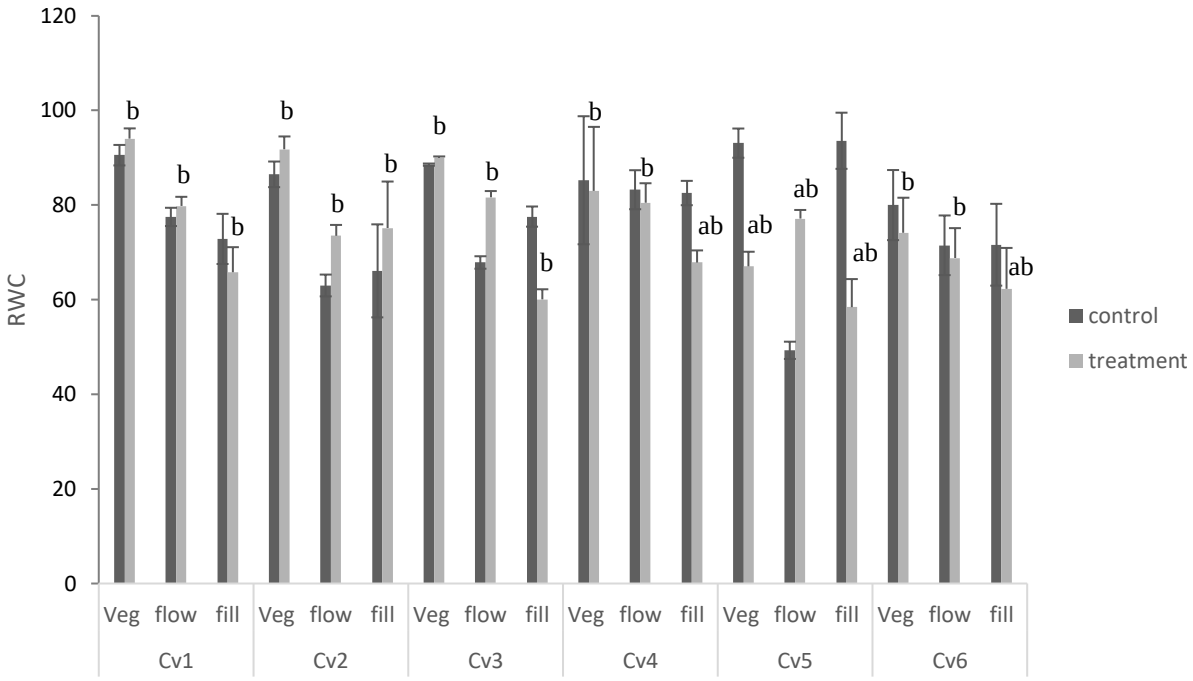


Figure 2. 6 Effect of water stress on R.W.C (%) in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193 Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. n=4, bars are standard errors.

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means. [b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

2.3.3 Proline content

Increase in free proline in shoots is often associated with water-stress responses in most plants. All wheat Cvs showed significant changes in proline content at all growth stages ($p<0.05$), compared to the control. Interestingly there was a big variation in the change between Cvs and growth stages. Cv1 and Cv2 proline content increased at all growth stages. In Cv1 there was a 1.9-fold increase at filling stage and 1.2-fold increase in vegetative stage. All changes were statistically significant ($p<0.05$) apart from the increase at the filling stage. In contrast, Cv5's proline content showed a decrease at all growth stages with 2-fold maximum significant ($p<0.044$) decrease at filling stage. Proline content in Cv3 and Cv6 decreased at the vegetative stage of growth and increased in the following stages while in Cv4, it increased at the vegetative stage and settled down close to the control in the next stages of growth. (figure 2.6, table S1. 8).

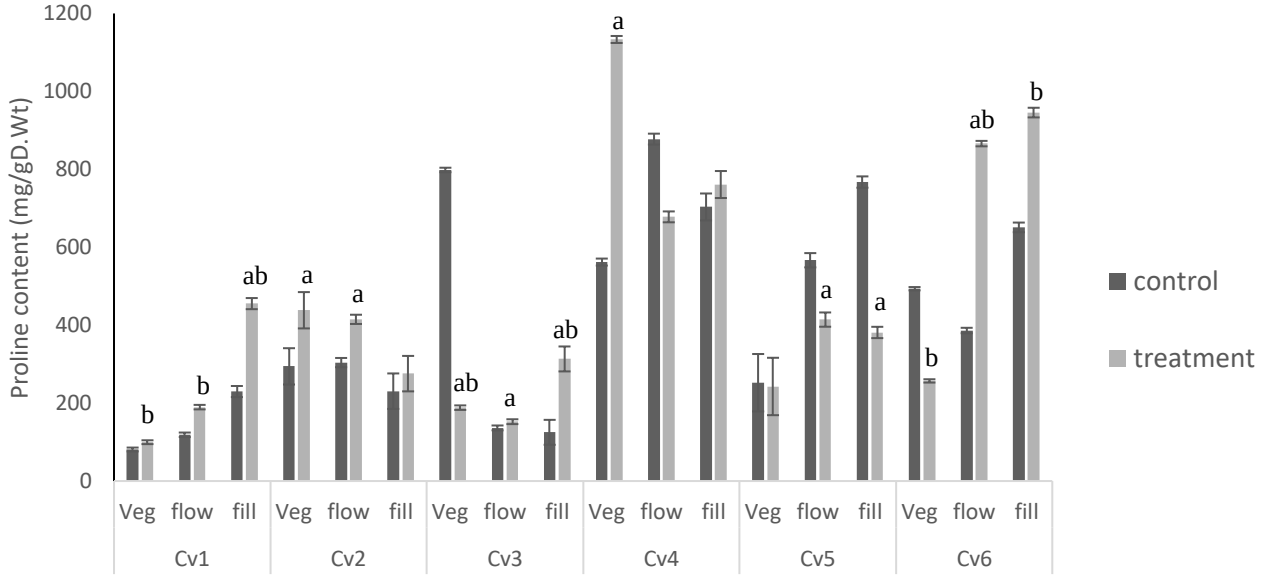


Figure 2. 7 Effect of water stress on Proline content (mg/gDWt) in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193 Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. n=4, bars are standard errors.

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means. [b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

2.3.4 Soluble sugars content

Soluble sugars content showed significant differential changes in shoots under PEG treatment depending on Cv and growth stage. At grain filling stage apart from Cv2 and Cv6, all Cvs have shown an increase in soluble sugars with a maximum significant ($p<0.001$) increase in Cv1 of 39.9%, and minor increase in Cv5 by 1.6%. Cv2 and Cv6 have shown a decline in soluble sugar content by 9.3% and 16.1% respectively at filing stage under PEG treatment. The vegetative stage of Cv3, Cv4 and Cv5 showed a decline compared to controls and Cv2 showed significant change ($p<0.011$) at the flowering stage (figure 2.7, table S1. 9).

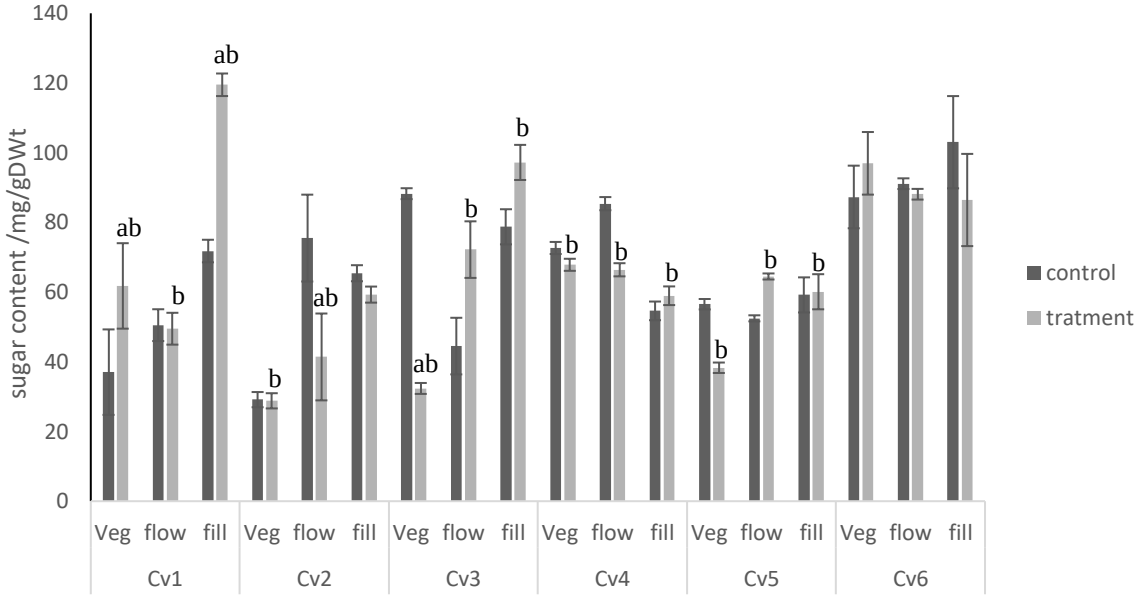


Figure 2. 8 Effect of water stress on soluble sugars content (mg/g) in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. n=4, bars are standard errors.

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means. [b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

2.3.5 Proteins content

Similarly, to soluble sugars, protein content changed under the effect of the PEG-imposed water-stress and the change varied depending on the Cv and the growth stage. Protein content in shoot of cultivars Cv1, Cv4 and Cv6 showed a relatively important reduction under PEG treatment at all growth stages with maximum reduction shown by Cv1 at flowering stage of 91.8% ($p<0.001$). In contrast Cv2 showed a relatively important increase in protein content under PEG treatment at all growth stages with maximum increase of 37.6% shown at filling stage (figure 2.9, table S1. 10). Protein content in Cv3 in plants subjected to water-stress remained close to that of the unstressed control plants at the 3 growth stages while in Cv5 it increased at vegetative and filling stages and declined at the flowering stage.

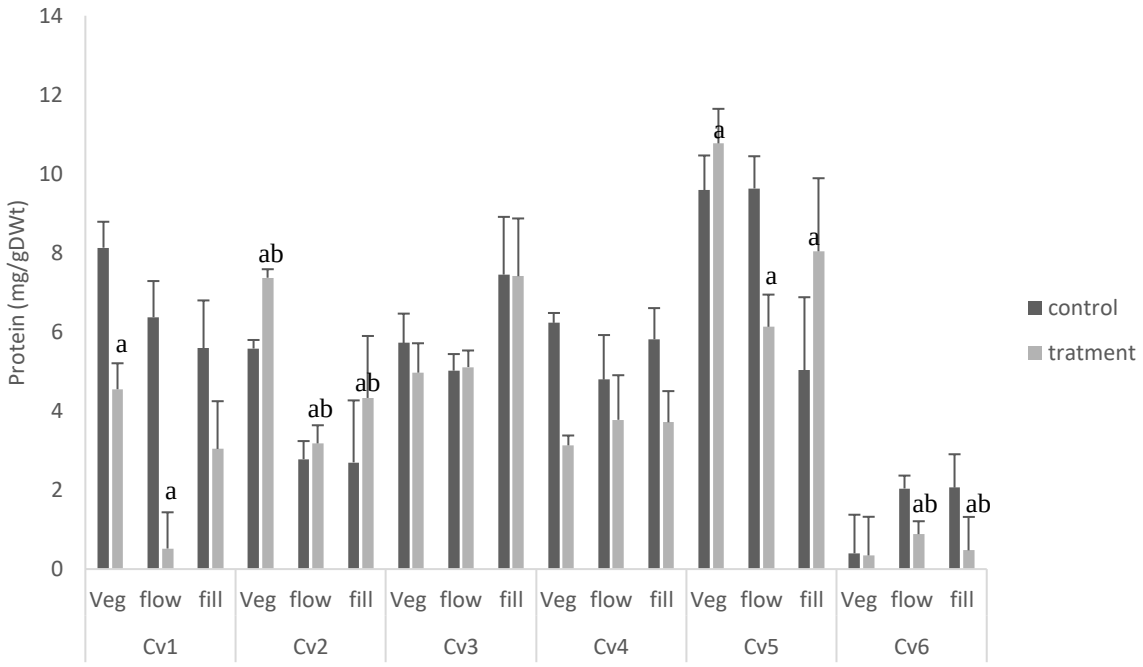


Figure 2. 9 Effect of water stress on Protein content (mg/g) in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193 Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15%PEG6000. n=4, bars are standard errors.

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means. [b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

2.3.6 Yield measurement

Grain yield traits (number of seeds per spike and weight of seeds) are most important criteria for selecting resistant and sensitive cultivars under water stress especially in wheats. Apart from Cv2 and Cv3 which have shown similar seed weight between water-stressed plants and unstressed control plants, all Cvs have shown decrease in seed weight (figure.2.9, table S1.11). Highest reduction of seed weight was recorded in Cv4 ($p<0.018$) and Cv1 ($p<0.05$) by 2.15 and 1.7 fold respectively. A similar response was recorded for seed numbers, seed number per spike declined significantly ($p<0.05$) under the effect of water-stress in Cv1, Cv4, Cv5 while it remained similar to that in control plants in Cv2 and Cv3 and Cv6 (figure 2.10, table S1. 11). The significant ($p<0.000$) reduction in number of seeds per spike in Cv4 and Cv5 was of 2.5 and 2.6-fold respectively (figure 2.10, table S1.11).

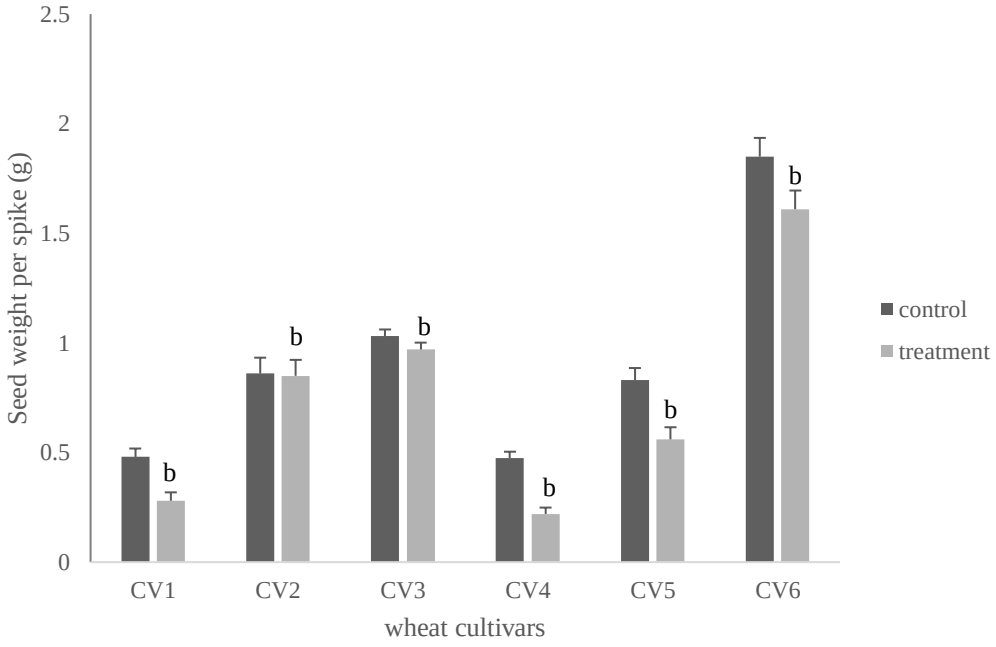


Figure 2. 10 Effect of water stress on Seed weight (g) in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia subjected to 15% PEG6000. n=4, bars are standard errors.

[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Figure 2. 11 Effect of water stress on Seed number per spike in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia subjected to 15% PEG6000. n=4, bars are standard errors.

Seed germination rate is an important treat, water-stress caused significant reduction in seed germination rate, however this reduction varied significantly between Cvs. Cv2 showed only minimal reduction in seed germination and Cv5 showed a significant ($p<0.000$) high reduction, which was 86% of the control. The best germination performance was shown by Cv2 in control and treated plants comparatively to the cultivars (figure.2.11, table S1. 11).

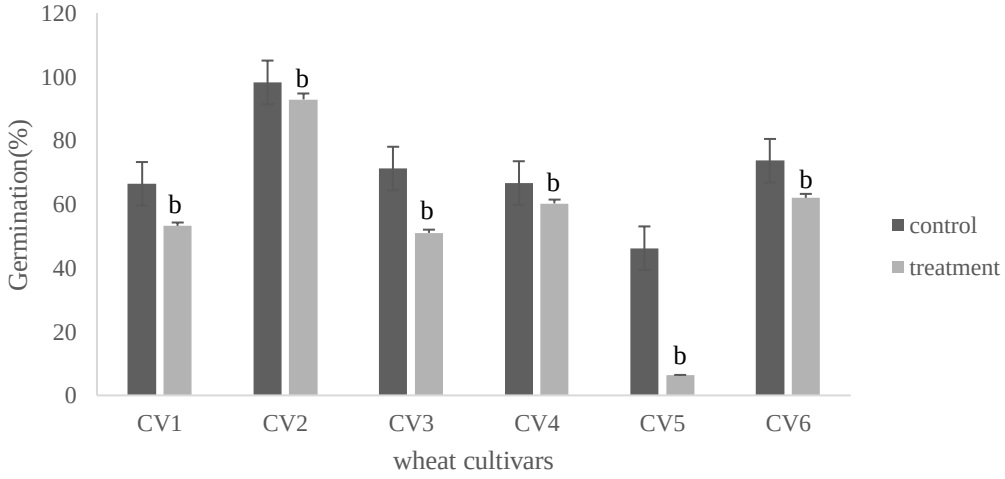


Figure 2. 12 Effect of water stress on Germination (%) in wheat (*Triticum aestivum* L) cultivars.; 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6) grown in different regions of Saudi Arabia subjected to 15% PEG6000. n=4, bars are standard errors.

2.4. Discussion

The findings of this study clearly show that water-stress affects differently growth of wheat depending on the cultivar and the growth stage of the plant. This is highly relevant from the practical point of view as it can allow better management of wheat cultivation in arid areas like Saudi Arabia. This would implicate the choice of the wheat Cv and the timing of cultures depending on predictions of dry preiods.. Plants respond variably to drought during their development, by reducing shoot and root growth, modulating gene expression and metabolism, closing stomata and adjusting osmatic pressure etc. (Chaves et al., 2003). These responses seem to be dependent on plant´s age and may vary between organs and cultivars. The findings of this research are in accordance with other studies, for example many researchers have found that when vegetative stage and flowering stages of growth are affected by drought, seeds quality and performance is reduced from poor grain filling (Kaya et al., 2006; Guo et al., 2009;' Sevik and Cetin, 2015; El-Nakhlawy et al., 2015; Swamy et al., 2017; Nagy et al., 2018,). Crops specially wheat are sensitive to drought at booting, flowering and grain filling stages (Ihsan et al., 2016) therefore studying wheat response to water-stress at different growth stages is essential for understanding the physiological response of this plant to drought (Kong et al., 2010). Growth and yield measurements of the wheat cultivars in this study showed substantially different responses to water-stress created by PEG6000 and these responses were dependent on growth stage. The identification of resistant and sensitive cultivars to water stress in this study was based on the shoot weight and length, root weight and length, the relative water content (R.W.C), proline content, soluble sugar content, protein content, number of seeds per spike and seed weight and germination rate.

All wheat cultivars in this study showed noticeable response to water stress and the variation in these responses revealed different strategies for resistance to water stress. Because in wheat the grain is the main crop seed quality and quantity is the main indictor of tolerance to water stress as discussed in first chapter (Obidiegwu et al., 2015, Belhassen, 1997). According to many research, usually the first response to water-stresses is shown by inhibition of shoot growth (Chaves et al., 2003; Khan and Naqvi, 2011; Ahmad et al., 2014).

2.4.1 Shoot and Root measurements

In the current study all cultivars showed a relatively important reduction in shoot dry weight and length under PEG treatment at all growth stages, Cv2 and Cv3 cultivars showed the lowest shoot dry weight reduction under water stress and Cv1, Cv4, Cv5 and Cv6 showed the highest decrease by an average of 90% of the control in shoot dry weight, and 20% of the control in

shoot length, the same results were found by Rauf et al. (2007) in sixteen Pakistani wheat cultivars. Also drought reduced the hight of Chinese bread wheat seedlings (Zhang et al., 2014b). Shoot dry weight and length reduction under water stress could be ascribed to several reasons, first, to the decrease in photosynthesis (Tezara et al., 1999; Guo et al., 2013; Saeidi et al., 2015) and the consequent reduction in carbohydrates necessary to build new cells or expand pre-existing cells (Rauf et al., 2007). Second, decrease in water in xylem that leads to reduction in cell elongation and expansion (Nonami, 1998, Anjum et al., 2011). These reductions could be considered as a strategic plant response to minimizing water loss by reducing the evapotranspirational area (Chaves et al., 2003, Obidiegwu et al., 2015), if they don´t negatively impact yield, otherwise, they would be considered as a lower plant performance under water stress, this was the case of Cv1, Cv3, Cv4, Cv5 and CV6 . Wheat response to water stress depends on the age and stage of development (Zhu et al., 2005), current results show an example of ages dependent response to water stress, the highest decrease of shoot dry weight and length was recorded at flowering and grain filling stages in all cultivars under water stress compared to the vegetative stage, this might be due to export of non-structural carbohydrates for the development of kernels (Plaut et al., 2004).

Root dry weight also showed noticeable decrease under PEG treatment. Most noticeable decrease was in Cv1, Cv4, Cv5 and Cv6 at all growth stages, similar findings were reported by Rauf et al. (2007), Ahmad et al. (2014) in fifty wheat genotypes from different Pakistan's regions. This reduction could be ascribed to the fact that plants tend to increase concentration of solutes in the cell instead of synthesizing new cells (Brady et al., 1995; Boyer, 1996), this would help water absorption under drought stress (Chaves et al., 2003). In contrast, Cv3 showed an increase in root dry weight under PEG treatment which shows the high performance of this cultivar under water stress and this increase was consistent with the decrease in shoot dry wheat, this, to improve water uptake by the plant under drought. Cv2 and Cv3 have shown increased root length under the effect of water stress at the three growth stages, the same result was found in synthetic hexaploid wheat (SHW) and four winter wheat cultivars (Becker et al., 2016). A water-stress signal is first detected by the roots as the primary event that leads to the physiological response of the plant to water stress (Nezhadahmadi et al., 2013).This induced increase in root length could be ascribed to a search for water in the soil (Babaie et al., 2014) which is a common response to drought stress in most plants. induced by the root impedance in drying soil (Ge et al., 2019). Also, Djanaguiraman et al. (2019) found that drought tolerance in winter wheat was associated with deep root system while in spring wheat a well branched root system was associated with higher drought tolerance. This emphasizes that Cv6 is sensitive

to water stress because its root length showed substantial decrease at all growth stages under PEG treatment, Rauf et al. (2007) and Muscolo et al. (2014) found that increased root length and branching was consistent with significant decrease in protein and sugar content which play an important role in the building of new cells. Extensive root system was associated with high yield under water stress and was well known since a long time to be related to drought resistance in wheat (Hurd, 1974). The root:shoot biomass ratio tend to be higher in plants adapted to drought stress (Hilbert and Canadell, 1995; Smirnoff, 1998; Qi et al., 2019). Wheat cultivars under water stress have shown increased root:shoot biomass ratio at most of the 3 growth stages, this response is due to root-shoot hormonal signaling such as signaling via Abscisic acid (ABA), ethylene and cytokinines which increase under low water potential. High levels of ABA decrease the gas exchanges (Zhang et al., 2006) by promoting the efflux of K^+ ions from the guard cells leading to the closure of stomata folwed by a decline in photosynthetic carbon uptake and increased photorespiration. negatively influencing total shoot weight. Similarly Cytokinines play an important role in the roots and their production in roots results in root expansion in the soil in response to water stress (Anjum et al., 2011). In this study, root: shoot biomass ratio increased in most Cvs and at all growth stages except in Cv1 and Cv 6 at vegetative growth stage. The same result was observed in previous studies where root:shoot biomass ratio was positively correlated with water stress in wheat (Hamblin et al., 1990) and other plants (Mokany et al., 2006, Wang et al., 2008; Mathew et al.,2018). In contrast, Cairns et al., (1997) at Reich et al., (2014) reached the opposite conclusion due to the fact that species and leaf age could regulate plant response to stress. (Blum and Ebercon, 1981; Sheoran et al., 2015; Daryanto et al., 2017).

2.4.2 Relative water content (RWC)

Relative water content (RWC) in shoot is an important indicator of plant water status, which would decrease during the plant progress in age under water stress (González and González-Vilar, 2001; Kameli and Lösel, 1995; Kundur et al., 2016). Thus RWC can be used as screening tool for drought tolerance in plant (Shivakrishna et al., 2018). RWC is controlled by both water loss by transpiration and water uptake by the roots (Anjum et al., 2011). In this study, RWC significantly declined in most wheat Cvs at different growth stages especially the filling stage due to decline in water level under PEG treatment. the same results were found in Iranian wheat cultivars (Saeidi et al., 2015) and a Chinese wheat cultivar (Guo et al., 2013). Rampino et al., (2006); Gregorová et al., (2015); Liu et al., (2015a); Sheoran et al.,(2015) observed a decrease in RWC in different drought sensitive wheat Cvs under drought stress. In our study Cv2 has

shown an increase in R.W.C. under PEG treatment at all life stages compared with control. It seems that this Cv has a high ability to control water loss by using different strategies including root extension, and/or increased accumulation of compatible solutes such as proline. This finding is in agreement with that reported by Bayoumi et al. (2008) which illustrates that tolerant wheat cultivars can keep RWC level high in their tissues.

2.4.3 Proline content

Accumulation of osmoprotectants in wheat tissues such as Proline in the cytoplasm and soluble sugars in vacuoles together with Late Embryogenesis Abundant proteins which are hydrophilic proteins can contribute to osmotic regulation and protect the plant cells from dehydration under water stress (Rathinasabapathi, 2000; Bayoumi et al., 2008; Foito et al., 2009; Hand et al., 2011; Farooq et al., 2014). Cv1 and Cv2 Wheat have shown significant increase in proline levels under water-stress at all growth stages. This result is in agreement with many research such as that of (Kosar et al., 2015) who found the same result in two Pakistani wheat cultivars. Furthermore, similar findings were reported by EL-TAYEB and Ahmed (2010), Keyvan (2010), Maralian et al. (2010), Liu et al. (2015a) and Rampino et al. (2006). However, the current study found that Cv3, Cv4 and Cv6 had significant reduction in proline levels at the vegetative and flowering stages under PEG-treatment, this might show an age dependent response to water stress. Free proline content in shoot is an important indicator of water-stress, accumulation of proline under drought stress is considered as a strategy against water stress (Sivamani et al., 2000). In addition to its osmotic role, proline plays an important role as anti-oxidant to protect plant cells from stress-induced damage caused by free radicals and might be used as protein-structure stabilizing agent (Ahanger et al., 2014; Islam et al., 2015b). Wheat Cvs that are tolerant to drought have high concentration of proline in their cells (Ashraf and Foolad, 2007). There was a significant decrease in proline levels at all growth stages of Cv5 with maximum decrease (50%) at grain filling stage, this could show that this Cv has poor osmo-regulation at all life stages, unless it might use a different osmoregulation system under water stress.

2.4.4 Soluble sugars content

An age dependent response to water stress was apparent for soluble sugar levels in shoots, on the contrary to proline content, Cv2 showed significant decrease in soluble sugar content at all growth stages with maximum decrease by 45% at flowering stage. Based on yield performance, this cultivar used most of photosynthetic production to enhance yield (seeds weight, seeds numbers and germination) under water stress. It is also possible that consequently to reduction

in stomatal conductance and the ensuing decrease in CO_2 assimilation under water stress, photosynthetic production (sugar) was decreased (Liu et al., 2015a; Rodrigues et al., 2019). On another hand, Cv1, Cv3, Cv4, Cv5 and Cv6 raised soluble sugar levels in their tissues at one or two growth stages, this results is in agreement with many studies such as that of Guo et al., (2013) and Turner, (2017). This increase could be attributed to the ability of these Cvs to maintain turgor under drought stress and higher accumulation of sugar from photosynthesis. However, the increase is soluble sugars levels in these Cvs is more likely to have resulted from increased starch degradation under water-stress, the activity of starch degrading enzymes are known to increase in most plant species in response to water stress (Yang et al., 2001; Lee et al., 2008; Thalmann et al., 2016; Zanella et al., 2016). Increasing proline and soluble sugars content were to protect macromolecular components from denaturation under drought stress (Mostajeran and Rahimi-Eichi, 2009). Kameli and LÖSELδ (1993) argued that carbohydrate accumulated more rapidly than proline in a drought resistant wheat cultivar. Therefore, carbohydrate such as glucose and fructose are more sensitive indicators of water stress level and potential tolerance in wheat than proline. However, the reduction of soluble sugar levels in some wheat cultivars such as Cv2 could be explained by the fact that this Cv might prefer a different osmoregulation strategy under water stress like increased proline level under decreased photosynthetic activity under drought (Jorge et al., 2016).

2.4.5 Proteins content

The last physiological criterion measured in this study was Protein level. According to Nagy et al. (2013b) protein level increased under drought in tolerant wheat Cvs and decreased in sensitive ones. In the current study Cv2 has shown substantial increase in protein level at all growth stages, this increased protein production might be involved in ROS scavenging and oxidative stress metabolism (Ford et al., 2011). Moreover to avoiding damage to photosystem II under oxidative stress resulting from water stress some compounds might increase to levels that can inhibit the turnover of D1 protein, a protein necessary for the repair of the damage to PSII (Nishiyama et al., 2001, Allakhverdiev and Murata, 2004). Cv1, Cv4 and Cv6 showed a relatively important reduction in protein levels under PEG treatment at all growth stages, this is consistent with low performance of these Cvs under water stress.

2.4.6 Yield measurements

Yield is one of the most important criteria for selecting resistant and sensitive cultivars under water stress especially in wheat (Fig 2.13). Three main traits were chosen to assess yield in the current study including grain filling, seed number and seed germination performance under

water stress. Grain filling traits changed under drought stress in all Cvs under study. Cv2 and Cv3 had the lowest grain filling reduction, for example, seeds weight decreased by 1.16% and 5.8% respectively compared with Cv1 (42%) and Cv4 (53%) reduction compared to control under water stress. Nezhadahmadi et al. (2013) reported 64% decrease in seed number per spike in wheat under drought which is consistent with current study results in all cultivars especially Cv4. The results are also in agreement with Sangtarash, (2010), Saeidi et al., (2015) and Zörb et al., (2017) . Moreover, the significant decrease in germination rate shown by all Cvs is consistent with many other studies such as that of Guo et al., (2013). This might be due to the reduced rates of photosynthesis caused by stomatal closure leading to metabolic limitation, oxidative damage to chloroplasts and poor grain setting and development (Farooq et al., 2014; Saeidi and Abdoli, 2018; Rodrigues et al., 2019). Interestingly, Cv2 which showed non-significant change under water stress in number of seeds per spike, seeds weight and only a slight decrease in germination in comparison with other Cvs is obviously consistent with the high performance shown by the Cv for all physiological traits at all life stages making it resistant to water-stress.

2.4.7 Assessing different criteria as screening tools for water stress tolerance

To evaluate the association of different morphological, physiological and biochemical measurements with water stress tolerance (WST) and to assess the suitability of these measurements for screening wheat Cvs for water stress tolerance, all recorded measurements in the 6 wheat Cvrs were ranked and scored based on the water stress tolerance indices according to El-Hendawy et al., (2007). One cultivar (Cv2) had the highest score for all measurements under water stress and was ranked as the most tolerant to water stress among the other cultivars. Cv4 had the lowest WST%, thus it was considered as the most sensitive Cv. This study found that wheat , Cv1, Cv2, Cv5 and Cv6 were more sensitive to water-stress at flowering and grain filling stages than vegetative stage. However Cv3 and Cv4. were more sensitive to water stress at the vegetative stage This result is in agreement with Farooq et al. (2014) who found that drought stress reduced wheat performance at all growth stages and the reduction was more sever at filling and flowering stages.

2.5. Conclusion

In this chapter,

1. Six different wheat cultivars from three main wheat growing areas in Saudi Arabia were investigated in terms of their response to water stress at morphological, physiological and biochemical levels at three growth stages. These wheat cultivars showed different responses to water-stress based on the shoot weight and length, root weight and length, the relative water content (RWC), proline content, soluble sugar content, protein content, number of seeds per spike, seeds weight and germination.
2. Among the investigated cultivars 193 Najran (Cv2) and 357 Sama (Cv3) were resistant to water stress, due to their high performance in terms of plant growth and productivity. In contrast, 181 Jizan (Cv1), 377 Rafha (Cv4), 562 Ma'ayah (Cv5) and 981 Najd (Cv6) were sensitive to water-stress.
3. The wheats under study were grouped into two groups, drought resistant (193 Najran (Cv2) and 357 Sama (Cv3)) and drought sensitive (181 Jizan (Cv1), 377 Rafha (Cv4), 562 Ma'ayah (Cv5) and 981 Najd (Cv6)).
4. To determine the physiological and molecular mechanisms underpinning drought-tolerance in wheat, two wheat cultivars from each group were selected randomly, 193 Najran (Cv2) as a drought resistant and 377 Rafha (Cv4) as drought sensitive and used for conducting a comprehensive analysis of changes in the transcriptome and the proteome to identify genes and proteins potentially responsible for the drought tolerance in wheat. The obtained results from these investigations are described in the following chapters.

Chapter 3 Differential gene expression profiles and associated metabolic pathways in two Saudi wheat cultivars under water stress at three growth stages.

3.1.Introduction

Wheat has a complex hexaploid genome (Kumar et al., 2015a; Kumar et al., 2015b; Poersch-Bortolon et al., 2016a), full of repetitive sequences. It is 17Gb in size, which is almost 6 times the size of the human genome ~3.3Gb (Gonzaga-Jauregui et al. (2012) and contains about 96000 genes. This genetic complexity is reflected in the physiological complexity and huge biological variation among wheat Cultivars (Cvs). Recent revolution in high throughput sequencing technologies combined with computational approaches has improved genetic studies in plants under different abiotic and biotic stresses (Liu et al., 2017; Bedre et al., 2019; Délye et al., 2020). Illumina sequencing platform (MiSeq, NextSeq 500 and the HiSeq series) is the most widely used of the high throughput technologies (Reuter et al., 2015, Bast Jr et al., 2017), it relies on fluorescent dye-labelled nucleotides that reversibly terminate the sequencing reaction; the main features of Illumina sequencing is that a single run can produce up to ten billion paired- end reads. These technologies with the development in bioinformatics software that can deal with huge quantities of sequencing information improved our understanding of the physiological and molecular responses in plant under stresses.

Based on above approaches, local wheat cultivars may offer sources of abiotic stress tolerance genes, for example against high temperature stress (Pradhan and Prasad, 2015) or water stress (Chorfi and Taıbi, 2011) because they grow in stressed habitats. Therefore, understanding the regulation of the genes involved in drought tolerance in some local wheat Cvs could pave the way for improving the physiological response to drought stress in other wheat Cvs. Under water stress, plants have to adjust its physiological and biochemical processes, involved in regulating ionic and osmotic homeostasis, as well as stress damage control and repair. Plants therefore may induce signalling pathways such as those initiated by non-hydraulic root signals (nHRS), which may result in increased accumulation of osmoprotectants, via protein kinases and plant hormones under water stress (Fan et al., 2008, Lv et al., 2019, Mellacheruvu et al., 2019). Many genes are highly regulated under drought stress, mainly those involved in protein processing in the endoplasmic reticulum, plant hormone signalling, photosynthesis, lipid metabolism and amino acid metabolism (You et al., 2019). In order to unravel these genetic and biological complexities, complementary approaches are needed. Transcriptomics deliver comprehensive qualitative and quantitative information about the complete set of mRNA molecules that result

in generating proteins; proteomics provide information about the complete collection of proteins; and metabolomics concerns the complete series of metabolites produced in a cell (van der Werf et al., 2005; van der Werf et al. 2005; Karakach T. 2010). Recently, the availability of genomic tools and resources has allowed researchers to understand transcriptome and proteome changes under different environmental stresses, which could unravel how plants respond and cope with different stresses. Major classes of genes have been suggested to assist with cellular adaptation to stress (Bray, 2004). For instance, the up-regulation of TaCRT transcripts was reported in wheat in response to drought stress (Islam et al., 2015a), and the transcription factor dehydration-responsive element binding protein (DREB) was shown to regulate the expression of genes in response to salt stress in wheat (Jiang et al. (2017b). Chen et al. (2016) found that high expression of the gene TaDlea3 regulates late embryogenesis abundant proteins (Schubert et al., 2015) and was related to drought stress in wheat. Raney (2012a) found from RNA-seq analysis of Chenopodium quinoa under water stress that there was an overlap between drought stress tolerance and other abiotic stress mechanisms. In *Arabidopsis thaliana*, 27 genes were shown to increase expression under water stress. The majority of genes regulated under stress were related to six different functional categories: transport, signalling, transcription, hydrophilic proteins, general metabolism or having unknown function (Bray, 2004). The preferred technique for identifying and characterising differentially expressed genes (DEGs) with their functional annotation under different environmental stresses in plants is high throughput cDNA sequencing (RNA-seq) using next generation sequencing (NGS) technology (Kumar et al. 2015b; Poersch-Bortolon et al. (2016a). High resolution and accuracy in genomic analyses are two of the advantages of NGS (Lee et al. (2013a). NGS has many applications such as whole genome sequencing, gene expression profiling, target sequencing and small RNA sequencing (Lee et al. (2013a). To obtain maximum information, selecting a suitable workflow and analysis tools for the massive amount of data generated by NGS is crucial and is often dependent on the biological questions or the aims of the study. For example, the most common workflow or experimental design to find DEGs based on RNA-seq. is alignment or mapping of short sequencing reads (FASTQ file) against related reference genomes from the National Center for Biotechnology Information (NCBI, the University of California, Santa Cruz, USA). This allows researchers to infer which transcripts are expressed by using advisable tools such as Bowite. Bowite2, which is an extremely fast, general purpose short read aligner that can be used with TopHat to align RNA-seq reads to a reference genome and to discover splice sites against de novo assembled RNA transcripts for novel species (Trapnell et al. 2012b; Križanović et al., 2018; Babarinde et al.,

2019). These software packages include SAMtools, which summarise the aligned results into BAM files, which are used to calculate differential Transcript levels after normalisation with DESeq, baySeq, edgeR or Cufflinks software. Read counts per transcript are determined with HTSeq or Cufflinks software (Lee et al., 2013a). This workflow could deliver trusted conclusions about genes or biological pathways in the plants under study. Due to the need for good knowledge and background in some programming languages such as Python, R language or any scripting language, many bioinformatics companies have tried to decrease the programming by combining all of these software tools into one piece of commercial software such as Geneious or CLC Genome Workbench. Researchers can gain the benefits without having any expertise in statistical programming languages. Gene transcript profiles could be accurately determined by RNA sequencing technology. Therefore, the mechanisms by which plants respond to stress conditions could be easily understood by profiling the plant transcriptome (Chung et al., 2017).

Based on the collected growth data in the first experiment, two cultivars – 193 Najran (Cv2) and 357 Sama (Cv3) – were shown to be resistant to water stress and the remaining cultivars,181 Jizan (Cv1), 377 Rafha (Cv3), 562 Ma'ayah (Cv5) and 981 Najd (Cv6) were sensitive to water stress. One Cv from each group (Cv2 and Cv4) were used to conduct a comparative analysis of the differences in the transcriptome and the proteome under both control (well-watered) conditions and water stress (15% PEG6000) conditions to identify the gene differences and differential transcript levels in the two Cvs under the effect of water stress at three growth stages: vegetative, flowering and grain filling.

3.2. Material and Methods

3.2.1. Plant growth conditions and water-stress treatments

a. Plant growth room conditions

This experiment was done in Newcastle University in a plant growth room under a light intensity of 250 µmol. $m^{-2}.s^{-1}$, 65% humidity, 12h light, 12h darkness photoperiod, and 22°C day/18°C night thermoperiod.

b. Plant growth

Seeds of the two wheat Cvs, 193 Najran (Cv2) and 377 Rafha (Cv4) were sown directly in pots filled with John Innes 2 compost and after germination divided into two batches having 9 plants per treatment: 1) control batches were watered with tap water only and 2) water-stress batches were watered with a PEG solution as shown in **c at P.32.** The two batches were divided into three groups, one for collecting leaf samples at the vegetative stage, the second for collecting leaf samples at the flowering stage and the last group for collecting the filling stage samples.

c. Plant sampling

Leaf samples were collected from both control plants and water-stressed plants at the vegetative, flowering and grain filling stages. Samples were placed in labelled foil bags (each sample separately but pooled replicates) as shown in (table 3.1), snap frozen in liquid nitrogen and stored at -80°C until analysis. Before analysis the plant material was ground under liquid nitrogen using a pestle and mortar and stored at -80°C until analysis. The powdered tissue was used for biochemical measurements, and for extracting RNA and proteins.

Table 3. 1 Samples abbreviated name description.

Abbreviated name	Description
Cv2Vegwater	193 Najran cultivar in vegetative stage under control condition
Cv2VegPEG	193 Najran cultivar in vegetative stage under treatment condition
Cv2Flowwater	193 Najran cultivar in flowering stage under control condition
Cv2FlowPEG	193 Najran cultivar in flowering stage under treatment condition
Cv2Fillwater	193 Najran cultivar in grain filling stage under control condition
Cv2FillPEG	193 Najran cultivar in grain filling stage under treatment condition
Cv4Vegwater	377 Rafha cultivar in vegetative stage under control condition
Cv4VegPEG	377 Rafha cultivar in vegetative stage under treatment condition
Cv4Flowwater	377 Rafha cultivar in flowering stage under control condition
Cv4FlowPEG	377 Rafha cultivar in flowering stage under treatment condition
Cv4Fillwater	377 Rafha cultivar in grain filling stage under control condition
Cv4FillPEG	377 Rafha cultivar in grain filling stage under treatment condition

d. Physiological parameters (Plant growth measurement)

The height (cm) and dry weight (g) were measured at three stages (vegetative, flowering and grain filling stage) and compared between the plants subjected to water stress and the controls. Plant height was taken one day before the plant was harvested (four biological replicates for each sample).

3.2.2. Biochemical measurements

a. Measurement of proline content

Ground shoot material (200 mg FWt) was homogenised in 1ml of 3% (w/v) aqueous sulfosalicylic acid solution, and proline content measured as in chapter 2, paragraph a, at P.33

b. Measurement of soluble sugar content

Phenol/sulphuric acid method was used for determination of sugars and related substances after Dubois et al. (1956). chapter 2, paragraph b, at P.33

3.2.3. Statistical Data analysis

Statistical analysis was performed using SPSS 2. The data represent the means calculated from four replicates for the measured growth parameters and physiological parameters. The analysis of variance was done using ANOVA followed by F-test analysis. The values are means ±SE and statistical significance was set to $p < 0.05$.

3.2.4. Transcriptomics

3.2.5.1. RNA extraction

About 100 mg of powdered leaf samples were homogenised in 1 ml of TRI reagent (Bioline, UK) in an RNase/DNase free 1.5 ml tube, and vortexing, the samples were left at room temperature for 10 min. A volume of 250 µl of chloroform were added to each sample and the samples were vortexed and span at 13000 rpm at 4 ºC. RNA was recovered in the aqueous upper phase and precipitated with the addition of 250 µl of isopropanol (ThermoFisher, UK) and centrifugation at 13000 rpm at 4 ºC for 30 min. The pellet was air dried and RNA resuspended in 20 µl of DEPc water. The RNA integrity was assessed using nanodrop spectrophotometer. RNA samples having an A260/280 ratio of 2.0 were checked for integrity using a Bioanalyzer following the manufacturer instructions (figure S2. 1; figure S2. 2) and sent to Admera, USA for Library preparation and QC, RNA integrity and sequencing as following.

3.2.5.2. Library preparation and QC

Next generation Hiseq illumina (SMART-Seq v4 Ultra Low Input RNA Kit for sequencing) was used to sequence the 12 RNA samples (two wheat cultivars in two conditions at three growth stages with three biological pooled replicates for each sample). The library preparation was designed to remove the ribosomal RNA prior to sequencing. This increases the depth of sequencing of the transcriptome since ribosomal RNA accounts for the vast majority of the transcriptome. Ribo-Zero protocol (ILLUMINA PROPRIETARY Part # 15065382 Rev A November 2014) was used for the qualification, and sequencing of RNA was carried out according to the procedure of Chenchik et al. (1993), Ramskold et al. (2012) and Picelli et al. (2014).

3.2.5.3. RNA integrity

RNA species of interests are Poly-A transcripts >170nt (mRNA sequencing) therefore the Oligo dT magnetic bead system was used. The length of the read was 85bp to keep the overall quality of the reads high (Q-score) on all the samples under study.

3.2.5.4. Sequencing

The library pool(s) to be sequenced were denatured and diluted/neutralised to the required concentrations. Then cluster generation was performed on the appropriate flow cell using single molecule clonal amplification. Finally, the high-throughput next generation sequencing was

performed using the Illumina sequencing technology platform. For a more detailed description of the sequencing process please visit the Illumina homepage at www.illumina.com

3.2.5.5. RNA-seq computational analysis

a. Quality Control

The quality of the FASTQ sequence files was assessed with FastQC, Version 11.7 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/. The FastQC results were passed to MultiQC to aggregate the results for all samples into a single report http://multiqc.info/. The tables and figures presented in the appendix contain an aggregated report for all FASTQ files (figure S2. 3; figure S2. 4). No read trimming was necessary, and all samples were retained for further analysis

b. Read Quantification

Reads were quantified against transcripts using Salmon tool which produce quantification estimates at the transcript level without need for alignment (Patro et al., 2016) and qualified reads were mapped to the reference genomes, using a program for quantifying expression of transcripts from RNA-Seq data: https://combine-lab.github.io/salmon/. The Salmon workflow can be summarised as follows:

1. Index file was build using the 'salmon index' command. The index was built from Ensembl Plants Triticum aestivum fasta files (release 40)http://plants.ensembl.org/Triticum_aestivum/Info/Index?db=core
2. The quantification algorithm 'salmon quant' was run for each FASTQ file against the index. Salmon quantifies reads against transcripts. To obtain gene-level counts, the R package 'tximport' was used http://bioconductor.org/packages/release/bioc/html/tximport.html

c. Differential Gene Expression Analysis (after normalisation of RNAa-seq data)

Differential transcript-level analysis was carried out with the R package DESeq2. http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html.

Samples have no replicates so no estimation of variability within groups was possible, thus statistical tests could not be carried out. Changes in expression were measured by changes in normalised gene-level counts and expressed as log2 fold changes. The comparisons shown in Table 3.2 were calculated. The results directory contains the raw and normalised count data for all genes, and tables of expression changes for all of the comparisons in table 3.2.

Notes:

- The tables of fold changes have been sorted by magnitude of log2 fold change
- Only genes which had non-zero counts in both samples have been included in these lists
- Naming the comparison ‘X’ vs ‘Y’ means that a positive log2FoldChange indicates upregulation in ‘X’ and vice versa.

The files ending .tsv are plain text tab-delimited files. The file top_genes.xlsx is an Excel workbook with a sheet for each comparison and includes hyperlinks to each region on the Ensembl plants website. In order that this file is not too large, each sheet is limited to the 200 genes with greatest absolute fold change. A list of genes to be differentially expressed between water stressed plants and control plants in each comparison were presented in the appendix with principle component analyses (PCA, Heatmap, Venn diagram). Heatmap showed the distances between samples measured with Euclidian methods also known as “Pythagorean” distance (the square root of the sum of the squared differences of the components), and based on normalised counts of gene-level data obtained in the previous step. Also, Principal Component Analysis (PCA) showed the sample-to-sample Euclidian distances based on gene-level and plot coloured by treatment (15%-PEG6000 and water control samples). Venn Diagrams was used to visualize overlaps in gene lists. Visualised results were done in R studio and saved in PNG format.

Table 3. 2 The differential expression comparison groups

Conditions Level	Growth stages Level	Cultivars level
Cv2VegPEG vs Cv2Vegwater	Cv2Vegwater vs Cv2Flowwater	Cv4Vegwater vs Cv2Vegwater
Cv2FlowPEG vs Cv2Flowwater	Cv2Vegwater vs Cv2Fillwater	Cv4Flowwater vs Cv2Flowwater
Cv2FillPEG vs Cv2Fillwater	Cv4Vegwater vs Cv4Flowwater	Cv4Fillwater vs Cv2Fillwater
Cv4VegPEG vs Cv4Vegwater	Cv4Vegwater vs Cv4Fillwater	
Cv4FlowPEG vs Cv4Flowwater		
Cv4FillPEG vs Cv4Fillwater		

d. Gene Annotation

In the same R package 'tximport' the annotations were added to the table results. Gene annotation was extracted from Ensembl Plants *Triticum aestivum* GFF file (release 40). http://plants.ensembl.org/Triticum_aestivum/Info/Index?db=core. by using 'wegt' and inserted to R using the following R-script, "results = left_join(sorted_results, grch38, by=c('ensembl_geneid'='ensgene'))"

e. Gene term enrichment (GO)

Association between wheat genes IDs and GO terms was based on high-confidence BLAST hits reported in The International Wheat Genome Sequencing Consortium (IWGSC) RefSeq Annotations

https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Annotations/v1.0/

Testing for enriched GO terms was carried out using the hyperGTest() function from the R package GO stats, with a p-value test at cut-off of 0.05.

f. Metabolic pathway by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of water stress regulated genes

The association between wheat gene IDs and KEGG genes was inferred from a BLASTX search of nucleotide sequences for the wheat genes against amino acid sequences for all UniProt entries for *Oryza Sativa*. UniProt entries list KEGG gene IDs where these have been established. BLASTX hits with an identity of at least 60%, a bitscore greater than 50 and an e-value less than 1e-10 were accepted as mappings between wheat genes and KEGG genes (these are the same criteria for high-confidence BLAST hits in the IWGSC RefSeq Annotations). Testing for enriched KEGG gene IDs was carried out using a one-tailed Fisher Exact Test with p-value cut-off of 0.05. Pathways for the enriched KEGG genes were determined using the R package KEGGREST, and pathway graphics were rendered using the R package Pathview. The colouring of the pathways was based on the mean log2 fold changes. The graphics were generated using the online tool KEGG Mapper–Colour Pathway (http://www.genome.jp/kegg/tool/map_pathway3.html).

3.3.Results

3.3.1. *Plant growth and biochemistry*

As shown in the previous chapter, the two wheat cultivars under study showed variable changes in morphological and biochemical measurements under water stress created by 15% PEG. To confirm previous conclusions of the first chapter, we repeated some morphological and biochemical measurements for 193 Najran (Cv2) and 377 Rafha (Cv4).

Cv2 and Cv4 showed an age-dependent response to water stress. The two Cvs have shown reduction in shoot and root weight under water-stress at all growth stages. The highest reduction (89%) in shoot dry weight was recorded at the grain filling stage in Cv4 and the lowest reduction was recorded in Cv2 (47%) compared to control samples at the same growth stage (figure 3.1). However, the highest reduction in root dry weight was recorded at the vegetative stage in Cv2 and Cv4 by 93% and 88% respectively, compared with the control sample, and the lowest reduction was found at the flowering stage in Cv2 and Cv4 by 12.5% and 73% respectively, compared with the control sample (figure 3.2). Shoot lengths of both cultivars (Cv2 and Cv4) decreased at all growth stages under water stress, with the highest reduction recorded in Cv2 at the flowering stage (21%) while in Cv4 the highest reduction was at the vegetative stage (33%), compared to the control samples (figure 3.4). Both cultivars significantly ($p<0.000$) increased in root length at the three growth stages ($p<0.000$) with the highest increase in the vegetative stage in Cv2 (21%) and the grain filling stage in Cv4 (65%) higher than the control samples. However, Cv4 at the vegetative stage showed a significant ($p<0.000$) reduction of 7.9% under water stress (figure 3.5). Root:shoot ratio and biomass ratio decreased significantly ($p<0.000$) in the vegetative stage and increased in the flowering and grain filling stages in both cultivars. The highest increase was in the flowering stage of Cv2 (102.9%) compared to the control samples and the lowest was in Cv4 at the same stage (0.39%) (figure 3.6).

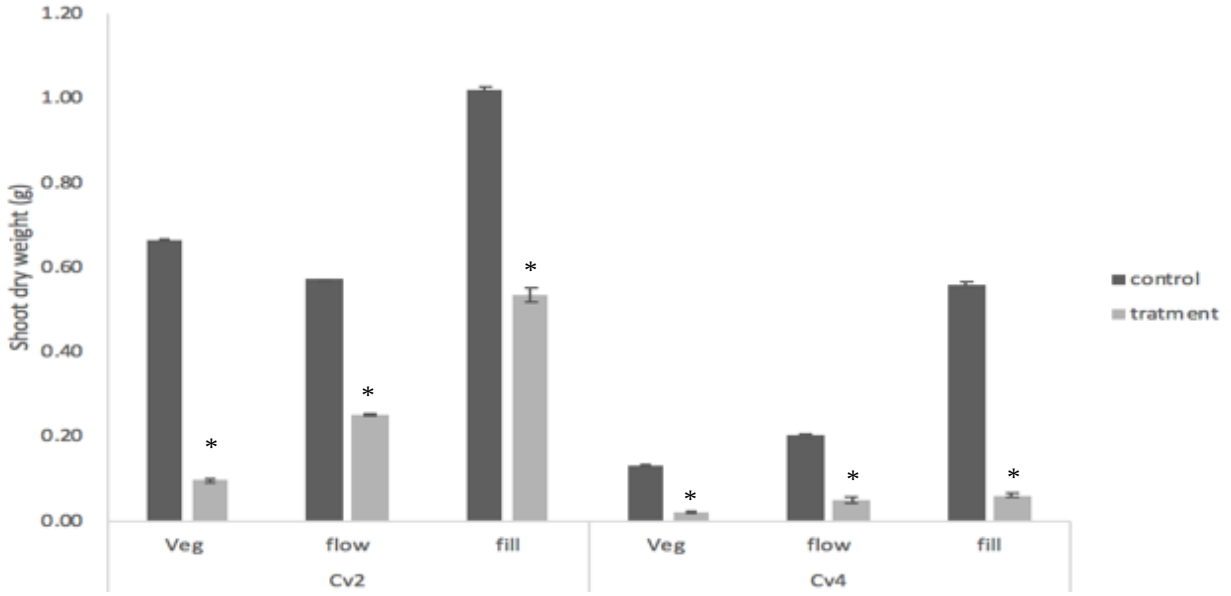


Figure 3. 1 Effect of water-stress on Biomass measured as Shoot dry weight (g) in two wheat (*Triticum aestivum* L.) cultivars: 193 Najran (Cv2); 377 Rafha (Cv4) at three growth stages: vegetative (Veg), flowering (flow) and grain filling (fill) stages subjected to 15% PEG6000. n=9, bars are standard errors.

* The mean difference between (cultivars*stages * water_stress) is significant by F-test at the .05 level.

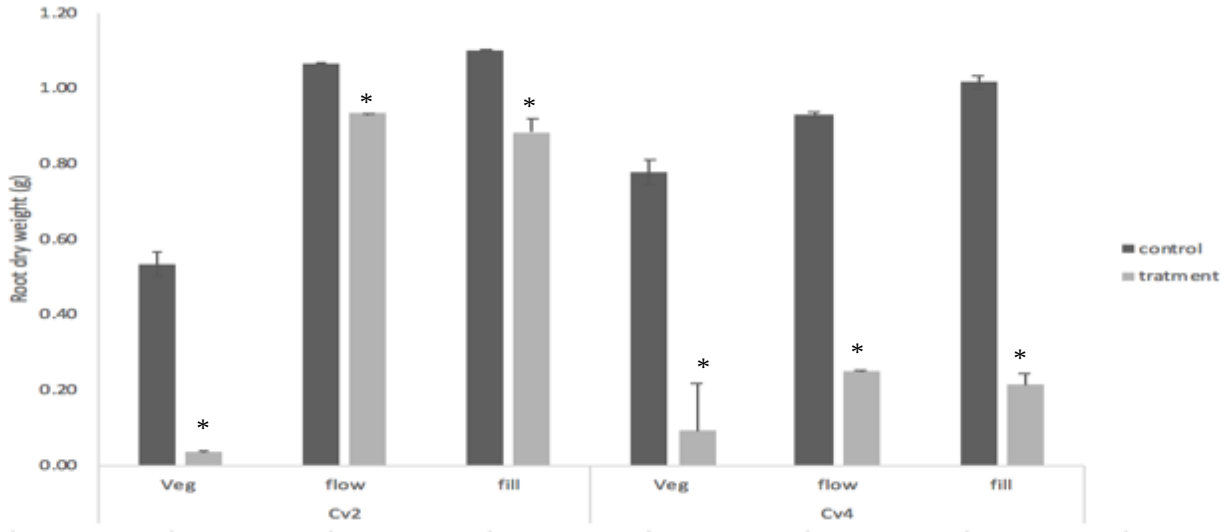


Figure 3. 2 Effect of water stress on Biomass measured as root dry weight (g) in two wheat (*Triticum aestivum* L.) cultivars: 193 Najran (Cv2); 377 Rafha (Cv4). at three growth stages: vegetative (Veg), flowering (flow) and grain filling (fill) stages subjected to 15% PEG6000. n=9, bars are standard errors.

* The mean difference between (cultivars*stages * water_stress) is significant by F-test at the .05 level.

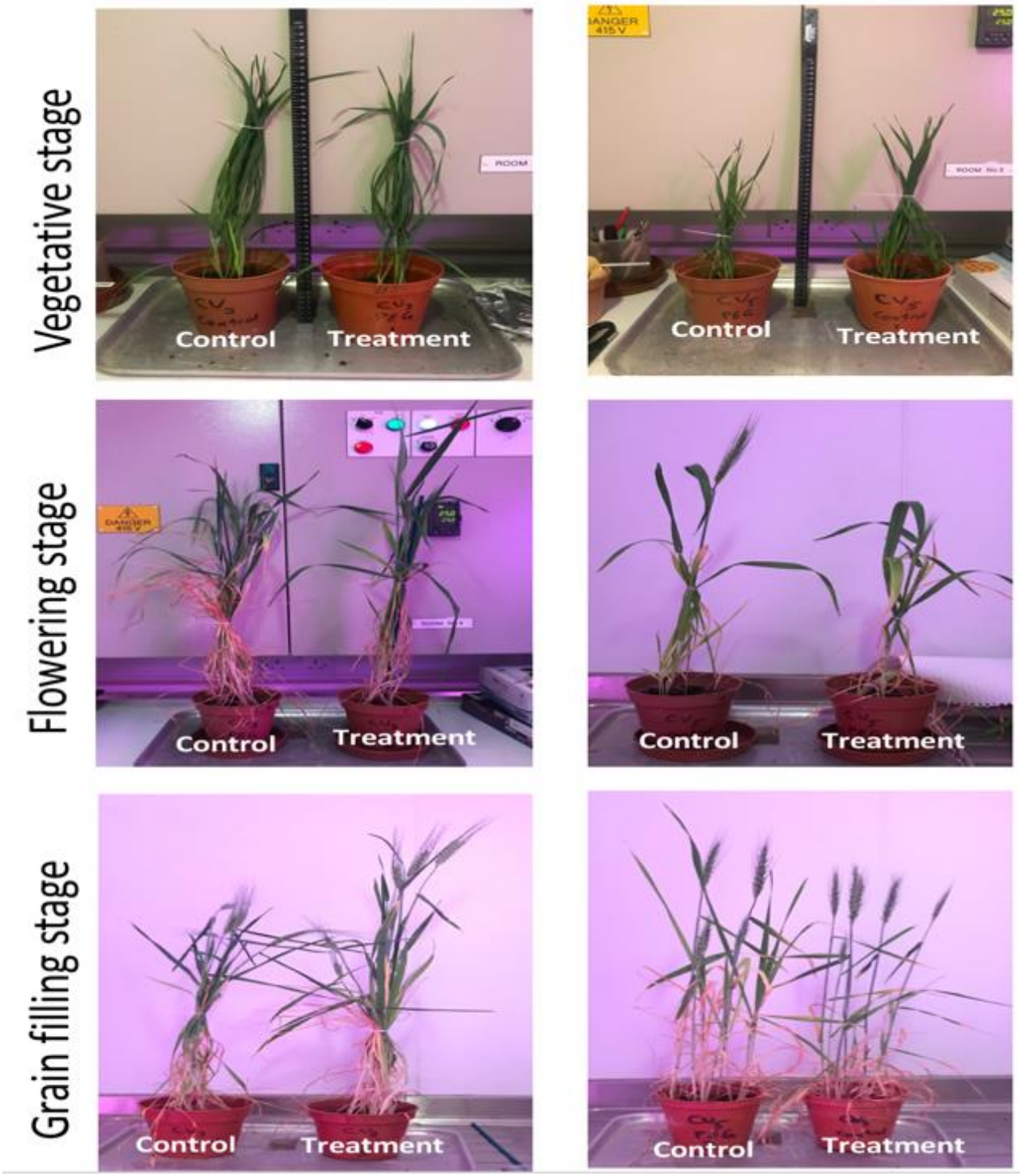


Figure 3. 3 Effect of water stress imposed by watering plant with a PEG solution for one week on plant morphology in two wheat (*Triticum aestivum*) cultivars:1. 193 Najran (Cv2) 2. 377 Rafha (Cv4).

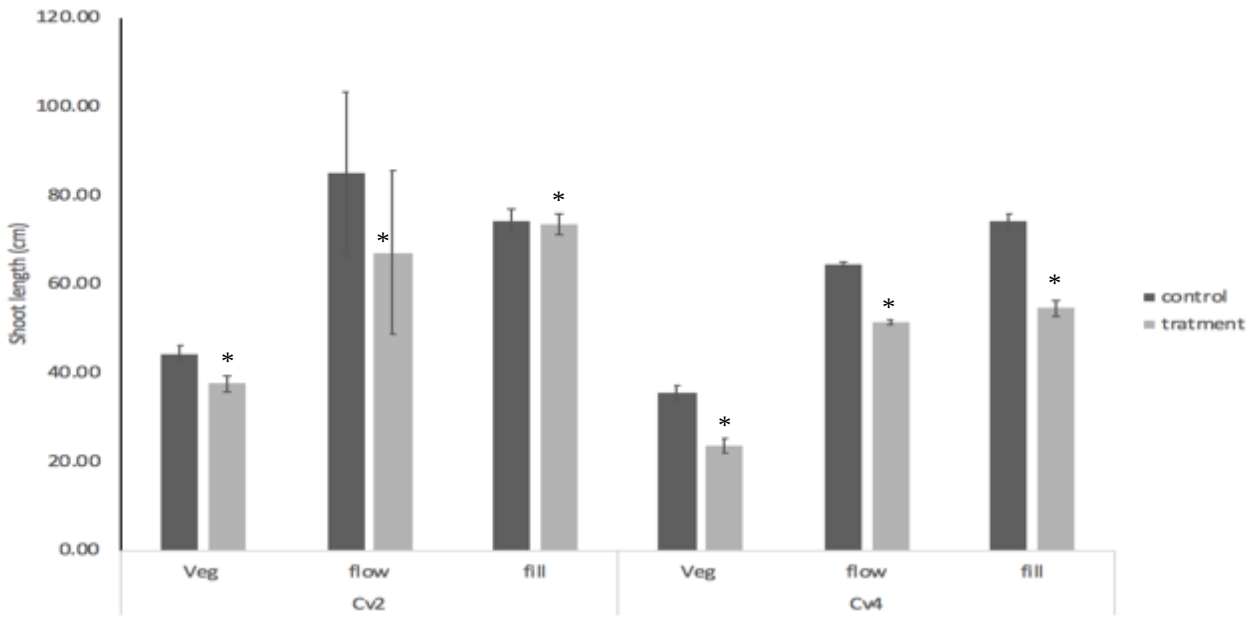


Figure 3. 4 Effect of water stress on Shoot length (cm) in two wheat (*Triticum aestivum* L.) cultivars: 193 Najran (Cv2); 377 Rafha (Cv4) at three growth stages: vegetative (Veg), flowering (flow) and grain filling (fill) stages subjected to 15% PEG6000. n=9, bars are standard errors.

* The mean difference between (cultivars*stages * water_stress) is significant by F-test at the .05 level.

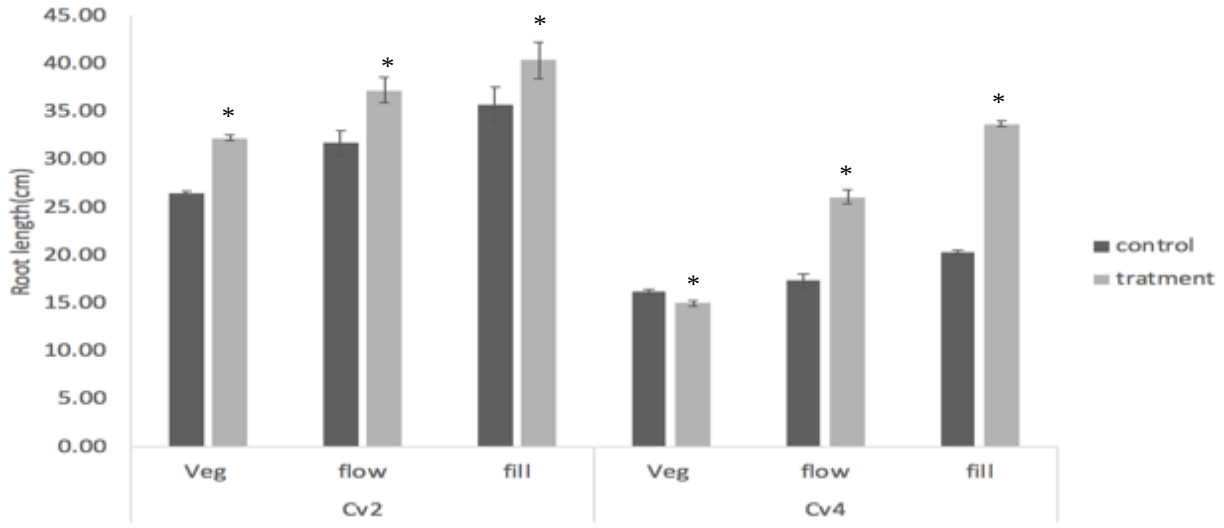


Figure 3. 5 Effect of water stress on Root dry weight (g) in two wheat (*Triticum aestivum* L): cultivars 193 Najran (Cv2); 377 Rafha (Cv4) at three growth stages: vegetative (Veg), flowering (flow) and grain filling (fill) stages subjected to 15% PEG6000 n=9, bars are standard errors.

* The mean difference between (cultivars*stages * water_stress) is significant by F-test at the .05 level.

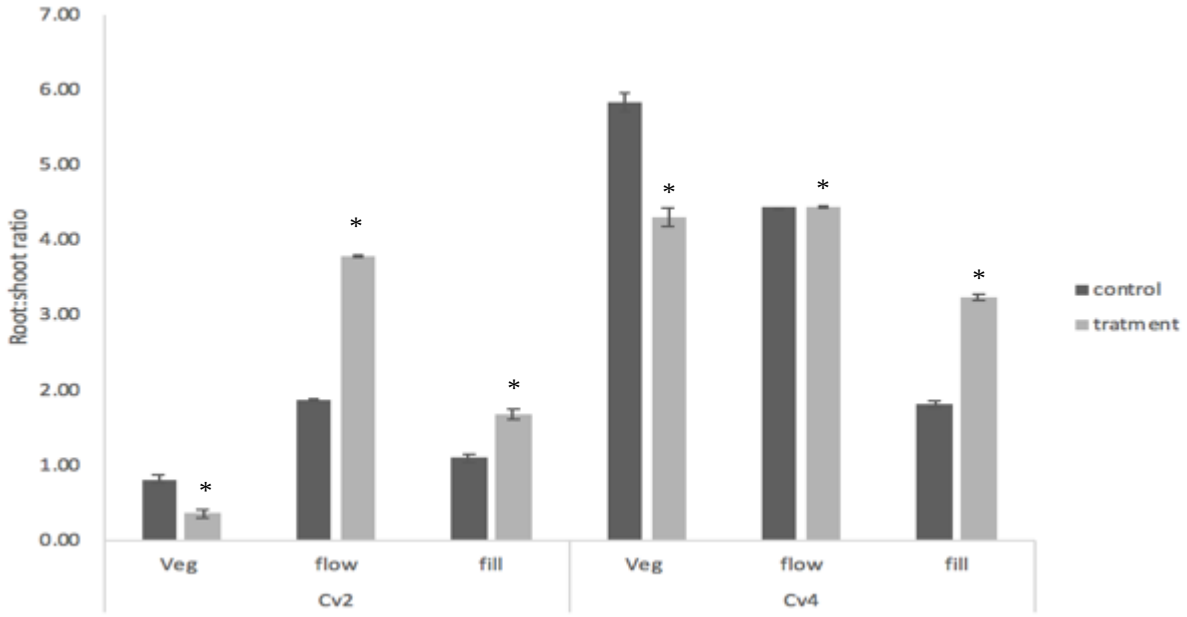


Figure 3. 6 Effect of water stress on Root/shoot ratio in two wheat (*Triticum aestivum* L) cultivars.: 193 Najran (Cv2); 377 Rafha (Cv4)) grown in different regions of Saudi Arabia at three growth stages: vegetative (Veg), flowering (flow) and grain filling (fill) subjected to 15% PEG6000 (treatment) n=4, bars are standard errors.

* The mean difference between (cultivars*stages * water_stress) is significant by F-test at the .05 level.

High free proline content in shoots is an important indicator of a stress-response including under PEG treatment. The two cultivars showed significant change in proline content at all stages ($p<0.027$). A significant increase was shown by Cv2 at all growth stages, but the highest increase was in the grain filling stage compared to the control samples. However, it was found that the flowering stage of Cv4 significantly ($p<0.027$) decreased (78%) compared to the control samples (figure 3.7).

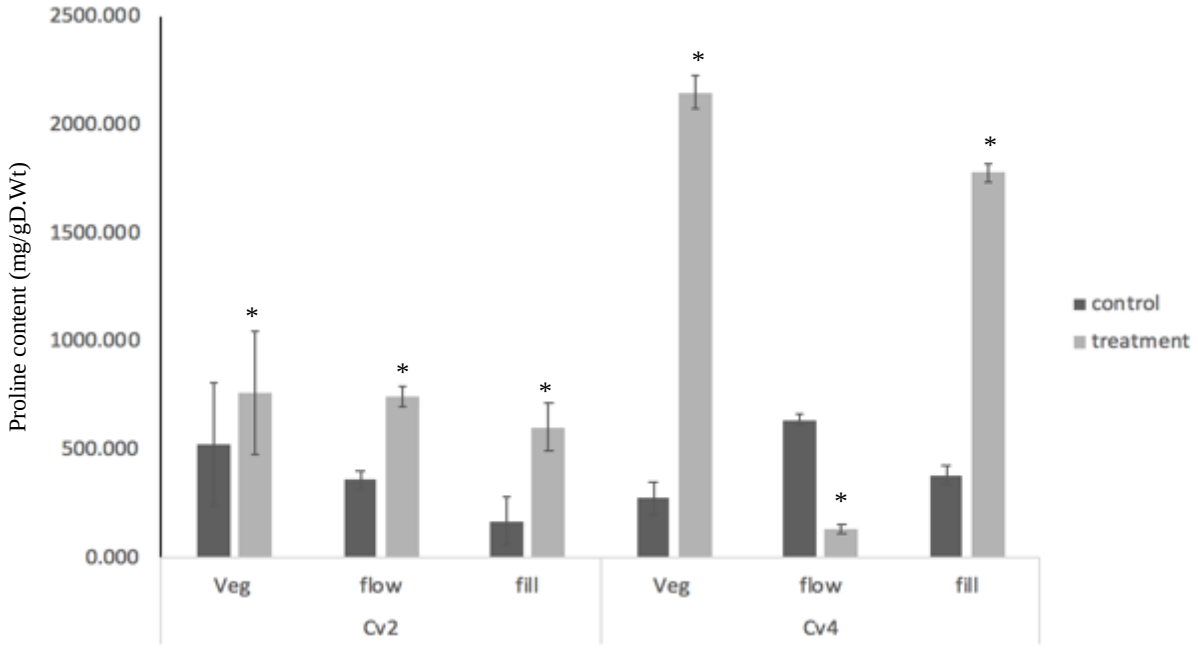


Figure 3. 7 Effect of water stress on Free proline content (mg/gDWt) in two wheat (*Triticum aestivum* L) cultivars: 193 Najran (Cv2); 377 Rafha (Cv4)) grown in different regions of Saudi Arabia at three growth stages: vegetative (Veg), flowering (flow) and grain filling (fill) subjected to 15% PEG6000 (treatment) n=4, bars are standard errors.

* The mean difference between (cultivars*stages * water_stress) is significant by F-test at the .05 level.

Soluble sugar content in shoots decreased significantly in both Cv2 and Cv4 at the flowering stage (71% and 65% respectively) compared to the control samples. However, there was a significantly increase at the grain filling stage of Cv4 ($p<0.000$) (30.6%) compared to the control samples (figure 3.8). There was no change in sugar content at the vegetative stage in both Cvs.

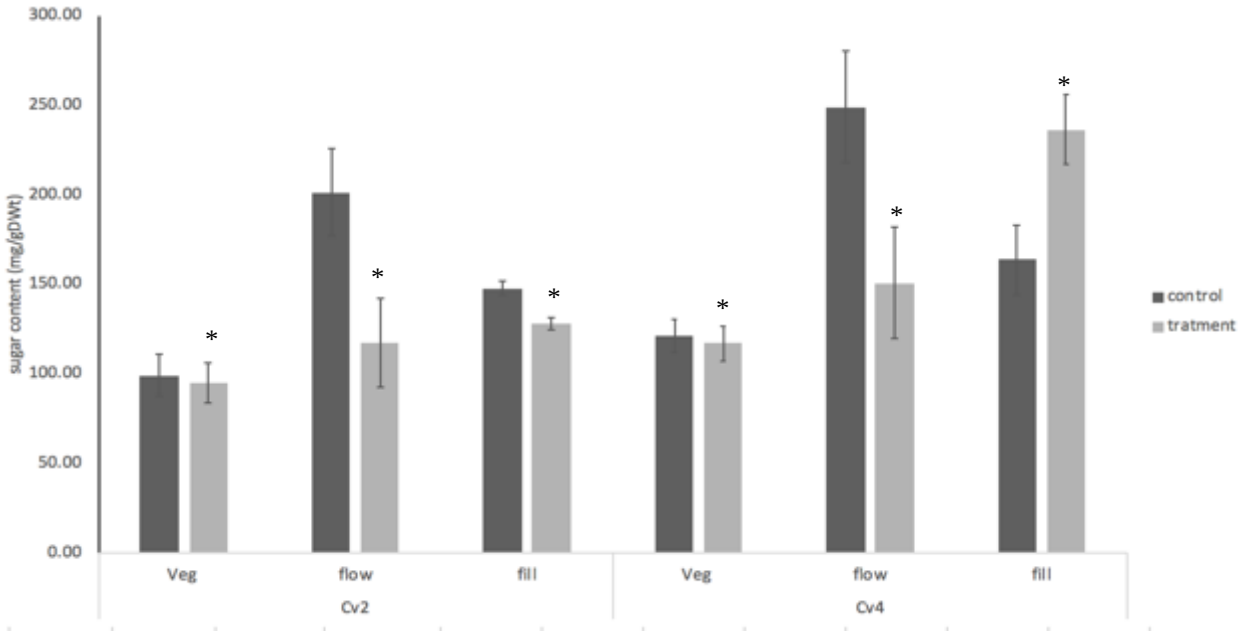


Figure 3. 8 Effect of water stress on Soluble sugar content (mg/gDWt) in two wheat (*Triticum aestivum* L). cultivars: 193 Najran (Cv2); 377 Rafha (Cv4) grown in different regions of Saudi Arabia at three growth stages: vegetative (Veg), flowering (flow) and grain filling (fill) subjected to 15% PEG6000 (treatment) n=4, bars are standard errors.

3.3.2. *RNA-seq analyses*

To understand the mechanism underpinning drought responses in Saudi wheat cultivars, RNA sequencing was carried out on12 RNA samples extracted from leaves harvested from control and PEG-treated plants from Cv2 (drought-tolerant) and Cv4 (drought-sensitive) at the vegetative, flowering and grain filling stages. The RNA sequencing yielded 24.2 GB of sequence clean read data, with at least 50 million reads of 85 bp in size for each sample (NCBI, Accession: PRJNA649099). All reads were mapped to wheat reference genome. The results showed that on average, about 36.5 to 60.9 -millions of clean reads could be mapped to the wheat reference genome (see table 3.3).

Total expressed genes in these samples were maximum 52,216,371 genes in PEG-treated Cv2 at flowering stage (Cv2 FlowPEG) and minimum 235,291 genes in PEG-treated Cv4 at vegetative stage (Cv4 VegPEG) (table 3.3).

Total expressed genes in these samples were maximum 52,216,371 genes in Cv2 PEG-treated at flowering stage (Cv2 FlowPEG) and minimum 235,291 genes in Cv4 PEG-treated at vegetative stage (Cv4 VegPEG) (table 3.3).

Table 3. 3 General statistics for 12 RNA sequencing data from two wheat Cultivars: 193 Najran (Cv2) and 377 Rafha (Cv4) under water-control and water-stress (15% PEG) conditions at three growth stages: vegetative, flowering and filling. M Seqs: total sequence per million, Length: average sequence length, %GC: average GC content per sample, %Dups: read duplication, DEGs: Differential Expression Gene.

Sample Name	**M Seqs**	**length (bp)**	**% GC**	**Unique reads**	**%Dups**	**Total expressed genes (No. reads)**	**Total DEGs**
Cv2Vegwater	40.7	75	43%	8,150,330	32560754	30,378,604.29	59205
Cv2VegPEG	36.5	76	45%	8888733	27618262	26,498,489.73	
Cv2Flowwater	36.5	76	46%	10616011	25858411	26,510,103.57	57790
Cv2FlowPEG	73.2	75	46%	17088306	56138353	52,216,771.56	
Cv2Fillwater	47.4	76	46%	12139329	35237110	32,238,157.75	51405
Cv2FillPEG	60.9	76	45%	13856162	47080770	44,012,731.72	
Cv4Vegwater	48.4	75	47%	9143054	39210269	33,026,893.93	64840
Cv4VegPEG	39.1	76	40%	1529874	37585087	235,516.9952	
Cv4Flowwater	45.9	76	45%	10745424	35184597	33,581,971.4	64759
Cv4FlowPEG	41.5	76	45%	11115161	30433944	29,597,794.3	
Cv4Fillwater	44.7	76	46%	12785644	31923565	32,428,510	4557
Cv4FillPEG	47.7	76	52%	19116587	28553227	22,477,251.67	

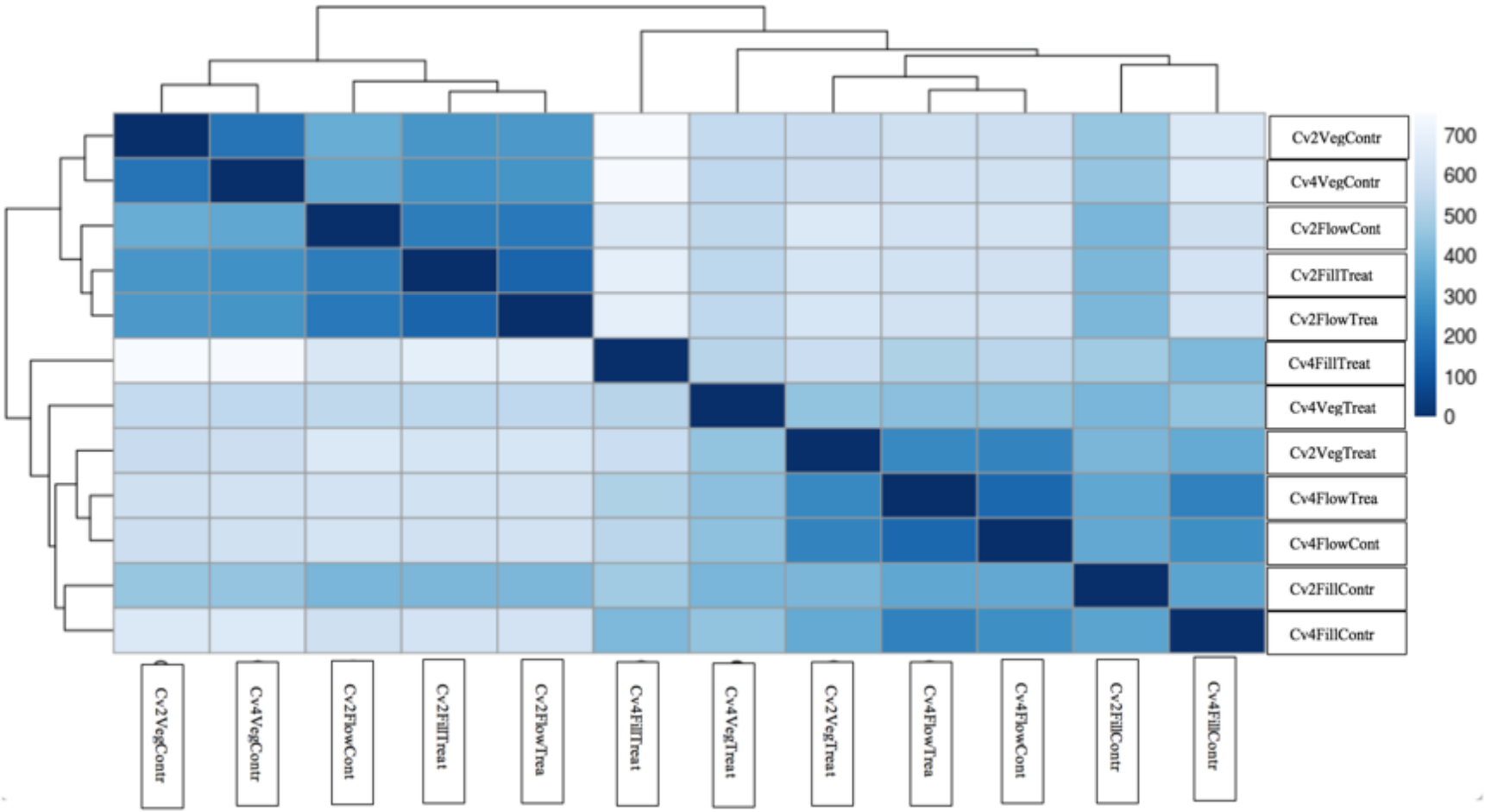


Figure 3. 9 The Hierarchical clustering (heat map), showing the sample-to-sample Euclidian distances based on normalized read count of samples under water stress at three growth stages. Cv4VegTret: Cv4 cultivar treated sample at Vegetative stage. Cv4VegContr: Cv4 cultivar water controlled sample at Vegetative stage. Cv4FlowTret: Cv4 cultivar treated sample at Flowering stage. Cv4FlowContr: Cv4 cultivar water controlled sample at Flowering stage. Cv4FillTret: Cv4 cultivar treated sample at Grain filling stage. Cv4FillContr: Cv4 cultivar water controlled sample at Grain filling stage

Figure 3. 10 Principal component analysis (PCA) of sample-to-sample Euclidian distances based on normalized read count of samples under water stress at three growth stages. Colour indicate the control (blue) and the 15%-PEG6000 treatment (red). Cv4VegTret: Cv4 cultivar treated sample at Vegetative stage. Cv4VegContr: Cv4 cultivar water controlled sample at Vegetative stage. Cv4FlowTret: Cv4 cultivar treated sample at Flowering stage. Cv4FlowContr: Cv4 cultivar water controlled sample at Flowering stage. Cv4FillTret: Cv4 cultivar treated sample at Grain filling stage. Cv4FillContr: Cv4 cultivar water controlled sample at Grain filling stage

3.3.3. *Clusterogram analysis of water-stress responses in the two wheat cultivars*

To explore the similarity and differences between RNAseq samples, we performed sample-level Principal Component Analysis (PCA) and hierarchical clustering methods. Principal component analysis showed the relationship between samples in the two wheat cultivars at three growth stages. Eighty- two percent of the total between-sample variance (figure 3.10, PC1 versus PC2) showed that the samples fell into two groups that were consistent with the results shown in the heat map. One of the groups was composed of Cv4Vegwater, Cv2Vegwater, Cv2FlowPEG, Cv2Flowwater and Cv2FillPEG, as shown on the left of the PCA plot, while Cv4VegPEG, Cv2VegPEG, Cv4Flowwater, Cv4FlowPEG, CV2Fillwater and Cv4Fillwater were located on the right of the plot. The heat map in figure 3.9 is a visualization of the sample-to-sample Euclidian distances based on normalized read count; it is consistent with the results of the PCA. These results indicate that most of the variation in gene expression among the two different cultivars is a consequence of the growth stage and water stress. This result is consistent with (Ma et al., 2017b) which found from PCA analysis that gene expression of commercial wheat varieties in the North China Plain were grouped into two groups according to their developmental stage and water stress under fileds condtions.

3.3.4. *Differentially Expressed Genes*

To identify the drought-responsive genes, the DEGs were analysed at different levels: water condition level, growth stage level and cultivar level with a p-value cut-off of 0.05 and Log2 fold change ≥ 2 as thresholds. At the water-condition level, in Cv2 (drought-resistant) 10,823 DEGs were revealed between the treatment and control samples, of which 8,491 genes were up-regulated, and 2,332 genes were down-regulated at the vegetative stage. At the flowering and grain filling stages 4888 and 8486 DEGs were revealed respectively, of which 29.4% and 15.02% of DEGs were up-regulated, and 70.5% and 84.7% of DEGs were down-regulated at the flowering and grain filling stages respectively (table 3.4, figure 3.11). The Venn diagram showed that 380 DEGs were overlapping or common to all DEGs lists between all growth stages of Cv2 under water stress (Figure 3.12).

Table 3. 4 The number of total DEGs, DEGs with fold change >2 or <-2 and up/down-regulated genes in a water-stress resistant, (Najran.Cv2) and water-stress sensitive (Rafha, Cv4) wheat cultivars under well-watered conditions (water) and water-stress conditions (PEG) at vegetative, flowering and flowering stages.

	Total DEGs	Up	Down	Total DEGs 2Log2 fold	Up	Down	Up_differences % ratio	Down_differences % ratio
Cv2 veg PEG vs. water	51405	25684	25721	10823	8491	2332	78.45	21.54
Cv2 Flow PEG vs. water	57790	26245	25160	4888	1441	3447	29.48	70.51
Cv2 fill PEG vs. water	59205	25996	25409	8486	1290	7196	15.20	84.79
Cv4 veg PEG vs. water	4557	2507	2048	1987	1339	649	67.38	32.66
Cv4 flow PEG vs. water	64759	31677	33080	8486	1359	1389	16.01	16.368
Cv4 fill PEG vs. water	64840	33465	31375	17328	8387	8942	48.4	51.60
Cv2 fill water vs. Cv2 Veg water	53677	21923	31753	10932	6685	4248	61.15	38.85
Cv2 flow water vs. Cv2 Veg water	51562	18658	32903	10530	5291	5239	50.24	49.75
Cv4 Fill water vs. Cv4 Veg water	51970	22906	29063	16507	9359	7149	56.69	43.3
Cv4 Flow water vs. Cv4 Veg water	52235	22814	29419	12632	8099	4533	64.11	35.88
Cv2 veg water vs. Cv4 veg water	52003	25684	26318	2823	963	1860	34.11	65.88
Cv2 Flow water vs. Cv4 Flow water	54130	22342	31786	15156	5367	9787	35.41	64.57
Cv2 Fill water vs. Cv4 Fill water	64325	25838	38487	13683	3393	10291	24.79	75.21

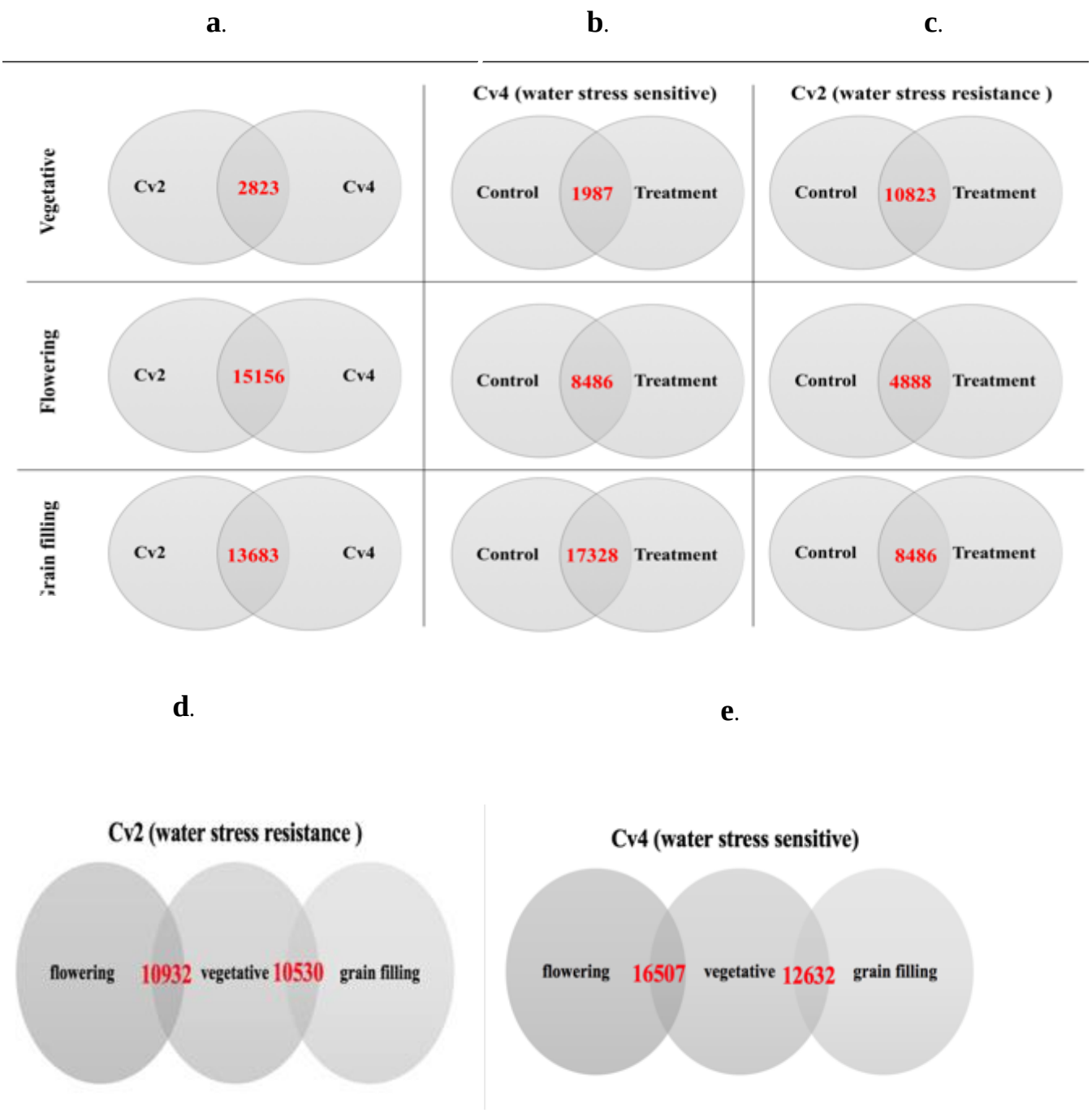


Figure 3. 11 Venn diagram representing the number of Differentially Expressed Genes in shoots at three growth stages which pass the fold change cut-off (fold change >2 or <-2 and False Discovery Rate-corrected p-value <0.05)) in 2 wheat cultivars: Najran (Cv2) and Rafha (Cv4). a. DEGs at between cultivars. b. DEGs between conditions in Cv4 r. c. DEGs between unstressed (water) and water-stress conditions (PEG) in Cv2 cultivar. d. DEGs between growth stages in Cv2. e. DEGs between growth stages in Cv4.

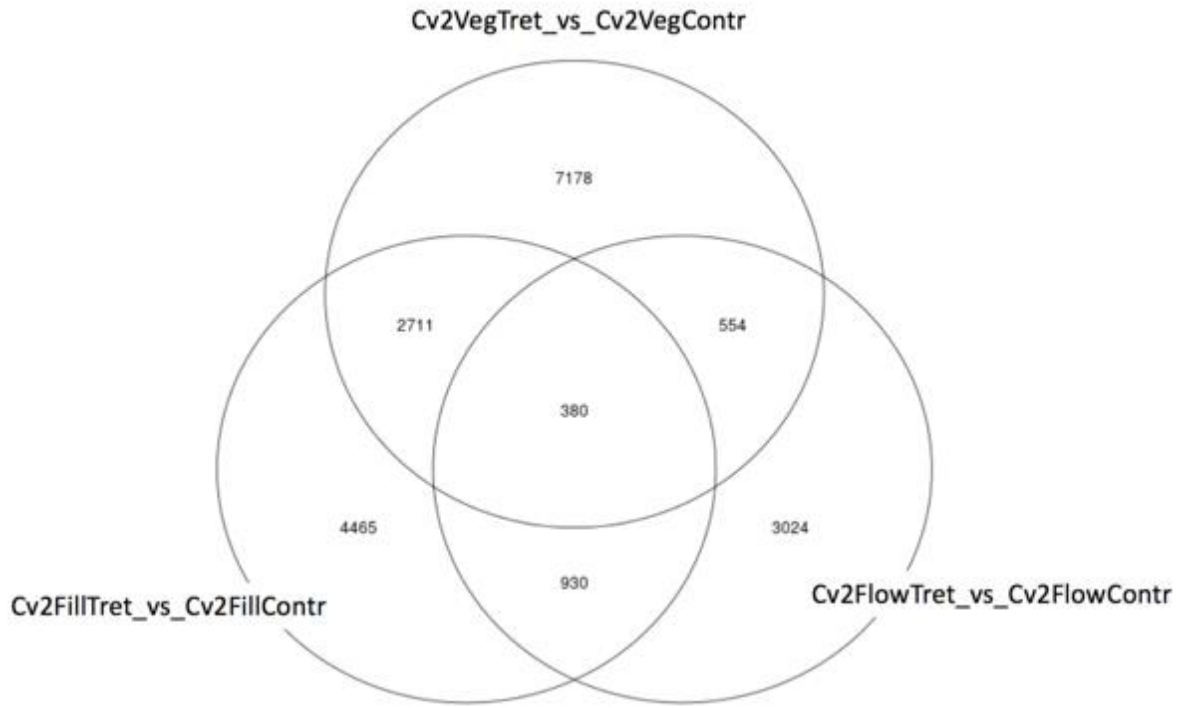


Figure 3. 12 Venn diagram representing overlaps between DEGs list of PEG-treated and water control plants of Najran wheat cultivar (Cv2) plants at three growth stages which pass the fold change cut-off (fold change >2 or <-2 and False Discovery Rate-corrected p-value (<0.05)). Cv2VegTret: Cv2 cultivar treated sample at Vegetative stage. Cv2VegContr: Cv2 cultivar water control sample at Vegetative stage. Cv2FlowTret: Cv2 cultivar treated sample at Flowering stage. Cv2FlowContr: Cv2 cultivar water control sample at Flowering stage. Cv2FillTret: Cv2 cultivar treated sample at Grain filling stage. Cv2FillContr: Cv2 cultivar water control sample at Grain filling stage.

In the Cv4, drought-sensitive cultivar, there were 1,987 DEGs, with 67.3% and 32.6% up-regulated and down-regulated genes respectively at the vegetative stage. Commonly 8,486 and 17,328 DEGs were indicated in the PEG-treated and control samples at the flowering and grain filling stages respectively, of which 16% and 48.4% were up-regulated, and 16.3% and 51.6% were down-regulated at the flowering and grain filling stages respectively (table 3.4, figure 3.11). The Venn diagram showed that 120 DEGs were overlapping between DEGs list of all growth stages of Cv4 under water stress (figure 3.13).

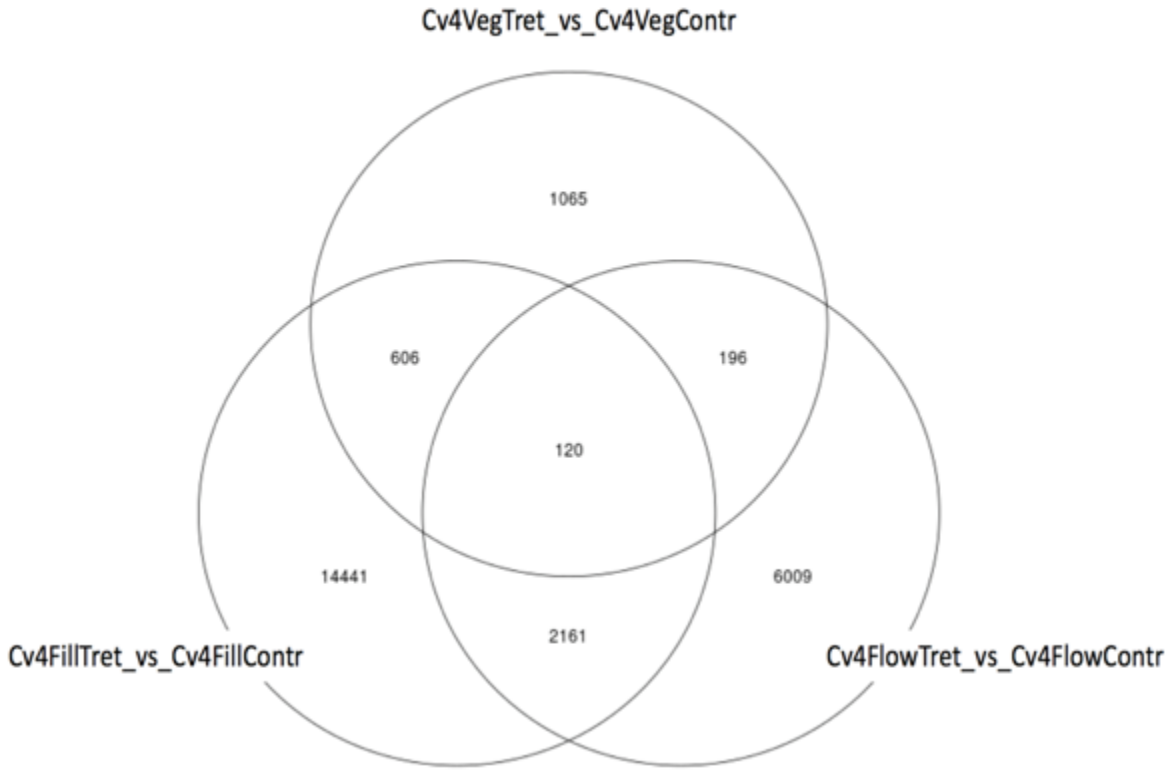
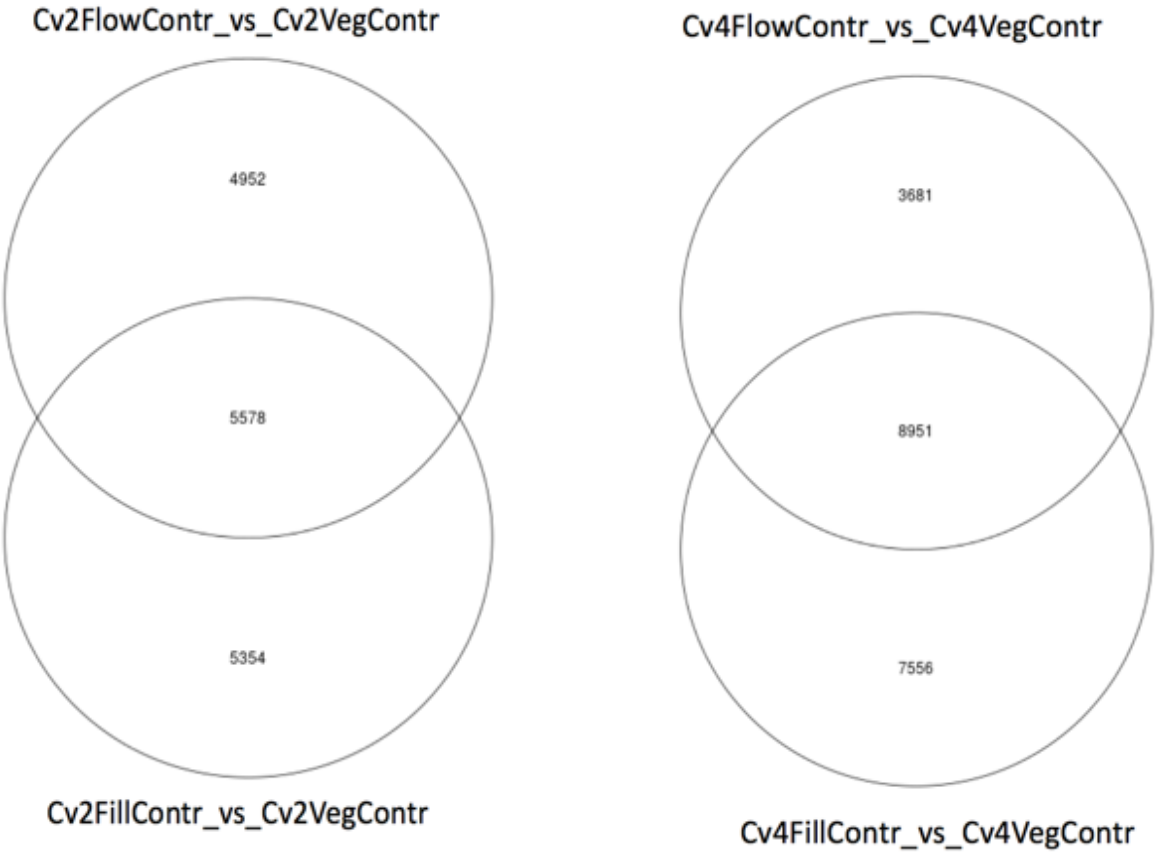


Figure 3. 13 Venn diagram representing overlaps between DEGs list of PEG-treated and water control plants of Rafha wheat cultivar (Cv4) at three growth stages which pass the fold change cut-off (fold change >2 or <-2 and False Discovery Rate-corrected p-value <0.05)). Cv4VegTret: Cv4 cultivar treated sample at Vegetative stage. Cv4VegContr: Cv4 cultivar water control sample at Vegetative stage. Cv4FlowTret: Cv4 cultivar treated sample at Flowering stage. Cv4FlowContr: Cv4 cultivar water control sample at Flowering stage. Cv4FillTret: Cv4 cultivar treated sample at Grain filling stage. Cv4FillContr: Cv4 cultivar water control sample at Grain filling stage.

Comparison between growth stages has shown 10,530 DEGs were indicated in the Cv2 at the flowering stage compared with the vegetative stage, of which 61.15% were up-regulated and 38.8% were down-regulated. 10,530 DEGs were indicated in Cv2 at the grain filling stage compared with the vegetative stage, of which 50.24% were up-regulated and 49.7% were down-regulated. Also, in the Cv4 flowering stage 16,507 DEGs, and 12,632 DEGs in the grain filling stage, were indicated compared with the vegetative stage, of which 56.7% (64.1%) were up-regulated and 43.3% (35.8%) were down-regulated at the flowering and grain filling stages respectively (table 3.4; figure 3.11). The Venn diagram showed that 5,578 DEGs were overlapping

between DEGs list of all growth stage of Cv2 in control conditions, and 8,951 DEGs were overlapping between all growth stages of Cv4 in control conditions (figure 3.14).



A. B.

Figure 3. 14 Venn diagram representing the number of Differentially Expressed Genes (DEGs) in shoots. A. Showing overlaps between DEGs lists in Najran wheat (Cv2) between flowering and vegetative growth stage and grain filling and vegetative growth stage. B. Showing overlaps between DEGs lists of in Rafha wheat (Cv4) between flowering and vegetative growth stage and grain filling and vegetative growth stage with fold change >2 or <-2 and FDR-corrected p-value <0.05).

At the cultivar level, compression was done between Cv2 (drought-resistant) and Cv4 (drought-sensitive) in control samples at each growth stage. 2,823 DEGs, 15,156 DEGs 13,683 DEGs were indicated at the vegetative, flowering and grain filling stages, respectively, of which approximately 35% DEGs were up-regulated and 70% DEGs were down-regulated (table 3.4, figure 3.11). The

Venn diagram showed that 313 DEGs were overlapping between DEGs list of both cultivars at all growth stages under control conditions (figure 3.15).

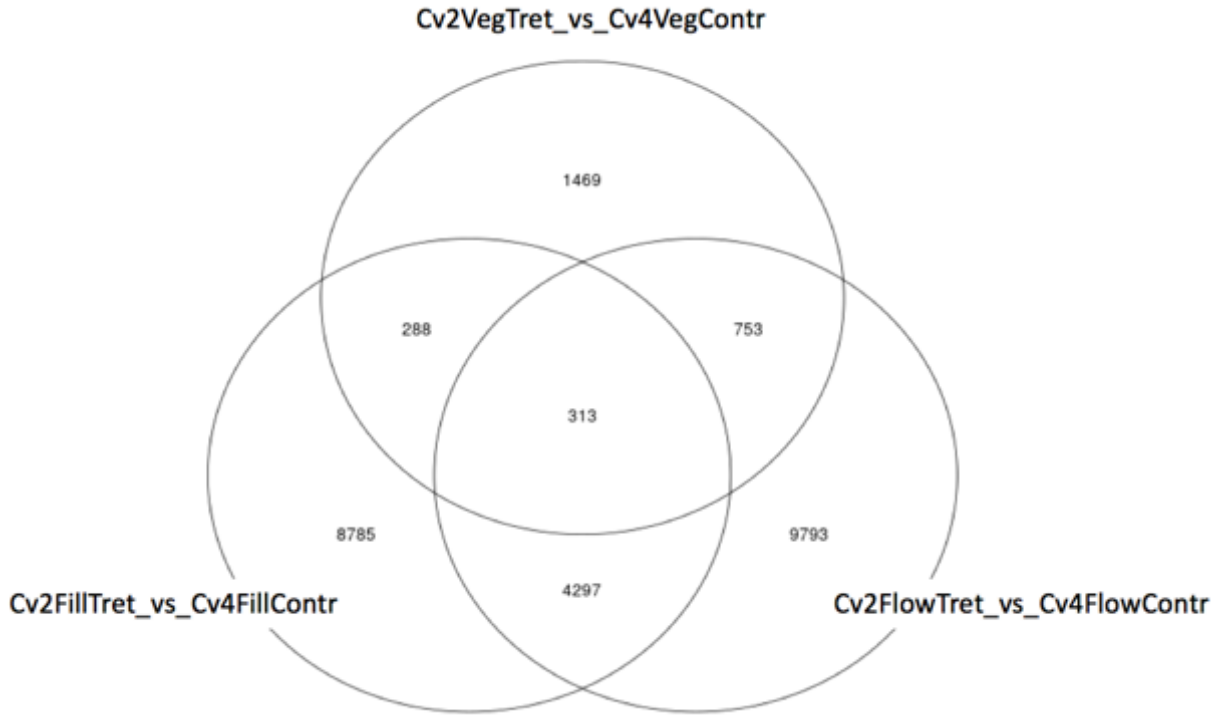


Figure 3. 15 Venn diagram representing the number of DEGs overlapped between DEGs lists of Rafha wheat (Cv4) cultivar and Najran wheat (Cv2) at three growth stages with fold change >2 or <-2 and FDR-corrected p-value (<0.05). Cv4VegTret: Cv4 cultivar treated sample at Vegetative stage. Cv4VegContr: Cv4 cultivar water control sample at Vegetative stage. Cv4FlowTret: Cv4 cultivar treated sample at Flowering stage. Cv4FlowContr: Cv4 cultivar water control sample at Flowering stage. Cv4FillTret: Cv4 cultivar treated sample at Grain filling stage. Cv4FillContr: Cv4 cultivar water control sample at Grain filling stage

3.3.5. *Gene Ontology (GO) Annotation of Differentially Expressed Genes (DEGs)*

The top 200 genes that were highly significantly regulated under water stress (<0.05) with fold change in most compressions >5 or <-5 were identified.

At the conditions level, it was found that the DEGs in Cv2 (drought-tolerant) at the vegetative stage that played a role in several molecular functions such as oxidoreductase activity (GO:0016702) and N,N-dimethylaniline monooxygenase activity (GO:0004499), which are part of the oxidation reduction process, were highly up-regulated under water stress. Also it was found that DEGs involved in deviance responses such as to bacterium (GO:0042742), to fungus (GO:0050832) and to other organism (GO:0051707), in addition to the carbohydrate metabolic process (GO:0005975) and the phenylpropanoid metabolic process (GO:0009698) were significantly up-regulated (figure 3.16).

BP GO term	Description
GO:0042742	defense response to bacterium
GO:0050832	defense response to fungus
GO:0051707	response to other organism
GO:0009605	response to external stimulus
GO:0009607	response to biotic stimulus
GO:0006952	defense response
GO:0007131	reciprocal meiotic recombination
GO:0061982	meiosis I cell cycle process
GO:0005975	carbohydrate metabolic process
GO:0009698	phenylpropanoid metabolic process
GO:0046274	lignin catabolic process
GO:0036211	protein modification process
GO:0006468	protein phosphorylation
GO:0006796	phosphate-containing compound metabolic process
GO:0055114	oxidation-reduction process
GO:0007264	small GTPase mediated signal transduction
GO:0016180	snRNA processing
GO:0034477	U6 snRNA 3'-end processing
GO:0008612	peptidyl-lysine modification to peptidyl-hypusine

CC GO term	Description
GO:0005576	extracellular region
GO:0016020	membrane

MF GO term	Description
GO:0016702	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen
GO:0004499	N,N-dimethylaniline monooxygenase activity
GO:0004867	serine-type endopeptidase inhibitor activity
GO:0008238	exopeptidase activity
GO:0030247	polysaccharide binding
GO:0004497	monooxygenase activity
GO:0052716	hydroquinone:oxygen oxidoreductase activity
GO:0005524	ATP binding
GO:0043168	anion binding
GO:0004672	protein kinase activity
GO:0016772	transferase activity, transferring phosphorus-containing groups
GO:0030554	adenyl nucleotide binding
GO:0016701	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen
GO:0097367	carbohydrate derivative binding
GO:0003824	catalytic activity
GO:0032555	purine ribonucleotide binding
GO:0003991	acetylglutamate kinase activity
GO:0060089	molecular transducer activity
GO:0000166	nucleotide binding
GO:0004857	enzyme inhibitor activity
GO:0030246	carbohydrate binding
GO:0097159	organic cyclic compound binding
GO:1901363	heterocyclic compound binding
GO:0004888	transmembrane signaling receptor activity
GO:0004970	ionotropic glutamate receptor activity
GO:0005230	extracellular ligand-gated ion channel activity
GO:0022835	transmitter-gated channel activity
GO:0005216	ion channel activity
GO:0015267	channel activity
GO:0022839	ion gated channel activity
GO:0004518	nuclease activity
GO:0020037	heme binding
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0043531	ADP binding
GO:0004521	endoribonuclease activity
GO:0005506	iron ion binding
GO:0016894	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 3'-phosphomonoesters
GO:0033897	ribonuclease T2 activity

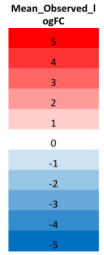


Figure 3. 16 Significantly enriched GO terms among the DEGs between 15%-PEG6000 treatmentvs. control sample (at conditions level) samples in Najran wheat (Cv2) at vegetative stage. Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

In the flowering stage, up-regulated DEGs were mainly involved in oligopeptide transport (GO:0006857). However, DEGs involved in carbohydrate processes such as the glucosamine-containing compound metabolic process (GO:1901071), the aminoglycan metabolic process (GO:0006022) and the one-carbon metabolic process (GO:0006730) were significantly down-regulated under water stress, in addition to DEGs involved in the L-phenylalanine catabolic process (GO:0006559), the L-phenylalanine biosynthetic process (GO:0009094) and the phenylpropanoid metabolic process (GO:0009698) (figure 3.17).

In the grain filling stage, water stress up-regulated DEGs that enabled RNA-DNA hybrid ribonuclease activity (GO:0004523) and hydroquinone:oxygen oxidoreductase activity (GO:0052716), which is part of the oxidation reduction process. In addition, the DEGs involved in the phenylpropanoid metabolic process (GO:0009698) and the lignin catabolic process (GO:0046274) were up-regulated. It was found that DEGs involved in response to water stress (GO:0009415) and the alpha-amino acid catabolic process (GO:1901606) were down-regulated under water stress, as were DEGs that were mainly part of the apoplast (GO:0048046) (figure 3.18).

In Cv4 (at conditions level) in the vegetative stage, water stress significantly up-regulated DEGs that were involved in the response to oxidative stress (GO:0006979) and some drug metabolic processes such as the drug catabolic process (GO:0042737), the regulation of neurotransmitter levels (GO:0001505) and the glycine metabolic process (GO:0006544). In contrast, water stress significantly down-regulated DEGs that were mainly involved in photosynthesis (GO:0015979) and carbon fixation (GO:0015977), and some other molecular functions regulated under water stress, as shown in figure 3.19.

In Cv4 at flowering (at condition level) it was found that water stress significantly up-regulated DEGs involved in the disaccharide metabolic process (GO:0005984) and the trehalose biosynthetic process (GO:0005992), which are involved in the carbohydrate metabolic process. However, the majority of DEGs that regulate the carbohydrate metabolic process were significantly down-regulated, such as those involved in the polysaccharide metabolic process (GO:0005976) and the sucrose metabolic process (GO:0005985), which is a consequence of significant down-regulation in DEGs involved in photosynthesis such as in the chlorophyll catabolic process (GO:0015996) and the tetrapyrrole catabolic process (GO:0033015), in addition to molecular functions that

enabled chlorophyllase activity (GO:0047746), sucrose alpha-glucosidase activity (GO:0004575) and glucosidase activity (GO:0015926). In addition, water stress significantly up-regulated molecular functions that enable inhibitor enzyme activity (GO:0004857) and protein kinase activity (GO:0004672) (figure 3.20).

In Cv4 at the grain filling stage (at conditions level), water stress significantly up-regulated DEGs involved in the response to water (GO:0009415) and wounding (GO:0009611), in addition to DEGS involved in the L-phenylalanine biosynthetic process (GO:0009094), the L-phenylalanine catabolic process (GO:0006559) and the proline biosynthetic process (GO:0006561), which is consistent with the increased proline content in Cv4 shoots at the grain filling stage under stress. In contrast, water stress down-regulated DEGs involved in glycine decarboxylation via the glycine cleavage system (GO:0019464), the chlorophyll biosynthetic process (GO:0015995) and protein stabilisation (GO:0050821), consistent with the observed decrease in protein content in Cv4 shoots (figure 3.21).

BP GO term	Description
GO:0006857	oligopeptide transport
GO:0009312	oligosaccharide biosynthetic process
GO:0005984	disaccharide metabolic process
GO:0032501	multicellular organismal process
GO:0051704	multi-organism process
GO:0000003	reproduction
GO:0034637	cellular carbohydrate biosynthetic process
GO:0009698	phenylpropanoid metabolic process
GO:0046274	lignin catabolic process
GO:0009856	pollination
GO:0048544	recognition of pollen
GO:0009094	L-phenylalanine biosynthetic process
GO:0005975	carbohydrate metabolic process
GO:0006576	cellular biogenic amine metabolic process
GO:1901607	alpha-amino acid biosynthetic process
GO:0050896	response to stimulus
GO:0006855	drug transmembrane transport
GO:0042493	response to drug
GO:0009832	plant-type cell wall biogenesis
GO:0010215	cellulose microfibril organization
GO:0016049	cell growth
GO:0043062	extracellular structure organization
GO:0070726	cell wall assembly
GO:0009073	aromatic amino acid family biosynthetic process
GO:0071554	cell wall organization or biogenesis
GO:0043436	oxoacid metabolic process
GO:0005976	polysaccharide metabolic process
GO:0042742	defense response to bacterium
GO:0050832	defense response to fungus
GO:0051707	response to other organism
GO:0055114	oxidation-reduction process
GO:0009309	amine biosynthetic process
GO:0005986	sucrose biosynthetic process
GO:0006596	polyamine biosynthetic process
GO:0006952	defense response
GO:0006979	response to oxidative stress
GO:0009607	response to biotic stimulus
GO:0017144	drug metabolic process
GO:0006527	arginine catabolic process
GO:1901361	organic cyclic compound catabolic process
GO:0019439	aromatic compound catabolic process
GO:1902221	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process
GO:0009072	aromatic amino acid family metabolic process
GO:0006422	aspartyl-tRNA aminoacylation
GO:1901606	alpha-amino acid catabolic process
GO:0046395	carboxylic acid catabolic process
GO:0044282	small molecule catabolic process
GO:0006559	L-phenylalanine catabolic process
GO:0006032	chitin catabolic process
GO:0016998	cell wall macromolecule catabolic process
GO:0046348	amino sugar catabolic process
GO:1901071	glucosamine-containing compound metabolic process
GO:0006022	aminoglycan metabolic process
GO:0006730	one-carbon metabolic process

CC GO term	Description
GO:0005885	Arp2/3 protein complex
GO:0048046	apoplast
GO:0031225	anchored component of membrane
GO:0000015	phosphopyruvate hydratase complex
GO:0005576	extracellular region

Mean_Observed_logFC
5
4
3
2
1
0
-1
-2
-3
-4
-5

MF GO term	Description
GO:0030247	polysaccharide binding
GO:0004674	protein serine/threonine kinase activity
GO:0016597	amino acid binding
GO:0043177	organic acid binding
GO:0052716	hydroquinone:oxygen oxidoreductase activity
GO:0016679	oxidoreductase activity, acting on diphenols and related substances as donors
GO:0016835	carbon-oxygen lyase activity
GO:0016740	transferase activity
GO:0004664	prephenate dehydratase activity
GO:0015297	antiporter activity
GO:0016747	transferase activity, transferring acyl groups other than amino-acyl groups
GO:0015238	drug transmembrane transporter activity
GO:0046872	metal ion binding
GO:0005507	copper ion binding
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0043565	sequence-specific DNA binding
GO:0005506	iron ion binding
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxyge
GO:0020037	heme binding
GO:0004634	phosphopyruvate hydratase activity
GO:0016491	oxidoreductase activity
GO:0050307	sucrose-phosphate phosphatase activity
GO:0004601	peroxidase activity
GO:0016758	transferase activity, transferring hexosyl groups
GO:0008792	arginine decarboxylase activity
GO:0003700	DNA-binding transcription factor activity
GO:0008061	chitin binding
GO:0010181	FMN binding
GO:0016829	lyase activity
GO:0004575	sucrose alpha-glucosidase activity
GO:0008725	DNA-3-methyladenine glycosylase activity
GO:0003905	alkylbase DNA N-glycosylase activity
GO:0004815	aspartate-tRNA ligase activity
GO:0004089	carbonate dehydratase activity
GO:0045735	nutrient reservoir activity
GO:0016841	ammonia-lyase activity
GO:0004568	chitinase activity
GO:0030145	manganese ion binding

Figure 3. 17 Significantly enriched GO terms among the DEGs between 15%-PEG6000 treatment vs. control sample (at conditions level) samples in Najran wheat (Cv2) at flowering stage. Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

BP GO term	Description
GO:0009698	phenylpropanoid metabolic process
GO:0046274	lignin catabolic process
GO:0019953	sexual reproduction
GO:0051103	DNA ligation involved in DNA repair
GO:0071897	DNA biosynthetic process
GO:0009856	pollination
GO:0048544	recognition of pollen
GO:0051704	multi-organism process
GO:0032501	multicellular organismal process
GO:0000003	reproduction
GO:0019439	aromatic compound catabolic process
GO:1901361	organic cyclic compound catabolic process
GO:0071554	cell wall organization or biogenesis
GO:0055114	oxidation-reduction process
GO:0005976	polysaccharide metabolic process
GO:0006073	cellular glucan metabolic process
GO:0044262	cellular carbohydrate metabolic process
GO:0006468	protein phosphorylation
GO:0030244	cellulose biosynthetic process
GO:0006355	regulation of transcription, DNA-templated
GO:0019219	regulation of nucleobase-containing compound metabolic process
GO:2001141	regulation of RNA biosynthetic process
GO:0006536	glutamate metabolic process
GO:0042221	response to chemical
GO:0006487	protein N-linked glycosylation
GO:1902221	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic
GO:0009072	aromatic amino acid family metabolic process
GO:0072488	ammonium transmembrane transport
GO:0070588	calcium ion transmembrane transport
GO:0006559	L-phenylalanine catabolic process
GO:0046395	carboxylic acid catabolic process
GO:1901606	alpha-amino acid catabolic process
GO:0044282	small molecule catabolic process
GO:0009415	response to water

CC GO term	Description
GO:0048046	apoplast
GO:0005618	cell wall
GO:0000015	phosphopyruvate hydratase complex
GO:0016020	membrane

MF GO term	Description
GO:0004523	RNA-DNA hybrid ribonuclease activity
GO:0052716	hydroquinone:oxygen oxidoreductase activity
GO:0005507	copper ion binding
GO:0003910	DNA ligase (ATP) activity
GO:0016886	ligase activity, forming phosphoric ester bonds
GO:0016679	oxidoreductase activity, acting on diphenols and related substances as donors
GO:0004674	protein serine/threonine kinase activity
GO:0045735	nutrient reservoir activity
GO:0046527	glucosyltransferase activity
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0005315	inorganic phosphate transmembrane transporter activity
GO:0016757	transferase activity, transferring glycosyl groups
GO:0005506	iron ion binding
GO:0030247	polysaccharide binding
GO:0046914	transition metal ion binding
GO:0016829	lyase activity
GO:0043169	cation binding
GO:0003824	catalytic activity
GO:0016301	kinase activity
GO:0016773	phosphotransferase activity, alcohol group as acceptor
GO:0016844	strictosidine synthase activity
GO:0016760	cellulose synthase (UDP-forming) activity
GO:0043565	sequence-specific DNA binding
GO:0003700	DNA-binding transcription factor activity
GO:0008519	ammonium transmembrane transporter activity
GO:0005388	calcium-transporting ATPase activity
GO:0016841	ammonia-lyase activity
GO:0004402	histone acetyltransferase activity
GO:0034212	peptide N-acetyltransferase activity
GO:0003830	beta-1,4-mannosylglycoprotein 4-beta-N-acetylglucosaminyltransferase activity
GO:0008409	5'-3' exonuclease activity

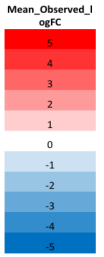


Figure 3. 18 Significantly enriched GO terms among the DEGs between 15%-PEG6000 treatment and control samples (at conditions level) in Najran wheat (Cv2) at grain filling stage. Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

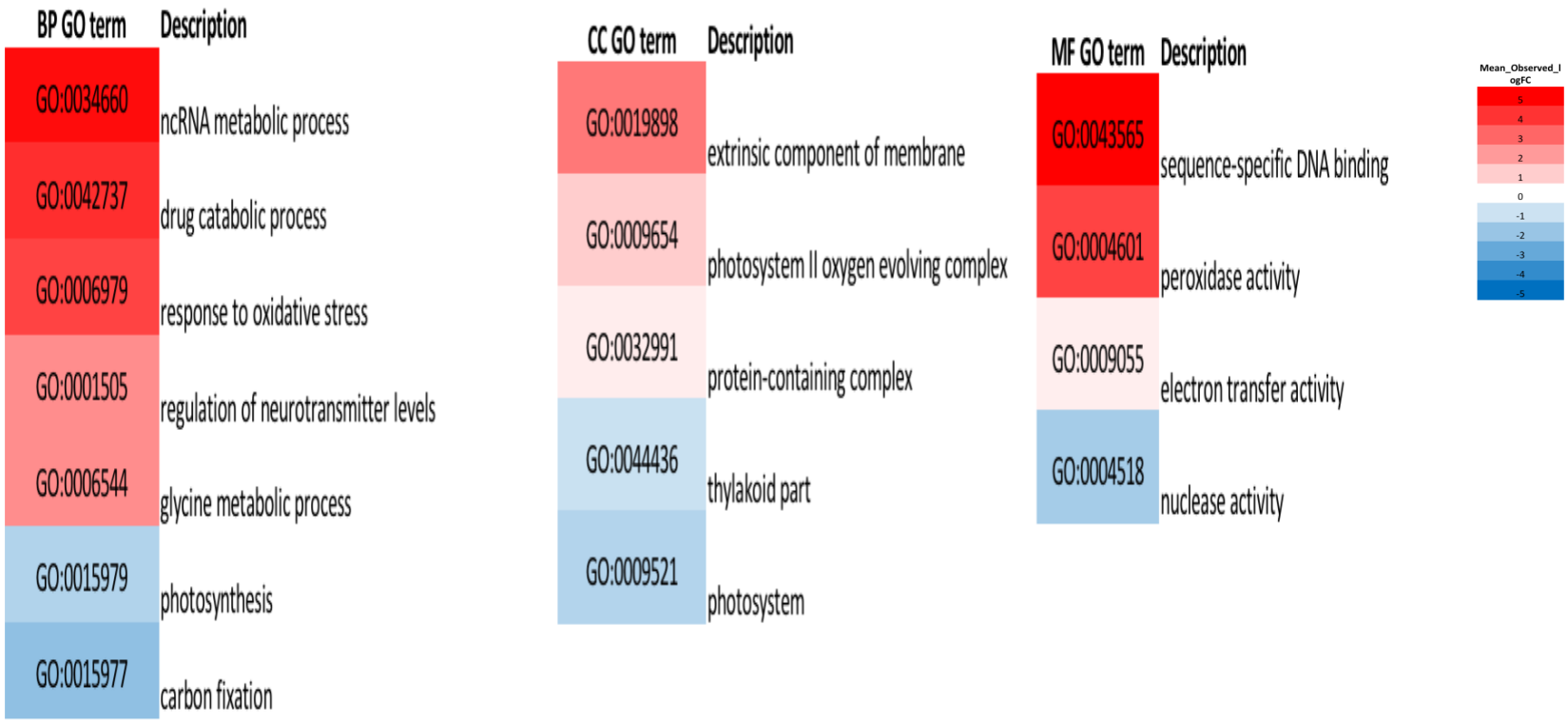


Figure 3. 19 Significantly enriched GO terms among the DEGs between 15%-PEG6000 treatment vs. control sample (at conditions level) samples in Rafha wheat (Cv4) at vegetative stage. Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

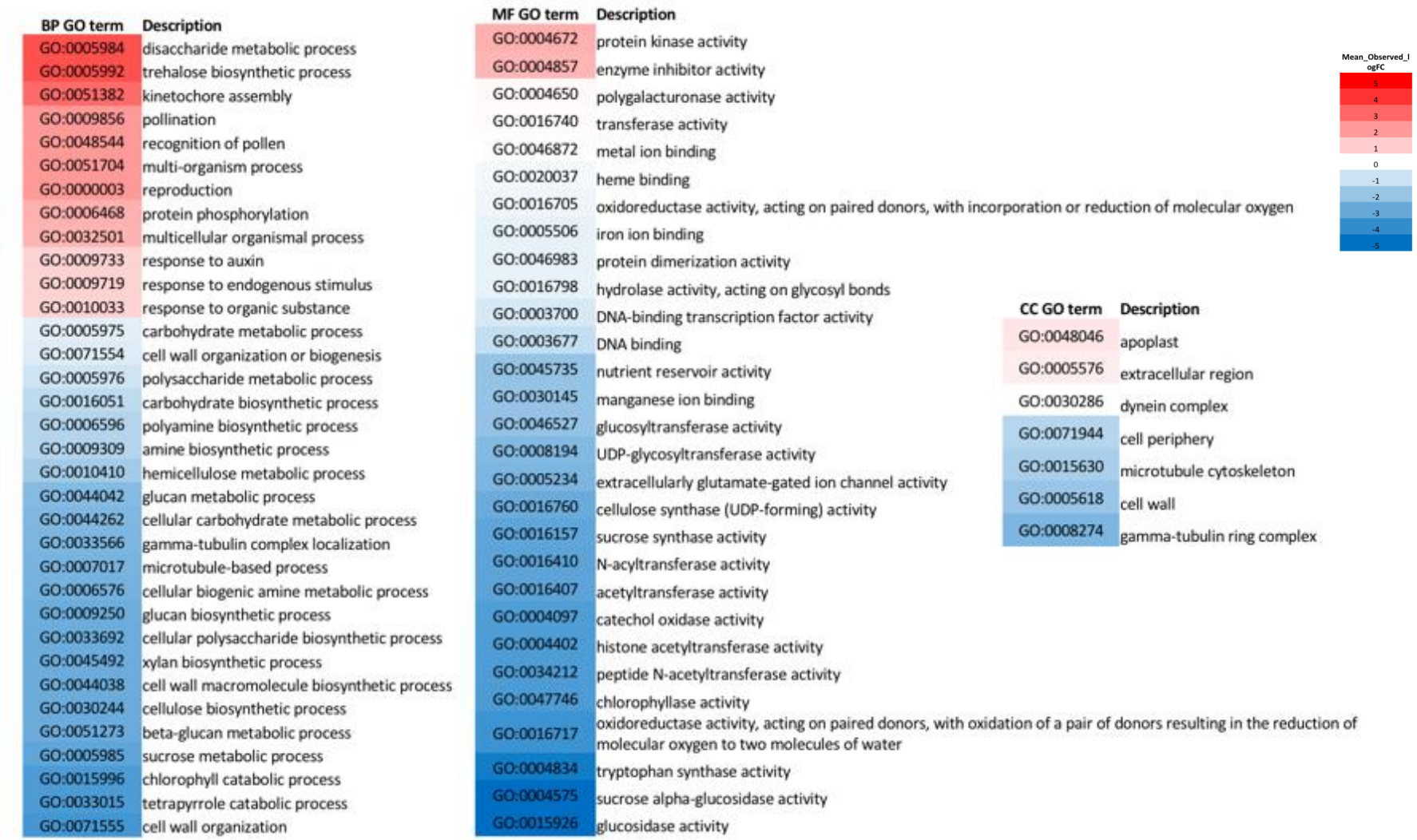


Figure 3. 20 significantly enriched GO terms among the DEGs between 15%-PEG6000 treatment and control samples in Rafha wheat (Cv4) at flowering stage. Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

Comparison between growth stage resulted in the following. In the flowering stage vs. the vegetative stage samples of Cv2, DEGs involved in plant deviance such as the response to wounding (GO:0009611), bacterium (GO:0042742), fungus (GO:0050832) and response to water (GO:0009415), were significantly up regulated. Also up-regulated were DEGs involved in the proline biosynthetic process (GO:0006561), the aromatic compound catabolic process (GO:0019439) and the L-phenylalanine catabolic process (GO:0006559). DEGs that enabled phosphopyruvate hydratase activity (GO:0004634), ammonia-lyase activity (GO:0016841) and oxidoreductase activity (GO:0016702) were also up-regulated. In addition, DEGs mainly related to the flowering stages such as pollination (GO:0009856), recognition of pollen (GO:0048544) and reproduction (GO:0000003) were up regulated. However, DEGs involved in carbohydrate processes such as the cellular polysaccharide catabolic process (GO:0044247) and the glucan catabolic process (GO:0009251) were significantly down-regulated (figure 3.22).

In the grain filling stage vs. the vegetative stage in Cv2 plants, it was found that the highly up-regulated DEGs were those involved in the response to water (GO:0009415), the L-phenylalanine catabolic process (GO:0006559), the erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process (GO:1902221) and the phenylpropanoid metabolic process (GO:0009698), along with DEGs that enabled oxidoreductase activity (GO:0016702) and phosphopyruvate hydratase activity (GO:0004634). DEGs that were involved in and which enabled the xyloglucan metabolic process (GO:0010411) and xyloglucan:xyloglucosyl transferase activity (GO:0016762) were also down-regulated (figure 3.23).

Figure 3. 21 Significantly enriched GO terms among the DEGs between 15%-PEG6000 treatment vs. control sample (at conditions level) samples in Rafha wheat (Cv4) at grain filling stage. Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

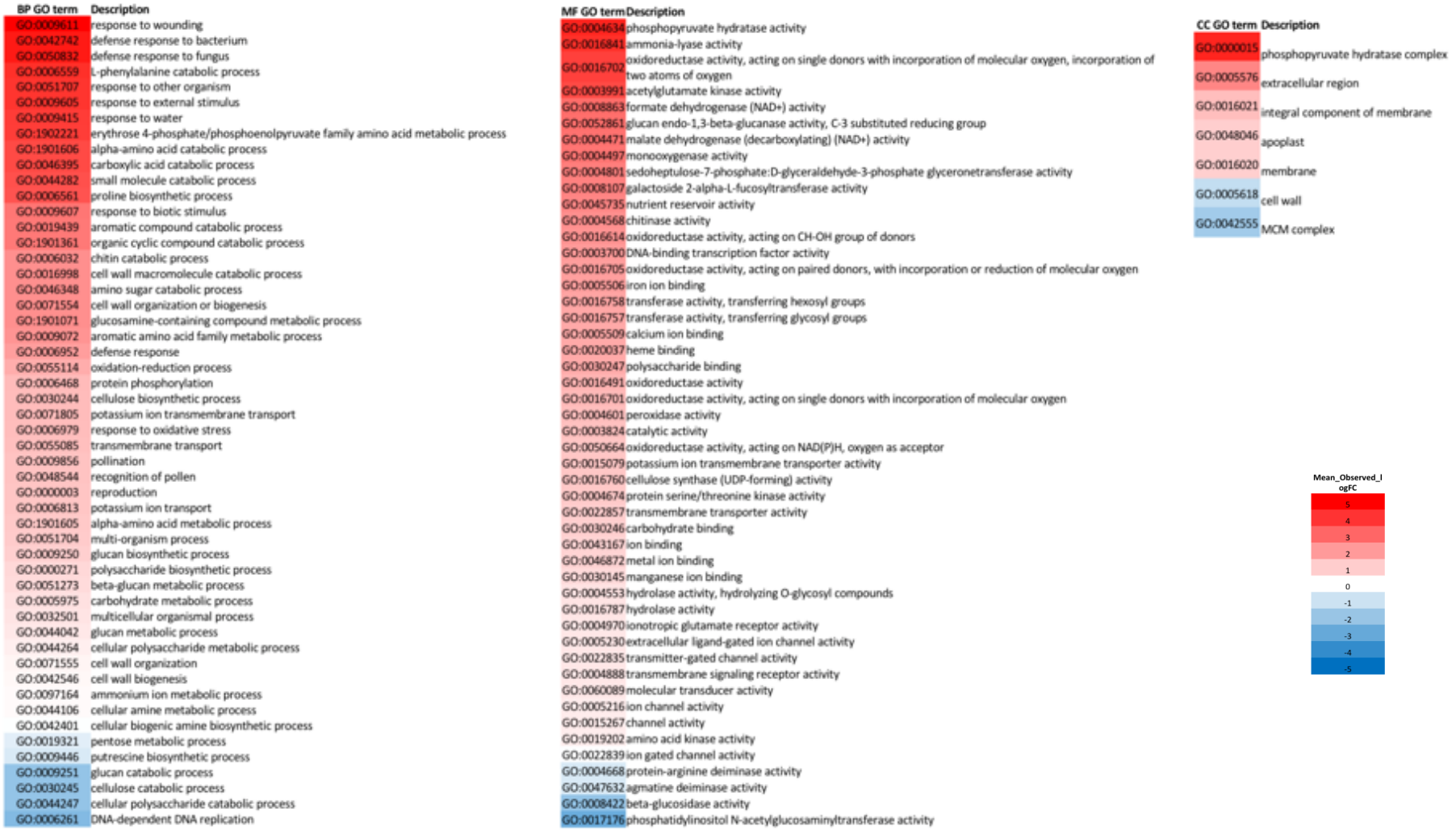


Figure 3. 22 significantly enriched GO terms among the DEGs at flowering stage vs. vegetative stage in Najran wheat (Cv2) samples (at stages level). Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

Figure. 3.22

Figure 3. 23 Significantly enriched GO terms among the DEGs at grain filling stage vs. vegetative stage in Najran wheat (Cv2) samples (at stages level). Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

Figure 3. 24 Significantly enriched GO terms among the DEGs at flowering stage vs. vegetative stage in Rafha wheat (Cv4) sample (At stages level). Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

Flowering stage vs. vegetative stage plants in Cv4: it was found that up-regulated DEGs were involved in the sucrose biosynthetic process (GO:0005986) and DEGs involved in the growth stage were up regulated, such as those involved in pollination (GO:0009856), the recognition of pollen (GO:0048544) and reproduction (GO:0000003). It was also found that DEGs involved in the phenylpropanoid metabolic process (GO:0009698), the proline biosynthetic process (GO:0006561) and the xyloglucan metabolic process (GO:0010411) were down-regulated, as were DEGs that enabled phosphoglycerate kinase activity (GO:0004618) and xyloglucan:xyloglucosyl transferase activity (GO:0016762) (figure 3.24). In grain filling stage vs. vegetative stage in Cv4 : DEGs involved in the L-phenylalanine catabolic process (GO:0006559) and the phenylpropanoid metabolic process (GO:0009698) were up-regulated. However, those involved in the cellular polysaccharide biosynthetic process (GO:0033692) and the xylan biosynthetic process (GO:0045492) were down-regulated, as were the DEGs that enabled homoserine dehydrogenase activity (GO:0004412) and the structural constituents of the cytoskeleton (GO:0005200) (figure 3.25).

DEGs between Cv2 vegetative stage and Cv4 vegetative stage (at cultivar level): DEGs mainly involved in the cell wall macromolecule metabolic process (GO:0044036) were up-regulated, as were the DEGs that enabled xyloglucan: xyloglucosyl transferase activity (GO:0016762). Moreover, the DEGs involved in the response to water (GO:0009415), the L-phenylalanine catabolic process (GO:0006559), the phenylpropanoid metabolic process (GO:0009698) and the lignin catabolic process (GO:0046274) were down-regulated, as were those that enabled hydroquinone: oxygen oxidoreductase activity (GO:0052716) and oxidoreductase activity (GO:0016679) (figure. 3.26).

In Cv2 flowering stage vs. Cv4 flowering stage: it was found that the DEGs involved in the response to water (GO:0009415), the phenylpropanoid metabolic process (GO:0009698), the lignin catabolic process (GO:0046274), the L-phenylalanine biosynthetic process (GO:0009094) and the thiamine metabolic process (GO:0006772), and DEGs that enabled phosphopyruvate hydratase activity (GO:0004634) and nutrient reservoir activity (GO:0045735) were up- regulated. However, the DEGs involved in the disaccharide metabolic process (GO:0005984) and the pyrimidine-containing compound biosynthetic process (GO:0072528) and those that enabled polysaccharide binding (GO:0030247) and alternative oxidase activity (GO:0009916) were down-regulated (figure. 3.27).

In Cv2 grain filling stage vs. Cv4 grain filling stage: it was found that DEGs involved in the response to water (GO:0009415) and carbohydrate metabolic processes such as the cellulose biosynthetic process (GO:0030244), the polysaccharide biosynthetic process (GO:0000271) and the beta-glucan metabolic process (GO:0051273) were highly up-regulated, as were those that enabled sucrose alpha-glucosidase activity (GO:0004575) and glucosyltransferase activity (GO:0046527). On the other hand, DEGs involved in the L-phenylalanine metabolic process (GO:0006558), the arginine catabolic process (GO:0006527) and protein ubiquitination (GO:0016567) were down-regulated (figure. 3.28).

BP GO term	Description
GO:0006559	L-phenylalanine catabolic process
GO:0009856	pollination
GO:0048544	recognition of pollen
GO:0051704	multi-organism process
GO:0000003	reproduction
GO:0006468	protein phosphorylation
GO:0036211	protein modification process
GO:0009698	phenylpropanoid metabolic process
GO:0046274	lignin catabolic process
GO:0032501	multicellular organismal process
GO:0006869	lipid transport
GO:0005975	carbohydrate metabolic process
GO:0044036	cell wall macromolecule metabolic process
GO:0042546	cell wall biogenesis
GO:0044262	cellular carbohydrate metabolic process
GO:0016051	carbohydrate biosynthetic process
GO:0051273	beta-glucan metabolic process
GO:0030244	cellulose biosynthetic process
GO:0044042	glucan metabolic process
GO:0009250	glucan biosynthetic process
GO:0009832	plant-type cell wall biogenesis
GO:0010215	cellulose microfibril organization
GO:0016049	cell growth
GO:0043062	extracellular structure organization
GO:0070726	cell wall assembly
GO:0033692	cellular polysaccharide biosynthetic process
GO:0045492	xylan biosynthetic process

CC GO term	Description
GO:0005576	extracellular region
GO:0016602	CCAAT-binding factor complex
GO:0000145	exocyst
GO:0005938	cell cortex
GO:0044445	cytosolic part
GO:0048046	apoplast
GO:0000439	transcription factor TFIIH core complex
GO:0031225	anchored component of membrane
GO:0005840	ribosome

MF GO term	Description
GO:0008061	chitin binding
GO:0016841	ammonia-lyase activity
GO:0030247	polysaccharide binding
GO:0005507	copper ion binding
GO:0016679	oxidoreductase activity, acting on diphenols and related substances as donors
GO:0004970	ionotropic glutamate receptor activity
GO:0005230	extracellular ligand-gated ion channel activity
GO:0022835	transmitter-gated channel activity
GO:0004888	transmembrane signaling receptor activity
GO:0004674	protein serine/threonine kinase activity
GO:0004672	protein kinase activity
GO:0004427	inorganic diphosphatase activity
GO:0008144	drug binding
GO:0052716	hydroquinone:oxygen oxidoreductase activity
GO:0016772	transferase activity, transferring phosphorus-containing groups
GO:0032559	adenyl ribonucleotide binding
GO:0020037	heme binding
GO:0019825	oxygen binding
GO:0016758	transferase activity, transferring hexosyl groups
GO:0030170	pyridoxal phosphate binding
GO:0016740	transferase activity
GO:0016831	carboxy-lyase activity
GO:0016835	carbon-oxygen lyase activity
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0008194	UDP-glycosyltransferase activity
GO:0046527	glucosyltransferase activity
GO:0004601	peroxidase activity
GO:0008184	glycogen phosphorylase activity
GO:0016760	cellulose synthase (UDP-forming) activity
GO:0004521	endoribonuclease activity
GO:0072341	modified amino acid binding
GO:0019843	rRNA binding
GO:0016894	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 3'-phosphomonoesters
GO:0033897	ribonuclease T2 activity
GO:0050661	NADP binding
GO:0004412	homoserine dehydrogenase activity
GO:0005200	structural constituent of cytoskeleton

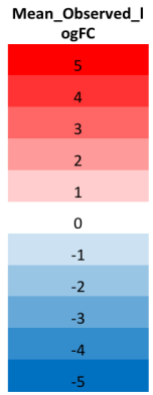


Figure 3. 25 Significantly enriched GO terms among the DEGs between grain filling stage vs. vegetative stage plants in Cv4. Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

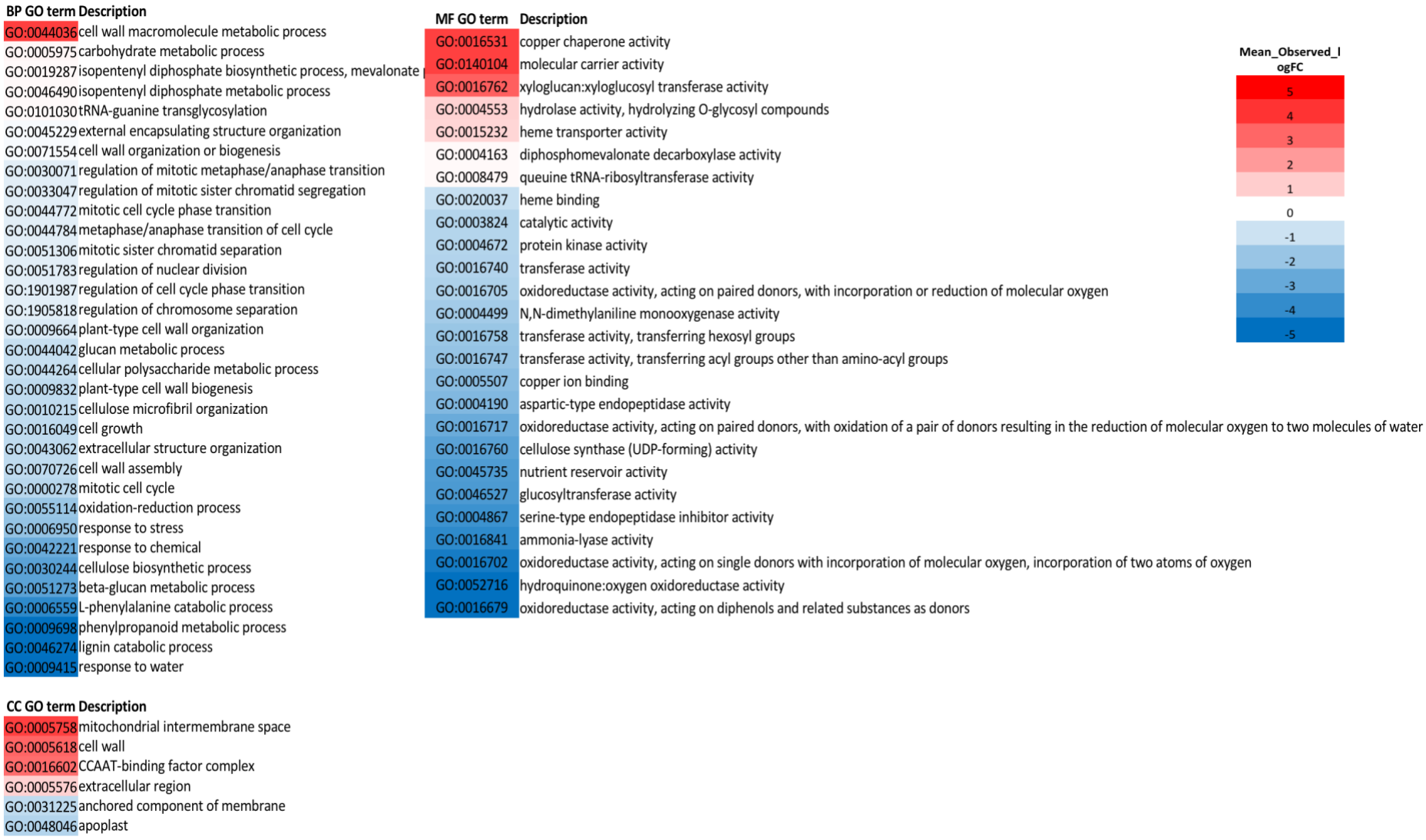


Figure 3. 26 significantly enriched GO terms among the DEGs at vegetative stage of Cv2 vs. Cv4 sample (at cultivar level). Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

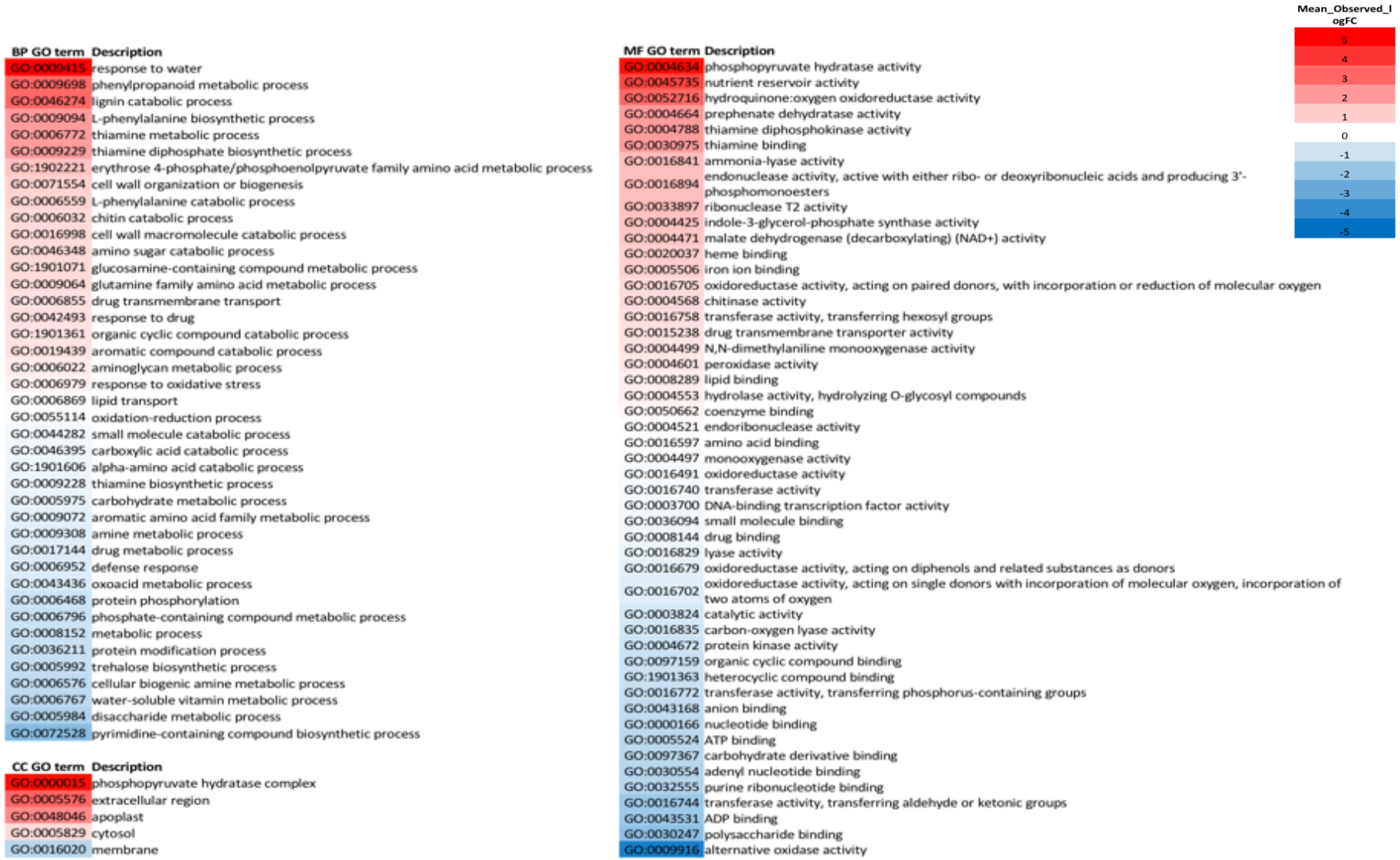


Figure 3. 27 Significantly enriched GO terms among the DEGs at flowering stage of Cv2 vs. Cv4 sample (at cultivar level). Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

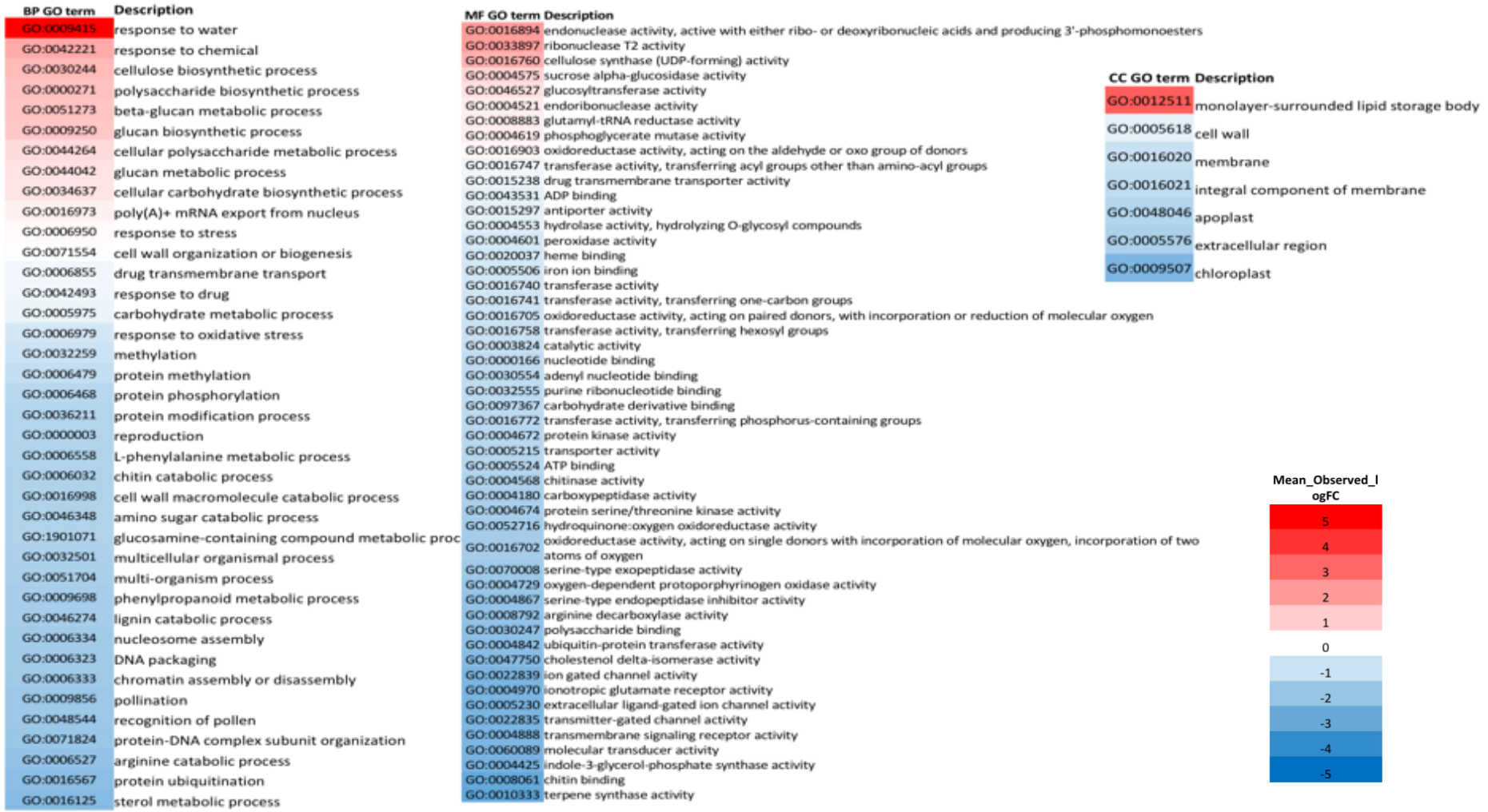


Figure 3. 28 Significantly enriched GO terms among the DEGs at Grain filling stage of Cv2 vs. Cv4 sample (at cultivar level). Up-regulated and down-regulated GO terms are indicated in red and blue, respectively, with gradients indicating different levels of significance.

3.3.6. *The enriched KEGG pathway results confirmed the GO enrichment results.*

Firstly, the comprarision at the condition level (15%-PEG6000 treatment vs. water control) found that in Cv2 under water stress at the flowering stage there were 22 different pathways involved in water stress response such as the ascorbate and aldarate metabolism, the starch and sucrose metabolism and the MAPK signalling pathway. Four different pathways were significantly regulated at the grain filling stage such as the phenylalanine metabolism and the biosynthesis of secondary metabolites (table S2. 2).

In Cv4 under water stress at the flowering stage, the DEGs were mainly involved in 7 different pathways such as flavonoid biosynthesis and protein processing in the endoplasmic reticulum. Twenty-three different pathways were identified under water stress at the grain filling stage in Cv4 such as photosynthesis, purine metabolism and phenylpropanoid biosynthesis) (table S2. 3). Secondly, the comprarision at the stage level (flowering vs. vegetative) found that 6 different pathways were identified in water controlled Cv4 at the grain filling stage compared with the vegetative stage, such as photosynthesis-antenna proteins, the lyoxylate and dicarboxylate metabolism and the carbon metabolism. The enriched KEGG pathways between the drought-tolerant cultivar (Cv2) and the drought-sensitive cultivar (Cv4) were 14, 9 and 4 regulated pathways at the vegetative stage, flowering stage and grain filling stage, respectively. They were mainly involved in the biosynthesis of secondary metabolites (table S2. 4).

The most frequently detected KEGG pathway under water stress in shoot transcriptome analysis was the amino acid metabolism (phenylalanine metabolism and Glutathione metabolism). In the biosynthesis of secondary metabolites, it was found that aromatic-L-amino-acid decarboxylase and phenylalanine ammonia-lyase were the most regulated in the phenylalanine metabolism under water stress, in addition to metabolic pathways, environmental information processing (MAPK signalling pathway) and environmental adaptation (plant-pathogen interaction).

Pathways that may relate to water stress are: the carbohydrate metabolism (ascorbate and aldarate metabolism, pentose phosphate pathway, fructose and mannose metabolism), the metabolism of cofactors and vitamins (ubiquinone and other terpenoid-quinone biosynthesis and thiamine metabolism), the lipid metabolism (alpha-linolenic acid metabolism), the metabolism of terpenoids and polyketides (terpenoid backbone biosynthesis and monoterpenoid biosynthesis), and the biosynthesis of other secondary metabolites (stilbenoid, diarylheptanoid and gingerol biosynthesis). Also involved are the energy metabolism

(oxidative phosphorylation, photosynthesis, photosynthesis - antenna proteins) and the nucleotide metabolism (purine metabolism). Pathways related to the growth stage are the biosynthesis of other secondary metabolites (flavonoid biosynthesis) and environmental adaptation (circadian rhythm). Pathways related to cultivar tolerance are: the biosynthesis of secondary metabolites (phenylpropanoid biosynthesis), the metabolism of cofactors and vitamins (porphyrin and chlorophyll metabolism) and the metabolism of terpenoids and polyketides (carotenoid biosynthesis) (table S2. 5; table S2. 6).

The current study found that the most regulated enzymes in phenylpropanoid biosynthesis (figure S2. 8) are phenylalanine ammonia-lyase, coumarate-CoA ligase, cinnamoyl-CoA reductase, peroxidase and cinnamyl-alcohol dehydrogenase.

3.4. Discussion

Water stress mainly affects crop growth and yield. Wheat is an important main-crop food for humans and several studies have shown that water stress has significant implications for wheat fields as it affects different stages of plant growth. In order to highlight the mechanisms of drought tolerance in Saudi wheat at the molecular level, we carried out RNA sequencing of the 12 RNA samples extracted from leaves harvested from the control and PEG-treated plants (Cv2 and Cv4) at the vegetative, flowering and filling stage. This yielded 24.2 GB of sequence data. At least 40 million reads of 85 bp each were obtained per sample.

3.4.1. RNA-seq and differentially expressed gene analysis

The traits of tolerance to water stress are considered to be more complex than any other plants traits because water stress or drought stress response involves anumber of cellular signal transduction pathways and many stress-related proteins and enzymes (Jiang et al., 2017a, Marček et al., 2019). In this project, we aimed to indicate the water stress response of each wheat growth stage, in terms of regulated physiological pathways. The effect of water stress on the wheat cultivars under study were discussed in the previous chapter and the current chapter has confirmed that 377 Rafha wheat cultivar (Cv4) was more sensitive than 193 Najran wheat cultivar (Cv2) to water stress created by 15% PEG6000, as assessed by morphological and biochemical measurements. The drought tolerance of wheat leaves decreases with plant age. Blum and Ebercon (1981), Chen et al. (2013) showed that the stomatal function, which is regulated by increasing levels of abscisic acid (ABA) in wheat leaves under water stress, was more responsive to ABA in young leaves than in old leaves under water stress. Consequently, young wheat leaves will decrease transpiration as a desirable response to decrease the impact of water deficit on leaves under water stress. The current study found that the vegetative stage and grain filling stage were resistant to water stress in Cv2 and were more sensitive in Cv4 than the flowering stage, which was ranked as moderately resistant to water stress in Cv2 and tolerant in Cv4. These results are in accordance with the findings of (Guo et al., 2009; Kaya et al., 2006; El-Nakhlawy et al., 2015; Sevik and Cetin, 2015;, Ihsan et al., 2016; , Swamy et al., 2017; Nagy et al., 2018) for different wheat cultivars. These related to the fact that plants can follow many physiological pathways to enhance their performance under stress (Ambawat et al., 2013; Tripathi et al., 2014;' Li et al., 2018b). Similarly, Maqbool et al. (2015) found that water stress induced several physiological pathways at the grain filling stage more than at other growth stages in the local wheat cultivar “Faisal-2008” (drought resistant) from Pakistan. Also,

AM et al. (2018) found that the Booting stage (which is the end of the vegetative stage according to the Feekes scale (Large, 1954)) was the most tolerant to salt stress out of other growth stages in the local wheat cultivar "Gimmiza 11" from Egypt, which may be consistent with the results at the flowering stage of the Cv4 cultivar.

Plants respond to stress through several changes in the transcript profile. This leads to several changes in the molecular and cellular mechanisms that protect plants from stress implications, which could negatively influence growth and reproduction (Ergen and Budak, 2009). In order to understand the mechanism of water stress response in Saudi wheat cultivars, RNA sequencing was carried out. Gene expression profiles could be accurately identified by RNA-seq. It can sequence the variants associated with DEGs in response to a treatment (Hübner et al., 2015). To identify the drought-responsive genes, the DEGs were analysed between conditions (15%-PEG6000 treatment vs. water control), growth stages (flowering vs. vegetative and grain filling vs. vegetative stages) and cultivars (Cv2 vs.Cv4) with a test p-value cut-off of 0.05, $\pm$ 2 $\geq$Log2 as thresholds. The high gene expression in Cv2 under water stress and the low gene expression in Cv4 may be related to the water stress resistance of Cv2, which is consistent with studies by Lv et al. (2018), Rampino et al. (2006) and Hu et al. (2018). It is also consistent with the results in the previous chapter, which showed the high tolerance performance of Cv2 under water stress. A higher number of overlapping DEGs between the comparison of Cv2 than that of Cv4 under water stress, which is also considered as a plant's response to abiotic and/or biotic stress (Chen et al., 2016b).

It was argued that increasing sequence depth (10 million–200 million reads) could lead to an increase in the detection of DEGs (Williams et al., 2014, Mirsafian et al., 2017, Ching et al., 2014, Baccarella et al., 2018, Tarazona et al., 2011). However, other studies such as Blencowe et al. (2009), Chen et al. (2016b), Liu et al. (2013) emphasized that the depth of sequences has a slight affect on DEG detection with respect to targeting tissue. For example, the DEGs between the treatment and control samples at the vegetative stage were higher than the DEGs in the flowering stage in the treated samples while the flowering stage samples had higher sequences than the vegetative stage in the same cultivar. Thus, higher DEGs are more related to stress than to the depth of sequencing.

The high DEGs that up- and down-regulated genes under water stress in this study could be compared in order to highlight the physiological pathways utilised under water stress. Wheat's adaptation ability to stress depends not only on the cultivar or genotype, but also on the developmental stage, tissue type and environment impact. In this study, the first comparison

found a highly up-regulated DEGs ratio in the vegetative stage of Cv2 and Cv4 under water stress. This was consistent with the second comparison that there were highly up-regulated DEGs between the grain filling stage and flowering stage, compared with the vegetative stage in Cv2 and Cv4. This could be evidence that the changes were related to the effect of water stress on the different wheat growth stages, not only the growth stages. In addition, highly down-regulated DEGs were indicated in Cv4 (water stress sensitive) compared to Cv2 (water stress resistant), which could be consistent with the results in the previous chapter. This is evidence of the abilities of the Cv2 cultivar under water stress, consistent with many physiological and biochemical studies on wheat such as (Chaves et al., 2003; Mahajan and Tuteja, 2005; Kaya et al., 2006; Ergen et al., 2009; Guo et al., 2009; El-Nakhlawy et al., 2015; Gregorová et al., 2015;, , , Sevik and Cetin, 2015; Sheoran et al., 2015, Ihsan et al., 2016; Swamy et al., 2017; Nagy et al., 2018).

3.4.2. Transcriptomic changes in the leaves of Saudi wheat plants under water stress

a. Response of photosynthesis in wheat under water stress

The number of DEGs was mapped to GO terms related to several metabolic and catabolic processes under water stress in both cultivars at all three growth stages under study, Plants' adaptations to water stress conditions require an extensive shift in metabolism (Less and Galili, 2008), including metabolic networks associated with carbohydrate metabolism, amino acid metabolism and secondary metabolites, due to the imbalance of ATP generation and utilisation under water stress (Dalal et al., 2018). Soluble carbohydrates (sugars) are important metabolites in plants under drought stress. Sugar status is used by plants as a signal to enhance growth and development in response to abiotic stresses. A significant difference in GO enrichment converge with KEGG analysis revealed that up-regulated DEGs were related to the carbohydrate metabolic process and molecular functions such as carbohydrate derivative binding and polysaccharide binding in the vegetative stage of Cv2 (drought tolerant). However, Cv2 has a decreased soluble sugar content in shoots and the dry biomass in shoots of all growth stages, which could explain the significantly extended root length instead of using sugar substances in the osmoregulation process. This could be associated with cultivar resistance or the growth stages, which may follow different osmoprotectant strategies under stress. In contrast, in the flowering and grain filling stages under water stress, down-regulated genes were involved in main enzymes in the carbohydrate metabolism such as chitinase in the amino sugar and nucleotide sugar metabolism, trehalose 6-phosphate phosphatase in the starch and sucrose metabolism, and ascorbate peroxidase in the ascorbate and aldarate metabolism. These

results agreed with (Zeng et al., 2011, You et al., 2019). Also, the large decrease in Cv4's (drought sensitive) biomass, sugar content and protein content was evident at the molecular level by the down-regulation of DEGs involved in the following: photosystem II P680 reaction centre D2 protein (psbD), photosystem II CP47 chlorophyll apoprotein (psbB), photosystem II cytochrome b559 subunit beta (psbF), photosystem II PsbI protein (psbI), photosystem II 13kDa protein (psb28), cytochrome b6-f complex subunit 4 (petD), apocytochrome f (petA), ferredoxin--NADP+ reductase (petH), light-harvesting complex II chlorophyll a/b binding protein 1 (LHCB1), and the light-harvesting complex II chlorophyll a/b binding protein 2 LHCB2. The latter is involved in photosynthesis and photosynthesis-antenna protein pathways, and plays a role in binding the prosthetic groups needed for energy and electron transfer, as well as in binding the multitude of plastid-encoded small subunits (Zouni et al., 2001, Komenda et al., 2004). Consequently, it has an effect on photosynthesis production and growth.

The flowering stage of the Cv2 phenotype showed less resistance to water stress than the vegetative and grain filling stages, whereas high levels of DEGs were observed in the flowering stage. However, the DEGs involved in biological processes such as the carbohydrate metabolic process were significantly down-regulated, which could be consistent with the significant decline in the soluble sugar content in the flowering stage of Cv2. Also, it was found that oligopeptide transporters (OPTs) were up-regulated in the flowering stage under water stress. This is related to membrane-localised proteins, which have the capability of transporting a wide range of substrates such as glutathione (Pike et al., 2009) and metals (Sasaki et al., 2011; Mendoza-Cózatl et al., 2014). It seems that OPTs help the wheat to maintain homeostasis in the cytoplasm under water stress, which is consistent with (Safdarian et al., 2019), who studied the transcriptional responses of wheat roots inoculated with *Arthrobacter nitroguajacolicus* under salt stress.

Based on yield performance, Cv2 (drought tolerant) uses most of the photosynthesis production to enhance yield (seed weight, seed numbers and germination) and to increase root length under water stress. This is accompanied by a reduction in stomatal conductance and this decreases CO_2 assimilation rates under water stress. The photosynthesis production is decreased (Liu et al., 2015; Rodrigues et al., 2019) or the subsequent photooxidative damage is induced by an accumulation of ROS under water stress. These results agreed with (Tezara et al., 1999; Guo et al., 2013; Saeidi et al., 2015; Liu et al., 2015a; Rodrigues et al., 2019,; Marček et al., 2019). However, the high tolerance to water stress shown by the flowering stage of the Cv4 cultivar

could be related to the highly up-regulated genes involved in the trehalose biosynthetic process, which is part of the metabolic pathway to produce energy and survive under water stress. The function of trehalose in plants is the storage of carbohydrate, the transport of sugar and plant protection under dry conditions. It is involved in the regulation of the carbon metabolism under stress rather than being directly involved in stress protection. Similar to sucrose, trehalose induces enzymes involved in the accumulation of carbohydrates in photosynthetic tissues (Wingler, 2002; Delorge et al., 2014; John et al., 2017; Liu et al., 2019a). These results agree with (Krugman et al., 2011; Ibrahim and Abdellatif, 2016).

Cv4's (water stress-sensitive) response to stress was to down-regulate DEGs in the carbohydrate metabolic process, photosynthesis and carbon fixation in all growth stages. This was obviously shown by the cultivar's performance under water stress, which agreed with (Lv et al., 2019, Marček et al., 2019). The drug catabolic process and response to oxidative stress genes were up- regulated in the vegetative stage. These are involved in the environmental adaptation pathway to break down the harmful substances accumulated under water stress. Under abiotic/biotic stress, plant cell organs such as plastids, mitochondria, and peroxisomes' initial response is to increase the toxic component in cells such as reactive oxygen species (ROS), which is produced by aerobic metabolism. That leads to an imbalance between the production and scavenging of ROS content, which is highly toxic and reactive. It causes large degradation in cellular energetics and inhibits physiological processes in plants, such as photosynthesis, respiration, transpiration, growth and development, which ultimately results in oxidative stress (Figure 1.12). This further affects plant growth and yield (Bailey-Serres and Mittler, 2006; Gill and Tuteja, 2010; Shah ZH, 2017; Sharma et al., 2019).

b. Response of amino acid metabolism and biosynthesis of secondary metabolites in wheat under water stress

Amino acids play an important role as a hydrogen ion buffer and a structural component of plant cells, due to their amphoteric nature. Amino acids can act directly on the ROS reduction under stresses (Teixeira et al., 2019). In addition, plants under stress reconstruct new proteins that have the ability to cope with the new abiotic stress. This alteration in transcription leads to the synthesis of new proteins and the degradation of existing ones that are less or not essential to the environmental effects of different cultivars' growing locations or water stress (Mahajan and Tuteja, 2005; Ergen et al., 2009; Marček et al., 2019). Noticeably, the results showed an increasing protein content and the up-regulation of DEGs involved in amino acid metabolism and the protein modification process at the vegetative and grain filling stages of the Cv2

cultivar under water stress. The Saudi cultivars under study were affected by water stress to a greater extent with several amino acid metabolisms significantly regulating the DEGs involved in the phenyalanine metabolism, the tyrosine metabolism, the tryptophan metabolism, betalain biosynthesis and isoquinoline alkaloid biosynthesis, which can reasonably result from enhanced stress-induced protein breakdown. The main up-regulated DEGs involved the following enzymes: aromatic-L-amino-acid decarboxylase, phenylalanine ammonia-lyase (PAL), 4-coumarate--CoA ligase, and aromatic amino acid deaminases such as phenylalanine ammonia-lyase (PAL). These are key enzymes mediating carbon flux from the primary to the secondary metabolism in plants (Barros and Dixon, 2020). The first committed step in the phenylpropanoid pathway (figure S2. 7) is the deamination of phenylene into cinnamate by PAL.

Our experiments on Saudi wheat cultivars showed that Cv2 (water stress-resistant) coped with water stress by regulating phenylpropanoid biosynthesis and the lignin catabolic process, which could be part of wheat's antioxidative system (Gholizadeh and Kohnehrouz, 2010). Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine and they have a wide variety of functions both as structural and signalling molecules. Phenylalanine is first converted to cinnamic acid by deamination. This is followed by hydroxylation and frequent methylation to generate coumaric acid and other acids with a phenylpropane (C6-C3) unit (Kanehisa, 2019, Kanehisa et al., 2019, Kanehisa et al., 2020). Reduction of the CoA-activated carboxyl groups of these acids results in the corresponding aldehydes and alcohols. The alcohols are called monolignols, the starting compounds for the biosynthesis of lignin. In the current study, it was found that the most regulated enzymes in phenylpropanoid biosynthesis were phenylalanine ammonia-lyase (Pal5), coumarate-CoA ligase, cinnamoyl-CoA reductase, peroxidase, and cinnamyl-alcohol dehydrogenase. This could help wheat to scavenge harmful oxygen species usually generated under abiotic stress (Sharma et al., 2019). It also plays a role in enhancing the structural defence barrier of cell walls under stress (Purwar et al., 2012). In detail, it was found that phenylpropanoid biosynthesis was highly up-regulated at the vegetative and grain filling stages of the Cv2 cultivar, which is consistent with the cultivar phenotype results under water stress in the current study and which agreed with (Purwar et al., 2012; Ma et al., 2014; Wei et al., 2017; Safdarian et al., 2019, Sharma et al., 2019, , ,).

One of the well-known biomarkers for water stress due to its osmoprotectant role is proline. It dramatically increases in the vegetative and grain filling stages of Cv4 (more than Cv2) due to

its high drought application in cultivars sensitive to drought. This was consistent with previous reports on other plant species (Less and Galili, 2008; Obata et al., 2015; Ma et al., 2016; Pires et al., 2016; Das et al., 2017; You et al., 2019). It was also found that glutathione metabolism, which is one of antioxidant metabolisms for maintaining cellular redox homeostasis and mediating plant abiotic stress resistance, was up regulated in Cv2 only, with the phenylpropanoid metabolic process and lignin catabolic process in the vegetative and grain filling stage of Cv2.

To sum up, most of the DEGs regulated under water stress were found to be involved in metabolic pathways and the biosynthesis of secondary metabolites in both cultivars at all growth stages under study. This relates to the fact that water stress induced plant cell dehydration (passive water loss) then accumulated the secondary metabolite substances (called active osmotic adjustments) to increase the cell water balance under drought stress. This result partially agrees with (Marček et al., 2019), who believe that it is difficult to determine which metabolic changes are involved in response to water stress; the response could be cultivar dependent only. We agree with the fact that the water response was mainly regulated in a cultivar-dependent manner. Otherwise, increasing some secondary metabolites such as flavonoid (Ma et al., 2014, Kaur and Zhawar, 2017) and lignin (Kaur and Zhawar, 2015, Santos et al., 2015) and the synthesis of some amino acid substances such as proline, phenylalanine, methionine, serine and asparagine were observed to contribute to the maintenance of energy homeostasis, protecting against over-reduction of PSII and consequent damage from oxidative stress (i.e. photo-inhibition) as expected under drought stress (Yadav et al., 2019b).

3.5. Conclusion

In this chapter

- Based on the collected growth data in the first experiment one Cv from each group (Cv2 and Cv4) were used to conduct a comparative analysis of the differences in the transcriptome under both control (well-watered) conditions and water stress (15% PEG6000) conditions to identify the gene differences and differential transcript levels in the two Cvs under the effect of water stress.
- Leaf samples were collected from both control plants and water-stressed plants at the vegetative, flowering and grain filling stages of tow cultivars (Cv2 water stress tolerance and Cv4 water stress sensitive) snap frozen in liquid nitrogen and stored at -80°C until analysis. RNA was extracted from the samples, then checked for quality using a bioanalyzer for RNA. The extracted RNA was sent to a professional company (Admera, USA) for sequencing.
- RNA sequencing of the 12 RNA samples extracted from leaves harvested from the control and PEG-treated plants (Cv2 and Cv4) at the vegetative, flowering and filling stage. and Transcriptomic analysis was done.
- This yielded 24.2 GB of sequence data. At least 40 million reads of 85 bp each were obtained per sample.
- Three main physiological pathways were indicted to be regulated under water stress at the tow Cvs are:
    - **Photosynthesis** founds to be down regulated in flowering of cv2 and up regulated in vegetative and grain filling stages, and it founds to be down regulated in Cv4's (water stress-sensitive) at all growth stages under water stress.
    - **Amino acid metabolism founds to be** up regulated at the vegetative and grain filling stages of the Cv2 cultivar (water stress-resistant) under water stress.
    - **Secondary metabolism such as** phenylpropanoid biosynthesis, Our experiments on Saudi wheat cultivars showed that vegetative and grain filling stages of Cv2 (water stress-resistant) coped with water stress by regulating phenylpropanoid biosynthesis and the lignin catabolic process, which could be part of wheat's antioxidative system.

Chapter 4 Proteomics analysis of water-stress induced changes in the proteome of two wheat cultivars, Najran and Rafha

4.1.Introduction

Water stress-tolerance response is considered to be a complex trait because it involves a large number of genes encoding components of cellular signal transduction pathways and proteins involved in adjustment to osmotic stress (Barnabás et al., 2008, Jiang et al., 2017a). Many studies have applied comparative transcriptomic approaches to determine the underlying molecular mechanisms of both biotic and abiotic stresses (Ergen et al., 2007; Kantar et al., 2011; Liu et al., 2015b;). Yet, transcripts and proteins are not always correlated, post-transcriptional, translational and post-translational regulations can lead to to levels of functional proteins which might not correlate with transcript levels (Stylianou et al., 2008). This means that transcripts levels are not sufficient to understand the molecular mechanisms of resistance to environmental stresses (Abdalla and Rafudeen, 2012, Budak et al., 2013). This non-correlation of mRNA with proteins implies that examining protein levels would provide a greater insight into gene functions (Xiong, 2006).

In conjunction with transcriptomics, proteomics provide an important opportunity to advance the understanding of the physiological-response mechanisms to water stress in wheat (*Triticum aestivum L.*) by determining the proteins that change in location, abundance, form or activity under water stress (Thelen and Peck, 2007; Budak et al., 2013; Liu et al., 2015a).“Proteome” is a term that refers to the total number of proteins encoded by the genome of an organism (Wilkins et al., 1996, Xiong, 2006). Proteins are the key controllers or regulators of a vast number of cellular processes due to their unique function, properties and dynamics, it is therefore important to combine transcriptomic and proteomic approaches to understand plant growth, development and responses to environmental stress (Caruso et al., 2009; Vu et al., 2017,).

Proteomics has become a major field in modern functional biology (Park, 2004), most of its approaches are generally based on the separation of individual proteins from complex mixtures using electrophoretic or chromatographic techniques followed by quantitation, characterisation and identification. The proteomic techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS), two-dimensional gel electrophoresis (2-DE) and capillary electrophoresis-mass spectrometry (CE-MS), among others. LC-MS/MS is a commonly used

technique that compares changes in the abundance and sequence of peptides. Its popularity is due to its accurate ability to identify proteins by improving protein/peptide resolution and facilitating subsequent identification and characterisation based on peptide resulting from enzymatic digestion with trypsin (Wolters et al., 2001).

Proteomic studies can deliver a complete and comprehensive understanding of the biological pathways linked to plant resistance to different environmental stresses (Salekdeh et al., 2002; Aryal et al., 2014a, Hossain et al., 2015; Nouri et al., 2015; An et al., 2016; Wang et al., 2016a, , ,). One such proteomic study found that some drought-related proteins were up-regulated in drought- tolerant wheat than in drought-sensitive wheat cultivars (Kong et al., 2010; Cheng et al., 2015). Form proteomic profiling at seeds germination stage of wheat (*Triticum aestivum* L.) under water stress, phenylpropanoid biosynthesis and fatty acid degradation were up regulated (Yan et al., 2020). Also, in Barley (Hordeum vulgare L.) under drought stress enzymes related to phenylpropanoid biosynthesis showed strong genotype×environment interactions (Rodziewicz et al., 2019). Proteomic analysis showed that the ability of plant to produce proteins involved in osmotic homeostasis under stresses will increase plant stress tolerance (Capriotti et al., 2014), for example, in young wheat seedlings, proteomic analysis showed an increase in the presence of proteins associated with stress and defence response were observed (Michaletti et al., 2018; Koobaz et al., 2020). Moreover, it can insight into importance of growth stages and environment interactions, to improve molecular study for breeding programs (Bennet et al., 2008; Chenu et al., 2011).

The general experimental workflow of proteomic studies is to extract proteins from shoots by homogenisation and precipitation. The proteins are then subjected to lysis and peptides are analysed by LC-MS/MS and identified using MASCOT database searches (Aryal et al., 2014a). This chapter of the research aimed to compare the changes in abundance and sequence of peptides in two Saudi wheat, drought tolerance (193 Najran cultivar) and drought sensitive (377 Rafha cultivar) at three growth stages under water stress. And the proteomic results together with transcriptomic results will confirm the physiological pathways regulated under water stress in the two Saudi wheat cultivars.

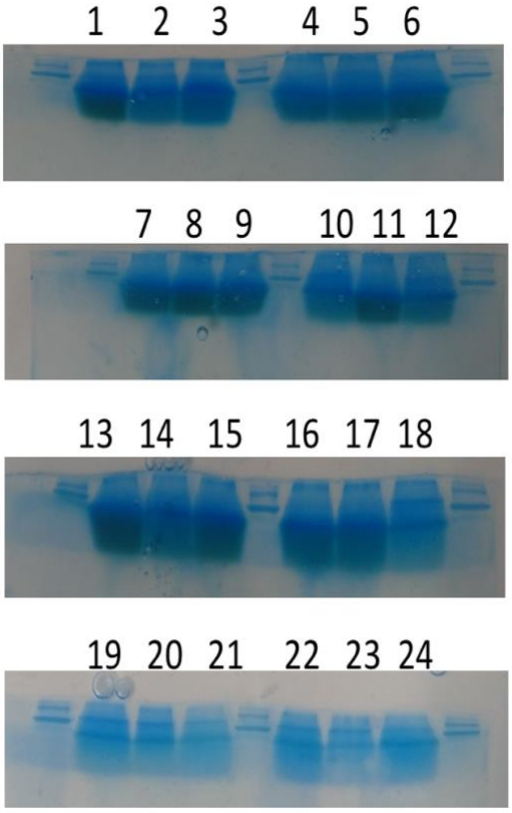
4.2.Materials and Methods

4.2.1 Plant growth conditions and water-stress treatments

plant growth conditions, Plant growth, Water-stress treatments and plant sampling were done similar to what had done in the third chapter (Differential gene expression profiles and associated metabolic pathways in two Saudi wheat cultivars under water stress at three growth stages). Three biological replicates of each fresh samples were grained with liquid nitrogen and sorted at -80 and sent to Protein Facility (NUPPA), Newcastle University, project id (#32), for LC/MS experiment and proteomic analysis. The experiment resulted in the ultimate comparison of 72 different samples (two cultivars, 6 technical replicates each, 2 different conditions, 3 time points).

4.2.2 Protein extraction

Powdered leaf tissue was homogenised in Tris buffer, and the extracted proteins were purified and concentrated using polyacrylamide gels, after an initial SDS-PAGE experiment to determine protein concentration extraction. Shoots were ground under liquid nitrogen and 100 mg of DWt ground material was homogenised in 1 ml of 20-50 mM, pH (7.0 - 9.0) Tris buffer. The protein extraction from each sample was loaded into polyacrylamide gels (40ul). Proteins were extracted from the gel as a single band (figure 4.1) and subjected to trypsin lysis. The complex peptide mixture was separated by liquid chromatography before peptide analysis by mass spectrometry.



No.	sample
1	Cv2 control vegetative1
2	Cv2 control vegetative2
3	Cv2 Treatment vegetative1
4	Cv2 Treatment vegetative 2
5	Cv2 control flowering 1
6	Cv2 control flowering 2
7	Cv2 Treatment flowering 1
8	Cv2 Treatment flowering 2
9	Cv2 control grain filling 1
10	Cv2 control grain filling 2
11	Cv2 Treatment grain filling 1
12	Cv2 Treatment grain filling 2
13	Cv4 control vegetative1
14	Cv4 control vegetative2
15	Cv4 Treatment vegetative1
16	Cv4 Treatment vegetative 2
17	Cv4 control flowering 1
18	Cv4 control flowering 2
19	Cv4 Treatment flowering 1
20	Cv4 Treatment flowering 2
21	Cv4 control grain filling 1
22	Cv4 control grain filling 2
23	Cv4 Treatment grain filling 1
24	Cv4 Treatment grain filling 2

Figure 4. 1 Partially run Polyacrylamide gels showing wheat protein samples as single bands that were used in current study to purify proteins before digestion and LC/MS analysis

The peptides are ionised to acquire the initial MS scan, and a spectrum of the mass-to-charge ratio of peptide ions in each sample is then acquired. Selected peptides from the MS scan are then individually fragmented for the MS/MS scan to collect amino acid sequence information about the peptides. Signal or peak integration of ions in MS scans has been used as a quantification technique for decades by small molecule analytical chemists, because theoretically the peak intensity of any ion should be proportional to its abundance (Rappsilber et al., 2007, Wiśniewski et al., 2009).

4.2.3 Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) experiment

a. In Gel Digestion

SDS-PAGE bands were excised with a clean scalpel (wiped with lint free tissue after each cut). Each band was diced into 1x1x1 mm cubes and transferred to a clean microcentrifuge tube. In gel digestion was done according to (Shevchenko et al., 2006) as flowing, Gel pieces were destained by excess addition of 50mM Ammonium Bicarbonate 50% Acetonitrile. The destain buffer was removed and exchanged 3 times, or until the gel pieces were clear. A molecular weight marker band was also excised as a digest control. Proteins were reduced with 10mM

dithiothreitol for 30 min at 60˚C to break disulphide bridges. This was followed by alkylation with 50mM iodoacetamide for 30 min at room temperature in the dark to prevent disulphide reformation. Gel pieces were washed in 50mM Ammonium Bicarbonate and then dehydrated with 3 washes of 100uL of acetonitrile. Residual moisture was removed from the gel pieces in a vacuum drier. Proteins were digested by the addition of trypsin added at a ratio of 30:1 (protein:trypsin), buffered with 50mM Ammonium Bicarbonate and incubated for 16 hours at 37˚C. The digest was stopped by the addition of 10% TFA to a final concentration of 0.5%, shaken for 30 min at 750 rpm. The liquid containing hydrophilic peptides was transferred to a fresh microcentrifuge tube. A solution of 80% Acetonitrile with 2% TFA was then added to the gel pieces and shaken for 30 min at 750 rpm. This dehydrates the gel pieces and removes hydrophobic peptides from the gel. The solution containing hydrophobic peptides was pooled with the hydrophilic peptide mix. The peptide solution was dried in a centrifugal evaporator and the peptides were dissolved in 3% acetonitrile, 0.1% TFA. The resulting peptide solutions were desalted using home-packed C18 stage tips (Rappsilber et al., 2007). The sample was dissolved in 50uL of 3% Acetonitrile, 0.1% TFA, giving the final concentration of ~1ug/uL.

b. Nano LC-MS/MS

Exactly 1 µg of a protein digest was separated with a 97 min nonlinear gradient (3-40%, 0.1% formic acid) using an UltiMate 3000 RSLCnano HPLC. Samples were first loaded onto a 300µm x 5mm C18 PepMap C18 trap cartridge in 0.1% formic acid at 5 µl/min and passed on to an in-house-made 75µmx25cm C18 column (ReproSil-Pur Basic-C18-HD, 3 µm, Dr. Maisch GmbH) at 400nl/min. The eluent was directed to an Ab-Sciex TripleTOF 6600 mass spectrometer through the AB-Sciex Nano-Spray 3 source, fitted with a New Objective FS360-20-10 emitter. For data-dependent data acquisition (DDA), MS1 data was acquired within a range of 400 – 1250m/z (250ms accumulation time), followed by MS2 of Top 30 precursors with charge states between 2 and 5 (total cycle time 1.8s). Product ion spectra (50ms accumulation time) were acquired within a range of 100–1500m/z, using rolling collision energy for precursors that exceed 150 cps. Precursor ions were excluded for 15s after one occurrence.

For Data Independent Acquisition (SWATH), MS1 data were acquired over a range of 400–800 m/z. The same m/z range was then covered with 83 variable SWATH windows, with a minimum size of 4Da. To calculate this, a DDA file was taken at random, and all MS1 data averaged into a single spectrum (spectral image). The ion current was then divided into variable bins containing roughly the same number of precursors (areas of low ion intensity

have wider windows and vice versa). Windows were overlapped by 0.5 Da to avoid losing data at the edges of a window. Ions were accumulated for 250ms in MS1. Each SWATH window was accumulated for 25 ms (high sensitivity mode) giving a total duty cycle of 2.325 s. SWATH and DDA were acquired with identical chromatography settings. This maximised the reproducibility of the data and improved the spectral library matching of the SWATH data (Schubert et al., 2015, Schilling et al., 2017, Sciex.com).

c. Protein identification to build spectral library

The acquired DDA data were searched against the protein sequence database available from https://www.uniprot.org, concatenated to the Common Repository for Adventitious Proteins v.2012.01.01 (cRAP, ftp://ftp.thegpm.org/fasta/cRAP), using ProteinPilot (Ab-Sciex, v5, parameters used: cysteine alkylation: iodoacetamide, digestion enzyme: trypsin, Parent Mass Error of 20ppm, fragment mass error of 30ppm). The individual search results were exported (using PeakView 2.2), in a spectral library format, as *.tsv files. The confidence cut-off representative to FDR<0.01 was applied to the search result file (Cox and Mann, 2008).

d. SWATH data processing

All 36 SWATH data files (*.d) were imported to PeakView2.1 SWATHmicroApp, along with the .tsv spectral library. Firstly, data from the spectral library were aligned to the SWATH data through chromatographic retention. This was done by manual selection of 50 peptides throughout the gradient. Each peptide had to be present in each SWATH file, in addition to having an intensity greater than 1e4. SWATH data were processed with the following settings:

Number of transitions per peptide: 6

Peptide confidence threshold %: 99

False discovery rate %: 1.0

XIC width: 50ppm

Data were exported in .txt format. .txt files were then uploaded to Purseus v1.6.2.3 (http://coxdocs.org/doku.php?id=perseus:start) for downstream analysis according to (Tyanova and Cox, 2018). The results were saved as .txt file including proteins ID, the gene annotation searched against the protein sequence database available from https://www.uniprot.org were added to the protein table.

4.2.4 Statistical analysis

It was carried out using Perseus software. Multiple-sample test (one-way ANOVA), controlled by Benjamini–Hochberg method–based FDR threshold of 0.01, was used to identify the significant differences in the protein abundance during water stress in wheat shoot.

4.3.Results

PEG imposed water stress caused important shifts in shoots protein complement sampled at three growth stages, vegetative, flowering and grain filling in two Wheat cultivars, Najran and Rafha. In total, more than 1,006 common differentially expression proteins (DEPs) were detected between PEG treated and control plants in both cultivars at three growth stages in each spectral image (DDA data files). Although, in 2019 approximately 306 proteins were delated form UniProtKB protein dataset, 700 proteins were still identified by the UniProt protein dataset (Consortium, 2018). The UniProt Knowledgebase (UniProtKB) contains only the latest Swiss-Prot and TrEMBL entry versions, Most UniProtKB/TrEMBL deletions are due to several reasons according to https://www.uniprot.org/help/deleted_accessions . From the 700 proteins existing, 390 proteins were uncharacterised and 309 proteins were characterised (table S3. 1). Among the 309 common DEPs, 162 and 153 proteins were up-regulated in Cv2 and Cv4 at the grain filling stage under water stress, respectively (figure 4.2; table S3. 1). Plotting the top two principal components revealed a clustering trend between all samples under water stress and between common DEPs (figure 4.5).

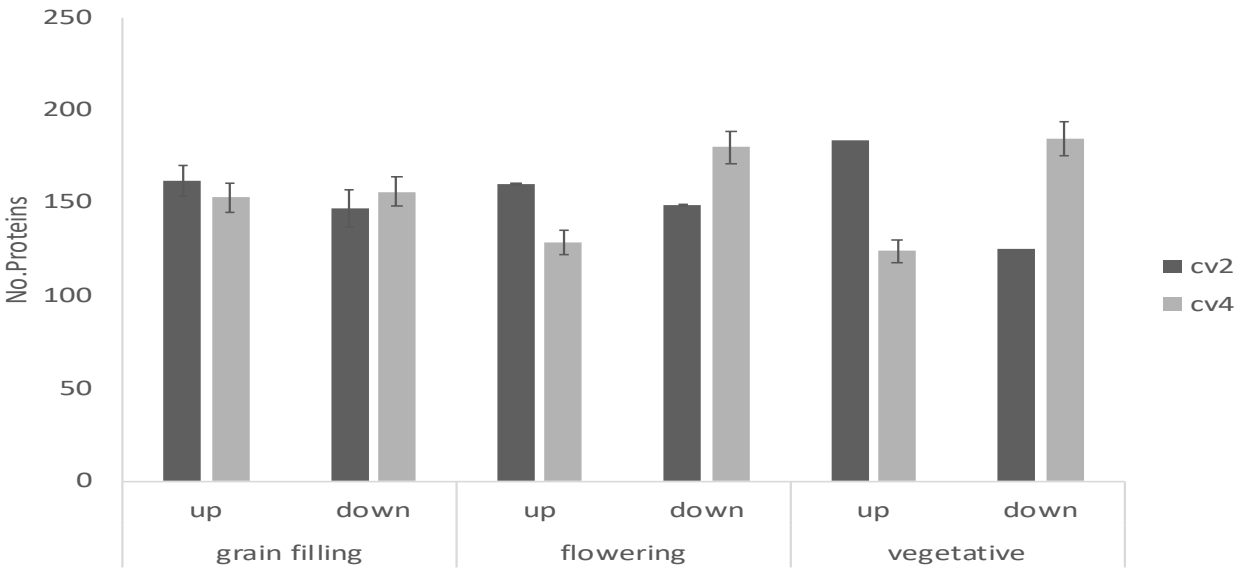


Figure 4. 2 Numbers of common DEPs regulated in wheat cultivars (*Triticum aestivum* L.; 193 Najran (Cv2); 377 Rafha (Cv4)) at three growth stages: vegetative, flowering and grain filling, subjected to 15% PEG6000 (n:6; P >0.05).

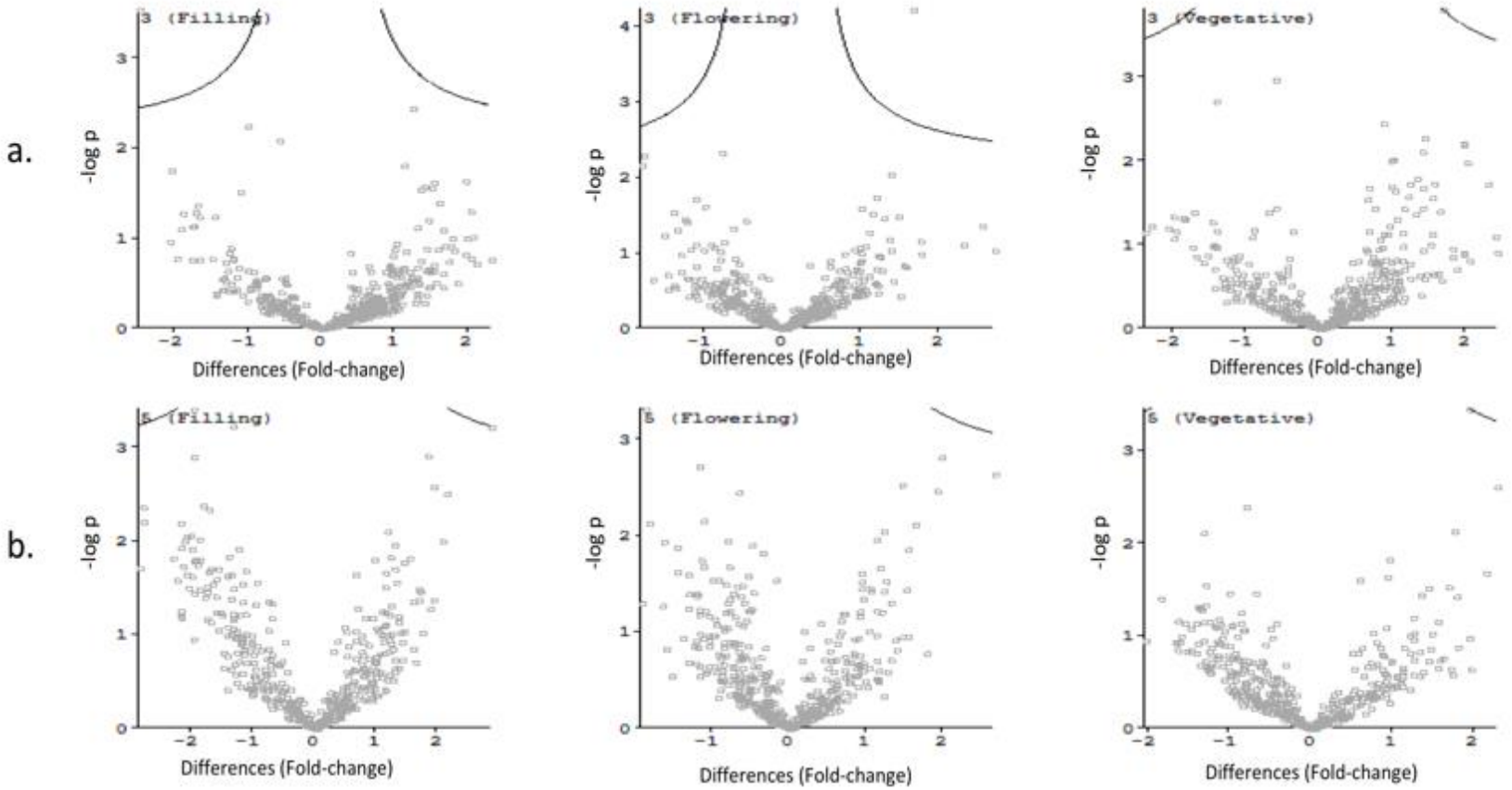


Figure 4. 3 Volcano plot showed number of differentially expressed proteins based on their adjusted p values (y) and log2 fold change (x) (FDR<0.01) in the analysis between wheat cultivars (*Triticum aestivum* L.; 193 Najran (Cv2); 377 Rafha (Cv4) at three growth stages: vegetative, flowering and grain filling, subjected to 15% PEG6000. a. DEPs in 193Najran (Cv2), Plot labelled with (3 filling): grain filling stage; (3 flowering): flowering stage and (3 vegetative): vegetative stage. b. DEPs in 377 Rafha (Cv4), (5 filling): grain filling stage; (5 flowering): flowering stage and (5 vegetative): vegetative stage.

To increase the focus, we narrowed down our investigation to highly fold change of proteins between the control and treatment (-Log10 >2<). Differential expression proteins (DEPs) are listed in table S3. 1 and figure 4.3. The current study found that Cv2 cultivar, which was previously characterised as being drought tolerant, had several proteins that were highly regulated under water stress at different growth stages. The analysis detected highly significant (p=-Log10 >2) up-regulated proteins in treated samples involved in photosynthesis such as Chlorophyll a-b binding protein, photosynthetic NDH and phosphoenolpyruvate carboxylase at all growth stages under water stress. Stress response proteins such as Pyruvate kinase were found to be up regulated in vegetative stage by 1.6 fold under water stress. Pyruvate kinase involved in reactions and pathways resulting in the breakdown of a carbohydrate into pyruvate (glycolysis). Carbohydrate metabolic proteins that contribute to the glycolysis process such as alpha-mannosidase was highly up regulated in vegetative and flowering stages by 1.07 and 2.03 fold change respectively. Glycosyltransferase was up regulated in vegetative stage by 1.2 fold change. Also, Stress response proteins such as peroxidase were up regulated in all growth stage specially flowering stage by 1.8 fold change under water stress. The curvature thylakoid 1D, chloroplastic-like (which responds to abscisic acid) was up regulated in all growth stage and the highest fold change was indicted in grain filling stage by more than 1.5 fold change under water stress. Serine hydroxymethyltransferase (SHMT) found to be up regulated in flowering stage by 1.2 fold change under water stress which might involve in the glycine metabolic process. Proteins related to alanine metabolic process were up regulated such as Aspartate aminotransferase by 1.7 fold change in grain filling stage under water stress. Phenylalanine ammonia-lyase (PAL) was found up regulated in vegetative and grain filling stages and down regulated in flowering stage by 0.747 fold change under water stress. NADPH-protochlorophyllide oxidoreductase was up regulated in flowering and grain filling stages and the highest fold change were indicted in grain filling stage by 1.6 fold under water stress. The data showed that malate dehydrogenase has four isoform some of them was up regulated in grain filling stage by 1.3 fold and the left were down regulated under water stress. also, Aldehyde dehydrogenase (ALDH) founds to be up regulated in grain filling stage (0.526 fold) and down regulated in all stages of Cv2 and Cv4 cultivars. It has also been shown that some chaperones such as Hsp70-Hsp90 were up regulated only in Cv2 at all growth stages.

The DEPs in the sensitive wheat cultivar (Cv4), shown by comparing PEG treated to control plants were mainly uncharacterised proteins including the uncharacterised oxidoreductase At4g09670, which is involved in the oxidation-reduction process, was mainly up-regulated in Cv4 under water stress at all growth stages, the highest fold change was in vegetative stage by 1.7 fold. However, the most of proteins involved in oxidation/reduction process were found to be down regulated such as dihydrolipoyl dehydrogenase and thioredoxin which had the highest reduction by 1.4 fold in flowering stage and 1.5 fold at grain filling stage. Also, Glutathione reductase which had the highest reduction by 0.248 fold change in vegetative stage. It found that CBS domain-containing protein involved in voltage-gated chloride channel activity (Consortium, 2019) was down regulated in flowering stage by 1.9 fold change under water stress. In the same line, it founds that proteins related to protein-folded and response to oxidative stress such as peptidyl-prolyl cis-trans isomerase (PPIase) were down regulated in vegetative and flowering stages under water stress. Proteins related to cold response (glycosyltransferase) in flowering stage by 1.5 fold change however it was down regulated in vegetative stage by 0.116 in treated samples. Carbohydrate biosynthesis proteins were found to be down regulated in all growth stages such as phosphoglycerate by 1.7 fold change in vegetative stage, phosphoribulokinase by 1.3 fold change in flowering and grain filling stages, and glucan endo-1,3-beta-glucosidase by 1.17 fold change in vegetative stage. Photosynthesis related proteins had variable change under water stress, such as NADPH-protochlorophyllide oxidoreductase which is involved in the chlorophyll biosynthesis pathway, was down regulated in all growth stages and the highest fold change were indicted in flowering and grain filling stage by 2.06 and 1.05 fold change respectively under water stress. Moreover, bisphosphate carboxylase large subunit decreased in all growth stages by approximately 1.5 fold change. There were a few proteins up regulation comparing with Cv2 wheat cultivar.In contrast, xylose isomerase showed up regulation in flowering stage and down regulation in grain filling and vegetative stages under water stress.

sample	name
1	cv2vegtwater1
2	cv2vegtwater2
3	cv2vegtwater3
4	cv2vegtPEG1
5	cv2vegtPEG2
6	cv2vegtPEG3
7	cv2folwwater1
8	cv2folwwater2
9	cv2folwwater3
10	cv2flowPEG1
11	cv2flowPEG2
12	cv2flowPEG3
13	cv2fillwater1
14	cv2fillwater2
15	cv2fillwater3
16	cv2fillPEG1
17	cv2fillPEG2
18	cv2fillPEG3

sample	name
19	cv4vegtwater1
20	cv4vegtwater2
21	cv4vegtwater3
22	cv4vegtPEG1
23	cv4vegtPEG2
24	cv4vegtPEG3
25	cv4flowwater1
26	cv4flowwater2
27	cv4flowwater3
28	cv4flowPEG1
29	cv4flowPEG2
30	cv4flowPEG3
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34	cv4fillPEG1
35	cv4fillPEG2
36	cv4fillPEG3

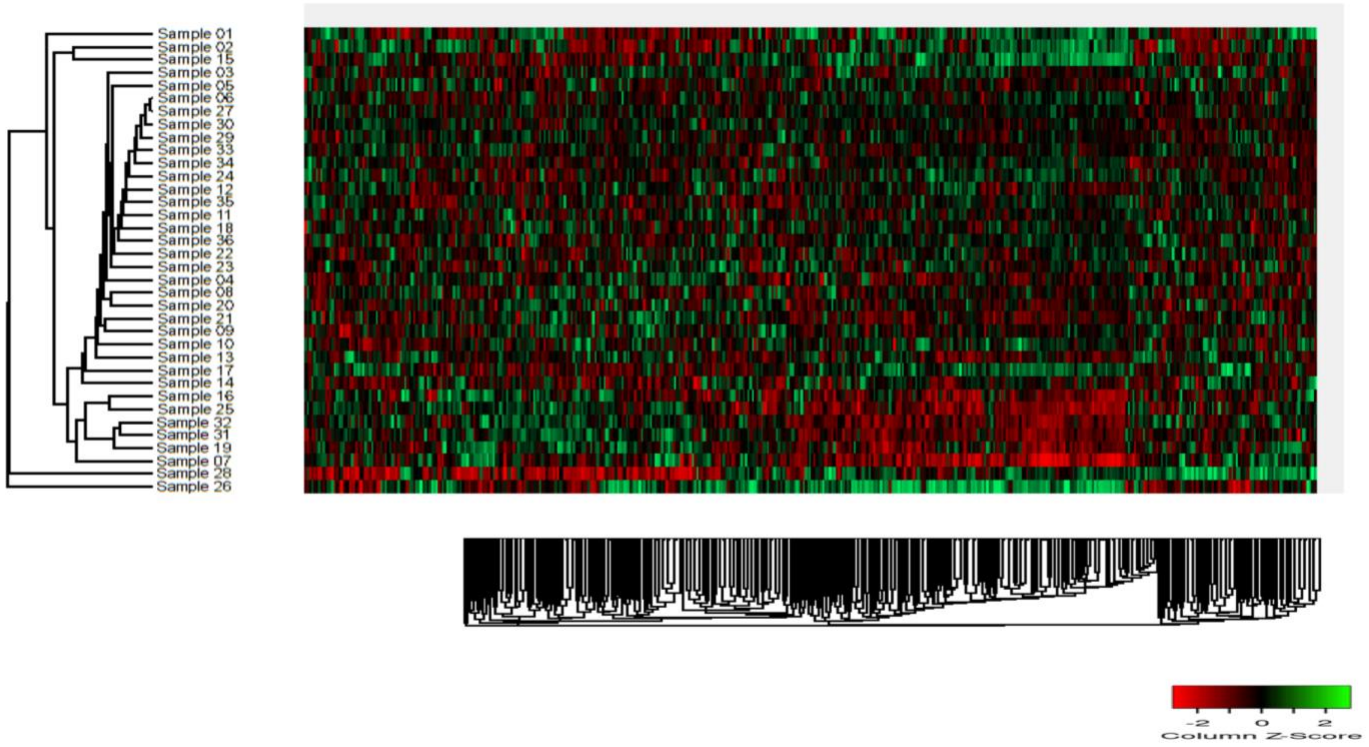
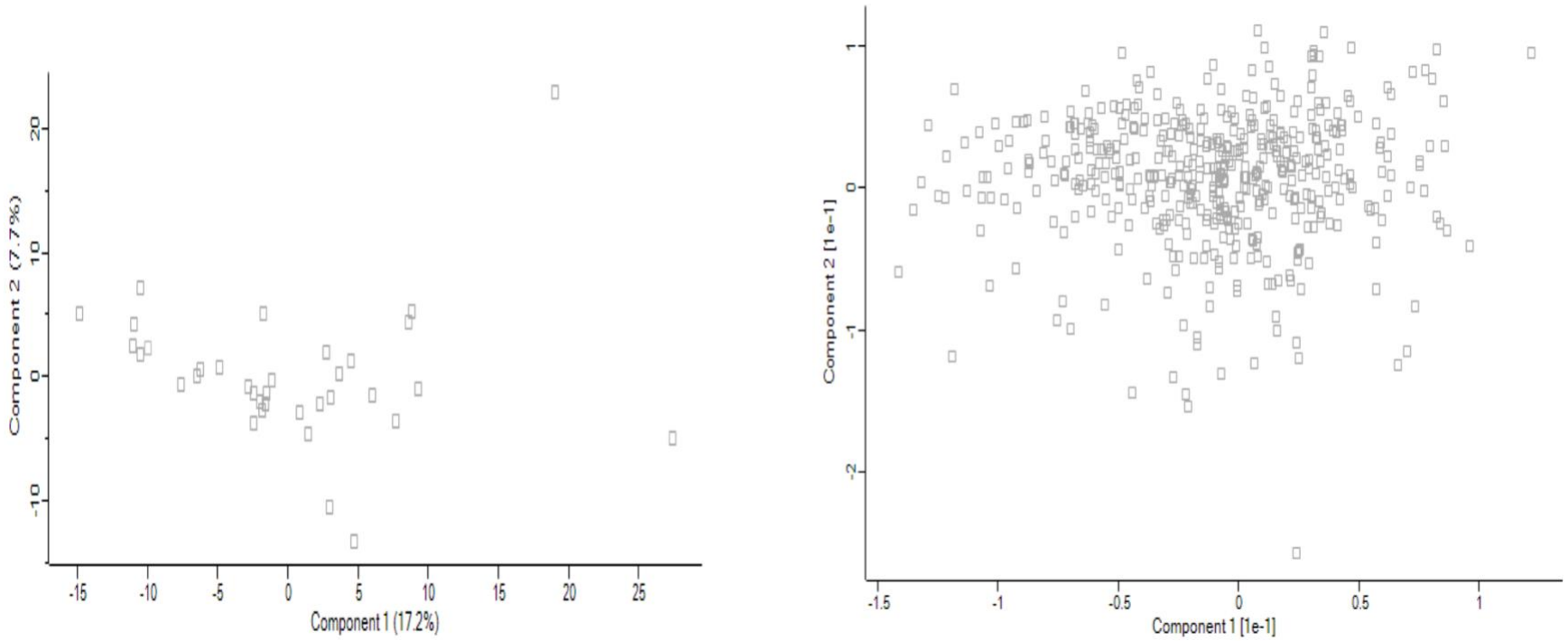


Figure 4. 4 Heat-map generated by PERSEUS®. representing the proteins with significant different abundances in the technical replicates with the respective biological replicates of two wheat Cultivars: 193 Najran (Cv2) and 377 Rafha (Cv4) under water-control and water-stress (15% PEG) conditions at three growth stages: vegetative, flowering and filling from the proteome analysis. Multiple-sample test (one-way ANOVA), controlled by Benjamini–Hochberg method–based FDR threshold of 0.01, Green fields indicate up-regulation and red fields down-regulation.



A B

Figure 4. 5 Principal components Scatter Plot showed the distance of variance among all samples under study (A) and between common list of differential expression proteins under water stress (B).

4.4.Discussion

The proteomic analysis identified 309 DEPs between Wheat (*Triticum aestivum*) samples under water stress, which is consistence with proteomic study were done wheat under heat stress (Wang et al., 2018). The highest up regulation DEPs were recorded in the drought-tolerant wheat cultivar (Cv2), precisely at the vegetative and grain filling stages. This might suggest higher ability of the tolerant cultivar to di-novo synthesise proteins that allow the plant to cope with the stress (Mahajan and Tuteja, 2005, Marček et al., 2019). This was particularly apparent mainly for proteins involved in the following biological process, photosynthetic, carbohydrate metabolism, stress/defence/detoxification and those involved in energy metabolism. These results are consistent with the transcriptomic results obtained in the current study and with many studies such as that of (Ge et al., 2012, Shan and Ou, 2018, Koobaz et al., 2020).

4.4.1 Stress/defence/detoxification proteins

In higher plants, antioxidant enzymes in water-stress sensitive cultivars are more sensitive to environmental stress than in tolerant cultivars. Compared with the control samples, ROS-detoxifying enzymes were generally more up-regulated in the drought-tolerant cultivar (Cv2) under water stress than the drought-sensitive cultivar (Cv4), as first line of defence. The antioxidant agents against ROS are essential for resolving H_2O_2 and preventing collapse of photosynthesis under stress conditions (Chakraborty and Pradhan, 2012, Mostofa et al., 2015, Koobaz et al., 2020). Thioredoxin, which is involved in ROS scavenging, was down-regulated in the Cv4 cultivar and up-regulated in Cv2 under water stress, which could improve the Cv2's resistance to stress, the thiol protease SEN102-like, which is also involved in ROS scavenging, was highly up-regulated in both cultivars under stress. This result is consistent with (Zhang et al., 2014a) who investigated in *Triticum aestivum* cv. KTC86211 seedlings.

Catalase (CAT) was founds to up regulated mainly in vegetative and grain filling stages of drought tolerant cultivar (Cv2) which also could involve in water stress deviance, this result consistence with study of Pakistani drought tolerant cultivar under drought stress(Nasim et al., 2017). Serine hydroxymethyltransferase (SHMT) is enzymes involved in the photorespiratory pathway (Cruz de Carvalho, 2008). It found to mitigate oxidative damage by minimizing production of reactive oxygen species (ROS) at the chloroplast in Arabidopsis thaliana under biotic and abiotic stress (Moreno et al., 2005). In current study, SHMT up regulated in flowering stage of both cultivars

under water stress, which consistence with studies that found that SHMT was up regulated in the flag leaves of drought tolerant Rice cultivar at reproductive stage (Raorane et al., 2015) and in leaves of *Triticum turgidum* ssp. dicocoides genotypes under drought stress (Budak et al., 2013). however, SHMT showed down regulated in vegetatve and grain filling stage of Cv2 may related to high regulation of several defence proteins, therefore the SHMT not signalling in these tissues. Current study found that aldehyde dehydrogenase (ALDH) was increased in the grain filling stage of the Cv2 cultivar only. This enzyme plays a role in the detoxification of toxic aldehydes from lipid peroxidation due to the formation of ROS during drought stress (Ford et al., 2011). This result is consistent with (Guo et al., 2009)'s results obtained in barley under drought stress and Ford et al. (2011) showed an increase in ALDH in drought-tolerant wheat cultivar but not in sensitive cultivars. In other hands, the most proteins involved in oxidation/reduction process were don regulated in drought sensitive cultivar (Cv4) such as dihydrolipoyl dehydrogenase and Glutathione reductase, which may improve the low performance of Cv4 under water stress. Although, oxidoreductase At4g09670 which identified as part of Cytosol in Arabidopsis thaliana cell and involved in oxidation-reduction process (Consortium, 2019), it found in current study oxidoreductase At4g09670 was highly up regulated in drought sensitive cultivar (Cv4) than in drought tolerant cultivar (Cv2), this could related to highly accumulation of oxidative stress productions under waters stress (Fracasso et al., 2016).

To sum up, my results demonstrate the importance of the proteins/genes responsible of H_2O_2 scavenging and detoxification in wheat cultivars at all growth stages under water stress (Koobaz et al., 2020) and possible role of increased efficiency of this process in the resistance mechanism to water-stress in wheat.

4.4.2 Photosynthetic proteins

Drought stress is known to reduce the photosynthetic rate and the extent of this decrease depends on osmotic adjustment and cultivar differences (Arnau et al., 1998; Liu et al., 2013; Gao et al., 2011; Gregorová et al., 2015). Restricted CO_2 diffusion occurs due to closed stomata and lower ATP content resulting from the loss of ATP synthase complex (Tezara et al., 1999). The photosynthesis rate reduces under water stress due to active down-modulation and fragmentation of RuBisCO (Michaletti et al., 2018) or/and photoinhibition (Johnová et al., 2016, Wang et al., 2016a). In the current study, the growth stage and the cultivars' differences played an essential role in the responses of the two cultivars to water stress. Photosynthesis-related proteins such as

Rubisco large subunit (Ribulose bisphosphate carboxylase) , photosystem I reaction centre subunit (psaK), light harvesting complex proteins (chlorophyll a-b binding protein) and phosphoribulokinase were highly decreased in the drought-sensitive cultivar (Cv4) comparing with drought-tolerant cultivar (Cv2) at all growth stages. Carbon fixation enzymes in the Calvin cycle such as glyceraldehyde-3-phosphate dehydrogenase that up regulated in vegetative and grain filling stage of Cv2 only. This down regulation in carbon fixation in drought sensitive cultivar (Cv4) could affect the collection of solar radiation in the thylakoid membrane of plant chloroplasts (Schmid, 2008) and consequently down-regulate Rubisco and photosynthesis (Michaletti et al., 2018).

It was noted that the probable plastid-lipid-associated protein 2, (PAP2) was up-regulated in Cv4 at all growth stages and in the grain filling stage of Cv2. This protein can decrease the photooxidative stress imposed by water stress, which could contribute to the ability of Cv4 to survive under water stress (Youssef et al., 2010). However, in the results from the current study this the up- regulation of this protein was not enough to maintain yield in Cv4 under water stress. In addition Curvature Thylakoid 1d was up regulated only in drought tolerant cultivar at all growth stages, which can assist photosynthesis under water stress by adjustment of grana diameter, the down regulation of Curvature Thylakoid 1d could effect on thylakoid plasticity of grana and will compromises regulatory mechanisms in plastid such as the photosystem II repair cycle and state transitions (Pribil et al., 2018, Johnson and Wientjes, 2020), current study is agree with studies on Cassava leaves (Chang et al., 2019), on maize leaves (Zea mays L.) (Shao et al., 2016) and on two wheat (*Triticuma estivum L*.) cultivars under drought stress.

NADPH-protochlorophyllide oxidoreductase is an essential enzyme that catalyzes the photoreduction of protochlorophyllide to chlorophyllide in chlorophyll biosynthetic process (Kwon et al., 2017, Schoefs, 2001), it was up regulated in flowering and grain filling stages of both cultivars under water stress, which would contribute in enhancing photosynthesis under water stress, that might be a common mechanism in response to drought stress in both drought-tolerant and sensitive cultivars (Yamazaki et al., 2006, Sakuraba et al., 2013). this results consistence with study in two wheat (*Triticum aestivum L*.) cultivars under drought stress (Cheng et al., 2016) and under heat stress (Lu et al., 2017).

It found that CBS domain-containing protein involved in voltage-gated chloride channel activity (Consortium, 2019) was up regulated in flowering and grain filling stage of drought tolerant

cultivar (Cv2) and highly down regulated in drought sensitive cultivar (Cv4), the increasing of CBS domain-containing protein could enhance photosynthesis in (Cv2) by regulate thioredoxin in chloroplast and reduce the H_2O_2 level (Shin et al., 2020).

Glucan endo-1,3-beta-glucosidase was found to involve in plant defence as maintaining cell wall shape and protecting them from osmotic lysis (Huntley et al., 2015). In current study, Glucan endo-1,3-beta-glucosidase was up in vegetative and grain filling stages of Cv2 cultivar which may play an important role in wheat resistance under water stress (Gupta et al., 2019). this results consistence with study on common bean under drought stress (Yang et al., 2011), on spinach (Spinacea oleracea L.) under cold stress (Hincha et al., 1997), on barly under salt stress (Mostek et al., 2016), on *Arabidopsis thaliana* under drought stress (Xu et al., 2020) and on wheat under drought stress (Faghani et al., 2015).

Photosynthesis proteins and light harvesting complex proteins increased more in Cv2 in the vegetative stage and the grain filling stage than in the Cv4 cultivar. This could be good evidence of growth stage dependent response to water stress. These differentially regulated proteins might be responsible for the stronger drought resistance of Cv2 compared to Cv4 and might explain the low growth performance of the sensitive cultivar (Cv4) shown in the second chapter. This result is consistent with (Aranjuelo et al., 2011; Ford et al., 2011; Ge et al., 2012; Kausar et al., 2013)

4.4.3 Carbohydrate metabolism proteins

Plants' carbohydrate metabolism proteins were measured to show the potential consequence of water stress on plants cell due to a significant reduction in photosynthetic rate and energy metabolism under stress (Basu et al., 2016). Stress can create an imbalance between photosynthetic carbon uptake and the use of photoassimilates, which influences the sugar concentration in the plant (Michaletti et al., 2018). In other words, the carbohydrate metabolism proteins are related to energy process in plant cell (Wang et al., 2016b). In the current study, it was found that the proteins involved in the carbohydrate metabolism regulated under water stress in both cultivars at all growth stages. Xylose isomerase is an important enzymes involved in the Xyloglycan biosynthesis (Kanehisa et al., 2019, Kanehisa, 2019). Xyloglycan is polysaccharide in (Choi et al., 2011; Pauly and Keegstra, 2016). In our case, it found that Xylose isomerase was up regulated in vegetative and grain filling stages of (Cv2) and flowering stage of (Cv4), which may contribute in cell wall adaptation under stress (Le Gall et al., 2015), this results consistence with study on early stages of wheat grain development (Nadaud et al., 2010) and rice under drought (Yang et al., 2006).

Triosephosphate isomerase is an enzyme of the glycolysis pathway, where it catalyses isomerisation of dihydroxyacetone phosphate and D-glyceraldehyde-3-P (Budak et al., 2013). Increased activity of triosephosphate isomerase by using up triose or glucose sugar via glycolysis prepares for the damages caused by oxidative molecules and increases the energy state of plants under stress (Gao et al., 2011; Kaur et al., 2015). In this study, it was found that the triosephosphate isomerase was more up regulated in the vegetative and flowering stages of the tolerant wheat cultivar (Cv2) than in the sensitive wheat cultivar (Cv4) at same stages. This could help the Cv2 to prevent the accumulation of the side products of primary metabolic pathways such as methylglyoxal (MG), which is generated through the breakdown of triose sugars (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate) under stresses (Kaur et al., 2015, Hoque et al., 2016, Kaur et al., 2016). However, Michaletti et al. (2018) found in two Iranian wheat cultivars that triosephosphate isomerase was more up-regulated in a drought sensitive wheat cultivar (Bahar) than in a drought tolerant one (Kavir). This is due to an inactivation of oxidative phosphorylation, and consequently increased glycolysis to compensate for the lower ATP yield in the wheat (Caruso et al., 2009). From another point of view, the differential response of this enzyme could be related to local cultivar differences or/and different strategies might be used by the tolerant wheat cultivar (Kavir) to prevent cell damage under stress. Other carbohydrate metabolism enzymes shown to be affected by water stress include alpha-mannosidase, Pyruvate kinase and Glycosyltransferase. Alpha-mannosidase is a key enzyme in N-glycan processing (von Schaewen et al., 2015), the TaMP gene that encodes α-mannosidase was shown to be induced in wheat (*Triticum aestivum* L.) under abiotic stresses (Wang et al., 2020). This is consistent with current results, which indicated that alpha-mannosidase was up-regulated to a greater extent at the vegetative and flowering stages of drought tolerant cultivar (Cv2) than in drought sensitive cultivar (Cv4) which would regulate cellulose biosynthesis, similarly, the analysis of glycosidase mutants in Arabidopsis thaliana under salt stress indicated that N-glycan modification affects salt tolerance (Kang et al., 2008). Pyruvate kinase involved in reactions and pathways resulting in the breakdown of a carbohydrate into pyruvate (glycolysis) and it is very important for the regulation of glycolysis pathway under stress conditions (Li et al., 2016). In current study, found that pyruvate kinase was up regulated in vegetative stage and down regulated in flowering and grain filling stages of both cultivars, although, the up regulated fold change at vegetative stage of drought tolerant cultivar more than drought sensitive cultivar, the pyruvate kinase regulation could related to drought

resistance of vegetative stage under water stress which consistence with (Guo et al., 2018) in 4 weeks old drought-tolerant wheat genotype (JingDong-17) under drought stress. Also, (Li et al., 2019b) study showed an increasing in the expression of pyruvate kinase provides a precursor and energy for rice drought response. This could explain the sugar production slightly change to decrease in vegetative stage comparing flowering and grain filling stage of Cv2 under water stress. Glycosyltransferase is one of carbohydrate metabolism proteins which required for the regulation of cellulose biosynthesis in the (Zhang et al., 2016) and strengthening of cell walls (Zhou et al., 2009). In current study Glycosyltransferase was up regulated in vegetative stage of drought tolerant cultivar (Cv2) which could help plant cell to reduce the stress generated from water stress through strengthening the synthesis of cell walls, this results consistence with (Zhang et al., 2020) in vegetative stage of plant drought tolerant maize. Beside up regulation of malate dehydrogenase in grain filling stage of drought tolerant cultivar (Cv2) which could contribute to accumulate malic acid in shoot under water stress to maintain intracellular ionic balance and nutrient uptake to resist drought stress (Guo et al., 2018; Cui et al., 2019) this results consistence with study on drought-tolerant wheat genotype (JingDong-17) and drought-sensitive wheat genotype (JingDong-8) by (Bartoli et al., 2005; Guo et al., 2018).

All regulation in carbohydrate metabolism proteins were done to re-establish homeostasis in wheat cell under water stress, that apparent in drought tolerant cultivar (Cv2) than drought sensitive cultivar (Cv4), this results consistent with (Cheng et al., 2016) that study two Chinas wheat cultivar under drought stress.

4.4.4 Amino acid metabolism

The amino acid metabolism notably responds to abiotic and biotic stresses (Choudhary et al., 2009, Manaa et al., 2011, Pandey et al., 2012). An increase in amino acid metabolism could involve osmoregulatory and ROS scavenging compounds, which may prevent damage to cell functions. Also, it may be involved in photorespiration for maintaining electron flow to prevent photoinhibition under stress conditions (Caruso et al., 2009). Therefore amino acid responses can be an indicator to water stress in wheat cultivars (Qin et al., 2016; Yadav et al., 2019a,). In current study, it was found that the glycine cleavage system P protein (glycine dehydrogenase) and the glycine cleavage system H protein were up-regulated in Cv2 in all three stages, and down-regulated in Cv4 in all three stages. This could explain the increase in expression levels of some proteins involved in osmo-regulator synthesis such as proline (Caruso et al., 2009).

The amino group was transferase into other amino acid during nitrogen assimilation by aminotransferase such as aminomethyltransferase, alanine aminotransferase and Aspartate aminotransferase which were indicted in both cultivars in current study. Aminomethyltransferase, was up-regulated in Cv2 in all growth stages, this result is in line with that found by (Caruso et al., 2009, Cheng et al., 2016) in wheat (*Triticum durum*) and (*Triticum aestivum* L.) respectively under drought stress. Aspartate aminotransferase was found to be down regulated in both cultivars which consistence with (Zhou et al., 2016) in wheat under drought stress, except, grain filling stage of drought tolerant cultivar (Cv2) that showed an up regulation on Aspartate aminotransferase level under water stress. These up regulation of aminotransferase could enhance the amino acid metabolism and the synthesis of other metabolites derived from amino acids under water stress (Wang et al., 2016a). Although, alanine aminotransferase 2 isoform X2 was found to be down-regulated in both cultivars at all growth stages, which could affect the reversible reaction of the conversion of alanine and 2–oxoglutarate into pyruvate and glutamate (Kendziorek et al., 2012), this in turn could regulate the respiratory oxygen consumption via the activation of the alternative oxidase in mitochondria (Gupta et al., 2009).

Degradation-related proteins such as proteasome subunit alpha type-6, proteasome and ATP-dependent were found to be up-regulated under water stress in both cultivars in all three growth stages. Proteases and proteasomes could play an important role in maintaining strict protein quality control and degrading specific sets of proteins to release amino acids might happen in response to water stress in both drought-tolerant and sensitive wheat cultivars, as shown by Hameed et al. (2011), Cheng et al., (2016); Stone (2019).

Chaperones proteins such as Hsp70-Hsp90 which known to be stress-responsive proteins that involved in processes associated with stomatal closure. Chaperones proteins could help plant to preserve water envelopes around biomolecules and prevent the target biomolecules from denaturation (Kosová et al., 2016). This protein was up regulated only in drought tolerant cultivar (Cv2) at all growth stages, this results consistence with (Budak et al., 2013; Hao et al., 2015, Cheng et al., 2015,) study on drought-tolerant wheat cultivar. Also, peptidyl-prolyl cis-trans (PPIases) which accelerate the folding proteins, It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides under stress (UniPort, 2019), PPIases was found to be up regulated in (Cv2) than (Cv4) at all growth stages, which could enhance folding proteins under stress, and maintenance the PS II activity (Wang et al., 2015). this results consistence with study on Sorghum

bicolor Seedlings under osmatic stress (Sharma et al., 2003), also consistence with study on Foxtail millet (Setaria italica L. P. Beauv) (Pan et al., 2018) and drought tolerant wheat cultivar (Ethos wheat cultivar) (Nykiel et al., 2019). also PPIases was identified in wheat seedling under heat stress (Singh et al., 2019).

Phenylalanine ammonia-lyase (PAL), one of the most important enzymes in the phenolic biosynthesis pathway, becomes more active during stresses (Wahid et al., 2007). In current study it founds that both cultivars at vegetative and grain filling stages were response to water stress by increasing PAL regulation which would enhance cell wall synthesis, that PAL is protein involved in lignin biosynthesis were generally increased under drought stress (Kosová et al., 2016; Wang et al., 2016a). PAL catalyzes the transformation of phenylalanine to cinnamylate in the first step of lignin biosynthesis (Baxter and Stewart Jr, 2013). this results consistence with results were found that activity of PAL was increased in the leaves of Trifolium repens (Lee et al., 2007), chilli (*Capsicum annuum* L.) (Jaswanthi et al., 2019) and tolerant wheat cultivars (Lugan et al., 2009) under the drought stress. Also, Phenylalanine ammonia-lyase (PAL) is involved in Phenylpropanoid biosynthesis which founds to be up regulated under many stresses (Vogt, 2010; Yan et al., 2020,), phenylpropanoid-derived compounds include Monolignols, flavonoids, various phenolic acids (Hodaei et al., 2018; Gharibi et al., 2019) and stilbenes (Chong et al., 2009). Monolignols which can polymerized to form lignin which is a major component in plant cell wall, that could enhance cell wall under drought stress (Yan et al., 2020). Phenylalanine ammonia-lyase (PAL) seem is to been contributed to plant growth, structural support, and various aspects of wheat responses under water stress.

4.5.Conclusions

- Based on the collected growth data in the first experiment one Cv from each group (Cv2 and Cv4) were used to conduct a comparative analysis of the differences in the transcriptome under both control (well-watered) conditions and water stress (15% PEG6000) conditions to identify the gene differences and differential transcript levels in the two Cvs under the effect of water stress.
- Leaf samples were collected from both control plants and water-stressed plants at the vegetative, flowering and grain filling stages, snap frozen in liquid nitrogen and stored at -80°C until analysis. Sent to Protein Facility (NUPPA), Newcastle University, project id

(#32), for LC/MS experiment and proteomic analysis was done in the Proteomic facility, Newcastle University.

- The experiment resulted in the ultimate comparison of 72 different samples (two cultivars, 6 technical replicates each, 2 different conditions, 3 time points).
- Proteomic analysis of two Saudi wheat cultivars have different drought tolerance under water stress at three growth stages showed tangible changes in protein levels indicated a general regulation trend of plant defence:
    - **Stress/defence/detoxification proteins**, most of proteins were up regulated in vegetative and grain filling stags of drought tolerant cultivar (Cv2).
    - **Photosynthesis proteins** were highly decreased in the drought-sensitive cultivar (Cv4) comparing with drought-tolerant cultivar (Cv2) at all growth stages. Carbon fixation enzymes in the Calvin cycle such as glyceraldehyde-3-phosphate dehydrogenase that up regulated in vegetative and grain filling stage of Cv2 only.
    - **Carbohydrate metabolism proteins** such as Xylose isomerase and Pyruvate kinase was up regulated in vegetative and grain filling stages of (Cv2).
    - **Amino acid metabolism proteins** were up-regulated in Cv2 in all three stages, and down- regulated in Cv4 in all three stages. and Phenylalanine ammonia-lyase up regulation in Drought tolerant wheat (Cv2) more than in drought sensitive wheat (Cv4).

Chapter 5 Differential regulation of targeted genes and pathways in six Saudi wheat cultivars under water stress (qRT- PCR).

5.1. Introduction

Water stress is a challenging factor affecting wheat production globally, a large variation in terms of level of drought tolerance exists among different wheat cultivars (Sallam et al., 2019; Khadka et al., 2020). Drought tolerance is a quantitative trait that involves a large number of genes encoding components of cellular signal transduction pathways and proteins and enzymes responsible for the stress-tolerance response per see (Barnabás et al., 2008; Jiang et al., 2017a). Understanding the regulation of the genes involved in drought tolerance in wheat could pave the way for improving the physiological response to drought stress in wheat cultivars, this can be informed by gene expression analysis and the identification of molecular changes under stress conditions in plants. Some Saudi local wheat cultivars are adapted to severe environments thus can be used as model to investigate the genes involved in the mechanisms of this adaptation. A such adaptation requires reprogramming of a suite of genes involved in protection of cellular functions under drought stress. To determine regulated genes, high throughput cDNA sequencing (RNA seq) using next generation sequencing (NGS) technology has become the preferred method over the last decade; a noticeable increase in transcriptomics data for various plant species occurred (Kumar et al., 2015a; Poersch-Bortolon et al., 2016b), The generated transcriptomes have helped to determine the global expression patterns of genes under a variety of conditions and start unraveling basic response mechanism to these conditions. To determine the expression pattern of single genes Northern blotting and Ribonuclease Protection Assays (RPA) were used, however, recently quantitative Real-time reverse transcription Polymerase Chain Reaction (qRT-PCR) has rapidly replaced these techniques (Sinha and Smith, 2014), due to its fast, specific and sensitive detection and quantification of targets (Bustin et al., 2009; Iehisa and Takumi, 2017). qRT-PCR is a quantitative method routinely used effectively to detect and quantify gene expression changes under environmental stress. It is also used to validate RNA-seq results (Remans et al., 2014, Bedre et al., 2019). qRT-PCR was used to validate the differentially Expressed Transcripts from RNA-Seq in maize (Kakumanu et al., 2012) and wheat (Hu et al., 2018) in response to water stress. Therefore, qRT-PCR can help to understand the underlying physiological and molecular mechanisms of tolerance to abiotic stress in plants (Wang et al., 2020). The technology is also used

to study the gene expression patterns and identification of transgenes in genetically modified crops. For instance, using qRT-PCR, Zeng et al. (2011) indicated that upregulation of expression of genes encoding chloroplastic enzymes may help increase freezing resistance in Chinese winter wheat under low temperatures.

In current study, using qRT- PCR, I have determined the change in transcript levels of 4 randomly selected genes found to be up-regulated in the Najran and Rafha wheat cultivars under water stress by RNA sequencing. The four genes included Dehydrin gene (DHn3), Bidirectional sugar transporter (Sweet), Phenylalanine ammonia-lyase (Pal5) and Serine hydroxy methyl transferase (Shmt). Dehydrin gene (DHn3) is associated with wheat response to drought stress and is one of the most studied drought-inducible gene families (LEA genes) (Suprunova et al., 2004, Li et al., 2018a, Krugman et al., 2011). Bidirectional sugar transporter (Sweet) belongs to SWEET proteins which play an important role in many essential developmental and physiological processes also was found to be highly regulated under drought stress in wheat cultivars (, Chen, 2014; Phukan et al., 2018; Gautam et al., 2019). Phenylalanine ammonia-lyase (Pal5) is involved in the biosynthesis of phenylpropanoids from phenylalanine or tyrosine and give rise to a large family of secondary metabolites (Vogt, 2010; Hu et al., 2018; Chaichi et al., 2019;Yan et al., 2020), such as phenolic compounds (Sharma et al., 2019). Elevated transcript levels of Pal have been reported in response to drought stress in plants (Gholizadeh and Kohnehrouz, 2010; Rezayian et al., 2018; Perin et al., 2019). Serine hydroxy methyl transferase (Shmt) is involved in amino acid transport and metabolism, and is mainly involved in the photorespiratory pathway to minimize production of reactive oxygen species (ROS) in the chloroplast (Moreno et al., 2005). Therefore, Shmt genes were found to be regulated under drought stress in wheat (Budak et al., 2013). I aimed in this part of the work to (1) confirm the transcriptomics results by comparing the change in transcript levels determined by qRT-PCR to transcriptomics results for the 4 genes (2) compare the expression patterns of these genes in six wheat cultivars 181 Jizan (Cv1), 193 Najran (Cv2), 357 Sama (Cv3), 377 Rafha (Cv4), 562 Ma'ayah (Cv5) and 981 Najd (Cv6) whose response to water stress was studied in this work. This would widen our prediction of regulated genes and pathways in different wheat cultivars under water stress.

5.2.Materials and Methods

5.2.1.Plant growth conditions and water-stress treatments

a. Plant growth conditions

Plant growth conditions was done similar to the previous experiment in chapter 3, paragraph (a) at P. 57

b. Plant growth

plant growth was done similar to the previous experiment in chapter 3, paragraph (b) at P.57

c. Water-stress treatments

Plants were grown under two treatments similar to what have done in chapter 2, paragraph (c) at P.29

d. Plant sampling

Leaf samples were collected from both control plants and water-stressed plants at three growth stage similar to what have done in chapter 3, paragraph c) P.57 . The powdered tissue was used to extract RNA (3.2.5.1) which was stored at -80°C until analysis. RNA samples having an A260/280 ratio of 2.0 were used in qRT--PCR.

5.2.2.qRT-PCR

Four genes associated with responses to drought in plants were randomly selected to analyse their transcript levels with qRT-PCR. The analysis was performed in triplicate biological samples. Synthesis of cDNA was done with the Maxima First-strand cDNA Synthesis Kit for RT-qPCR with dsDNase #K1671 (ThermoScientific, UK) according to the manufacturer's protocol. Gene-specific primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). qRT-PCRs were performed in three technical triplicates using a Bio-Rad Real-Time PCR System with CFX Manager Version 3.1.1517.0823 following the manufacturer instructions. The qRT-PCR programme was as follows: 20 min at 50 °C, 2 min at 95 °C, followed by 39 cycles of 15 sec at 95 °C, 30 sec at 55°C, 30 sec at 72°C, read, and a final extension at 72 ºC for 5 min and finally a melting curve analysis between 45 ºC and 95 °C with a hold for 5 sec at every one ºC increment. The optimal annealing temperature was determined by running a gradient from 55 to 62.3 ºC (gel images for Pal, Ubq and EF1 as an example in the appendix (figure S5.1). Reactions were performed in a final volume of 20 uL, containing 10 uL of Powerup Sybr Master Mix (Fisher, UK), 8 uL of diluted cDNA (1:10), 1 uL of Forward primer, 1 uL Reverse primer (10 mM each). The reference gene used for the normalisation of expression

was *T. aestivum* Ubiquitin gene (GenBank: AY862401.1) (Table.S5.1). The qRT-PCR primers designed for the four genes of interest were evaluated for PCR amplification efficiencies by carrying out real-time PCR using a five-fold serial dilution of cDNA template from all biological replicate mixed of control and water stress samples. PCR percentage efficiency was calculated with efficiency equation,

Equation 3 $E = 100 * (-1 + 10 - 1/\text{slope})$

(Ferreira et al., 2006, Park et al., 2020, Schriewer et al., 2011). (table.S4. 1). Relative expression data analyses were performed by comparative quantification of the amplified products using The Pfaffl method (Pfaffl., 2002), Gene expression Equation 4.

Equation 4 $\text{Ratio} = \text{Efficiency amplification} \wedge - (\text{Ct target gene (control} - \text{treatment)}) /$ $\text{Efficiency amplification} \wedge (\text{Ct reference gene (control} - \text{treatment)}$

5.2.3.Statistical Data Analysis

Statistical analysis was performed using SPSS 2. The analysis of the main effects and interaction effects of the four genes under study within the three methods (RTqPCR, RNAseq and Proteomic) was done using ANOVA followed by F-test analysis. Statistical significance was set up to $p < 0.05$ for both analyses. The values in tables are means ±SE.

5.3.Results

5.3.1 Comparison of water-stress induced change in transcripts levels measured by qRT-PCR and transcriptomics together with protein levels estimated by semi-quantitative proteomics in Najran and Rafha wheats

qRT-PCRs were performed to validate the RNA-Seq data (see chapter 3) and potentially the proteomic results in the 193 Najran (Cv2) (drought resistance) and 377 Rafha (Cv4) (drought sensitive) wheat Cvs. Also, the change in transcript levels for these genes was checked in the remaining four wheat Cvs used in this study to widen our prediction of genes and pathways regulated under water stress in different wheat cultivars. In General, high consistency between qRT-PCR results and the RNAseq results in terms of change in transcript levels under water-stress of the Dhn3, Sweet, Pal5 and Shmt genes was seen the Najran and Rafha Cvs. (figure 5.1). However, a lower correlation between water-stress induced change in relative protein levels and transcripts levels for the four genes (figure 5.1) (table S.4 1).

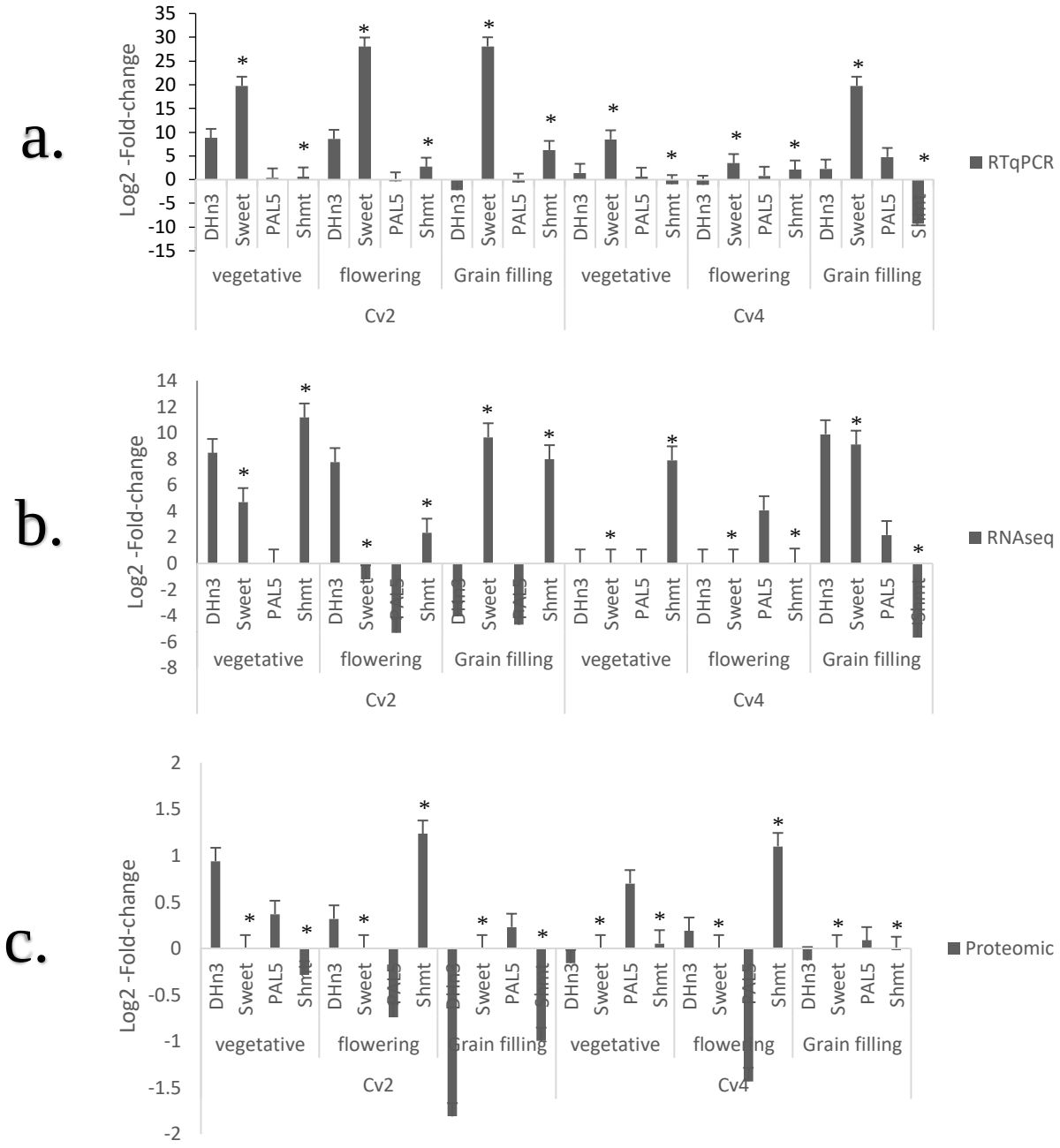


Figure 5. 1 Water-stress induced fold change (Log2) in the transcript levels of Dehydrin 3 (DHn3), Bidirectional sugar transporter (Sweet), Phenylalanine ammonia-lyase (Pal5), Serine hydroxy methyl transferase (Shmt) genes. Transcript levels were monitored by a. qRT-PCR and compared to b. RNAseq and c. Proteomic quantifications for the four genes in wheat (*Triticum aestivum* L) cultivars: 193Najran (Cv2) and 377 Rafha (Cv4)) at three growth stages: vegetative, flowering and grain filling. Water-stress was imposed by watering plants with 15% PEG6000. n=3, bars are standard errors. The mean difference between four genes within the three methods (RTqPCR, RNAseq and Proteomic) are significant by F-test at the .05 level.

5.3.2 Expression patterns of Dehydrin gene (DHn3) in the six wheat cultivars under water stress.

qRT-PCR results have shown that DHn3 transcript levels have changed in Cv2 and Cv4 wheat Cvs in the same direction to that indicated by transcriptomics results under water-stress. However, the amplitude of change in transcript levels measured by qRT-PCR and transcriptomics was not exactly the same. Moreover, there was a low correlation between transcript levels and protein levels measured by proteomics with respect to level and trend of change (figure 5.1).

Significant differences were observed between samples (control and treatment) for dehydrin expression in all cultivars under study. It was found that at least one growth stage had an increase in DHn3 expression under water stress. Transcript levels for Dehydrin 3 (DHn3) increased under water-stress in all cultivars at the vegetative stage except for Cv6 which has shown a 4.05-fold down regulation. The highest up regulation of Dhn3 occurred at the vegetative and flowering stages in Cv2 (figure 5.2).

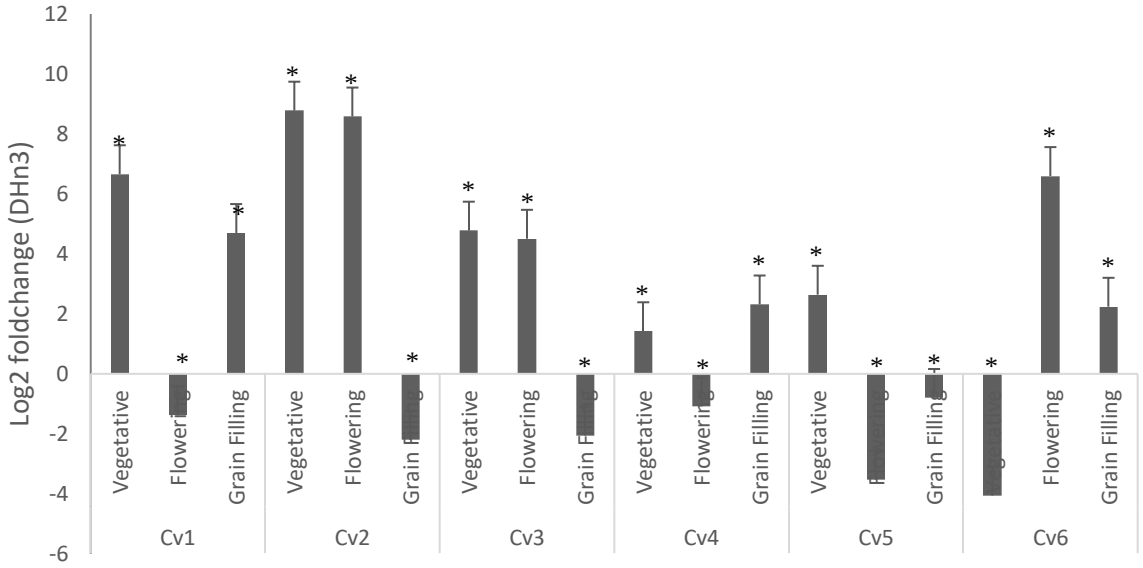


Figure 5. 2 Water-stress induced fold change (Log2) in transcript levels of Dehydrin 3 (DHn3) gene in four Saudi wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) monitored by qRT-PCR at three growth stages, vegetative, flowering and filling. Water-stress was imposed by watering plants with 15% PEG6000. n=3, bars are standard errors.

*The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 10.626, P value > 0.000

5.3.3 Expression patterns of the bidirectional sugar transporter (Sweet) in the six wheat cultivars under study.

Analysis of transcript levels by RT-qPCR clearly indicates that Sweet gene was induced in the 6 studied wheat cultivars under water stress. The highest transcript levels were recorded in Cv1 with 32 and 34 log2-folds increase at vegetative and flowering stage respectively, and 28 log2-folds increase at the flowering and grain filling stage of Cv2 cultivar (figure 5.3). The lowest change in Sweet transcript levels was recorded in Cv3, Cv4 and CV5 with no change (0.04 log2-fold increase) at vegetative stage of Cv3 under water stress (figure 5.3). Comparing with transcriptomic and proteomic results of Cv2 and Cv4, qRT- PCR results showed that Sweet expression pattern showed good correlation with gene expression profiles obtained from transcriptomics data with respect to trends of regulation (figure 5.1). However, the gene expression in vegetative stage of Cv4 was not detected in transcriptomics data nor proteomic data. Also, qRT- PCR results showed that Sweet transcript levels at flowering stage of Cv2 were inconsistent with transcriptomics and proteomics data.

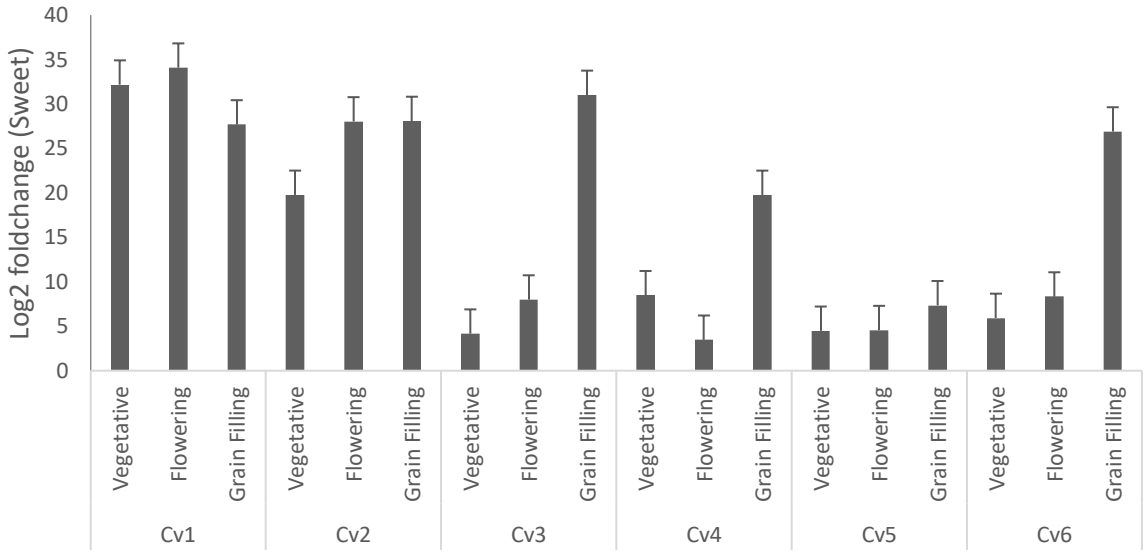


Figure 5. 3 Water-stress induced fold change (Log2) in transcript levels of of Bidirectional sugar transporter (Sweet) monitored by qRT-PCR in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages: Vegetative, Flowering and Grain Water-stress was imposed by watering plants with 15% PEG6000. n=3, bars are standard errors.

*The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 10.626, P value > 0.466

5.3.4 Expression patterns of Phenylalanine ammonia-lyase (Pal5) in the six wheat cultivars under study.

Transcript levels for the Pal5 gene were found to be unchanged under water stress in Cv2 (0,38 and 0.65 Log2-folds decrease at flowering and grain filling stage respectively). While in a slight decrease was observed in Cv1 and Cv5 at the vegetative and grain filling stages (1.58 Log2-folds decrease at the grain filling stag). Also, Cv3 showed low expression at all growth stages under water stress. In contrast, high up regulated fold-change of Pal gene detected in flowering stage of Cv1 (0.84 Log2-fold) and Cv5 (6.1 Log2-fold). also, high up regulated fold-change of Pal gene detected in vegetative stage of Cv2 (0.455 Log2-fold) and Cv6 (3.77 Log2-fold) (figure 5.4). Results showed that Pal5 gene showed good correlation with differential expression profiles obtained from transcriptomics data with respect to trends of regulation. However, some inconsistencies between the qRT-PCR and transcriptomics data were detected in vegetative stage (figure 5.1).

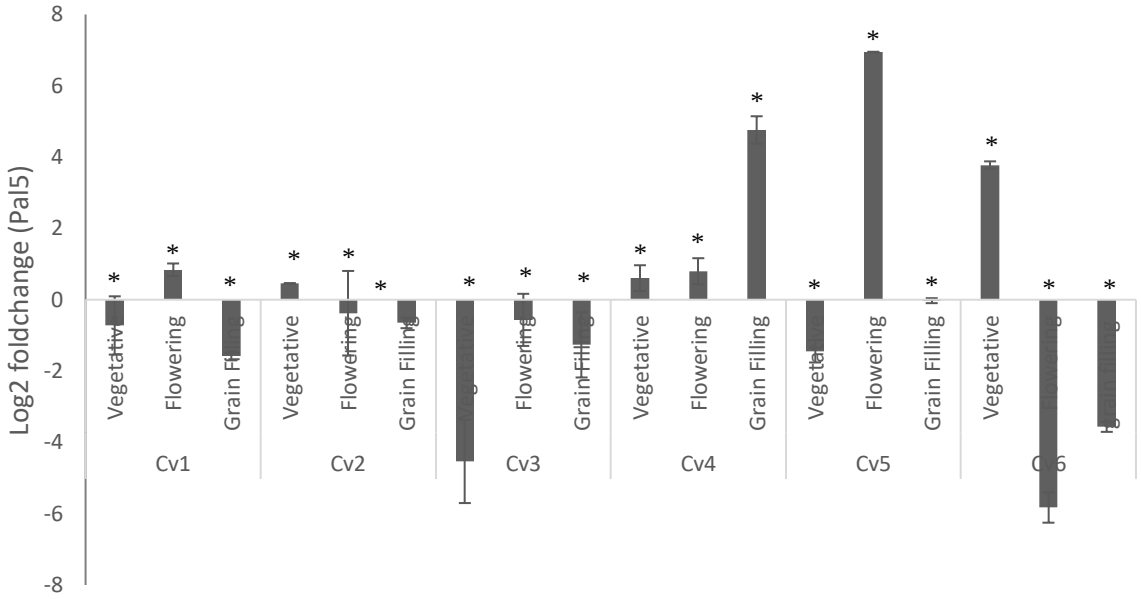


Figure 5. 4 Water-stress induced fold change (Log2) in transcript levels of Phenylalanine ammonia-lyase (Pal5) monitored by qRT-PCR in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages: Vegetative, Flowering and Grain filling. Water-stress was imposed by watering plants with15% PEG6000. n=3, bars are standard errors.

*The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 10.626, P value > 0.000

5.3.5 Expression patterns of Serine hydroxy methyl transferase (Shmt) in the six wheat cultivars under study.

Expression patterns of Serine hydroxy methyl transferase (Shmt) under water stress was highly up regulated in Cv1 and Cv2 at grain filling stage by (6.24 and 17.79 Log2-Fold). and highly decrased level at grain filling stage in Cv3, Cv4, Cv5 and Cv6 (2.96, 9.28, 8.03 and 3.16 Log2-fold respectivly) (figure 5.5). At the flowering stage there was moderate to high increasein all cultivars. Results showed high consistency of expression patterns of Shmt gene at all growth stages of Cv2 and Cv4 between transcript and proteins data.

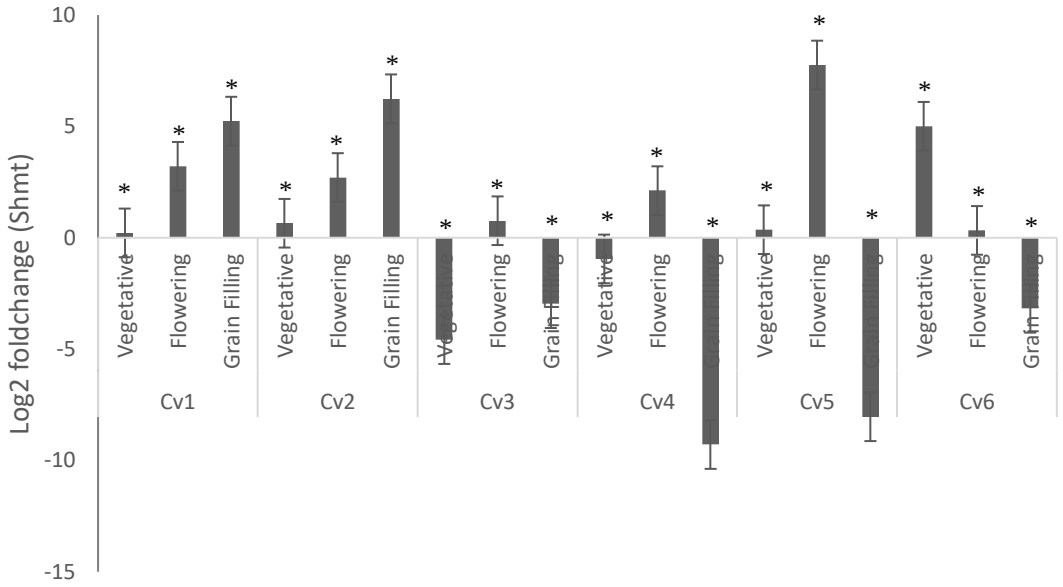


Figure 5. 5 Water-stress induced fold change (Log2) in transcript levels of Serine hydroxy methyl transferase (Shmt) monitored by qRT-PCR in wheat (*Triticum aestivum* L) cultivars: 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages: Vegetative, Flowering and Grain filling. Water-stress was imposed by watering plants with15% PEG6000. n=3, bars are standard errors.

*The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 10.626, P value > 0.000

5.4.Discussion

To validate the results from the transcriptomic data, four differentially regulated genes (Dehydrins gene (YSK2 dehydrin 3), Bidirectional sugar transporter (Sweet), Phenylalanine ammonia-lyase (Pal5) and Serine hydroxy methyl transferase (Shmt), which represented up-regulated, unchanged, and down-regulated genes identified through the RNAseq studies, were selected and specific primers were designed for analysis using quantitative real-time PCR. These four selected genes were involved in stress defense biological process such as L-phenylalanine catabolic process, phenylpropanoid metabolic process, cinnamic acid biosynthetic process, carbohydrate metabolism, carbohydrate transport, amino acid metabolism and secondary metabolites. The results showed that most selected genes showed good correlation with differential expression genes profiles obtained from the transcriptomic results with respect to trends of regulation.

5.4.1 Expression patterns of Dehydrins gene (YSK2 dehydrin 3) in the six wheat cultivars under study.

Most of the Dhn genes are up regulated by various stresses that cause cellular dehydration such as drought, salt and extreme temperature (Lopez et al., 2003; Suprunova et al., 2004; Peng et al., 2008; Yu et al., 2018). The up regulation of transcript or protein levels of Dehydrin in plant tissues has been shown to be related to increased drought tolerance (Suprunova et al., 2004; Škodáček and Prášil, 2011). It has also been shown that Dehydrin transcripts respond more rapidly to changes in environment while proteins had a more steady change (Kosová et al., 2014). Wheat seedlings are highly tolerant to water-stress and have the capacity to withstand long dehydration period (Koobaz et al., 2020), this was obviously shown in the up regulation of YSK2 Dhn 3 at the vegetative stage in the six studied wheat cultivars under drought. Structural YSK2 dehydrins usually respond with up regulation under strong dehydrative stresses such as drought, frost and salt as well as under ABA in common wheat (Kosová et al., 2014). The qRT-PCR analysis has shown that the "YSK2 Dhn 3" was up regulated in both tolerant and sensitive wheat cultivars; Suprunova et al. (2004) indicated the same results in resistant and sensitive wild barley genotypes. The qRT-PCR analysis has shown that 193 Najran (Cv2) had an increase in Dehydrin3 transcript levels under water stress which is consistent with the highest water-stress tolerance shown by this cultivar among the wheat cultivars under study. The expression levels of YSK2 DHn3 were higher in vegetative and flowering stages in Cv2 and flowering stage in Cv6 than the remaining cultivars, these (Cv2 and Cv6) cultivars showed highly to moderate drought resistance based on the physiological and

morphological measurements in the second chapters of my thesis. The accumulation of DHn3 in drought resistance wheat cultivars is consistent with study was done by (Hassan et al., 2015) in Egyptian wheat cultivars, which showed an increase in DHn expression in vegetative stage of drought tolerant wheat cultivar than in the sensitive wheat cultivar under drought conditions. Also, agreed with many studies such as (Lopez et al., 2003) at grain filling stage of winter wheat growing in the U.S. Pacific Northwest (PNW) and with (Kumar et al., 2014) at rice (Oryza sativa L.). Some of Egyptian and American wheat cultivars were widely sowing in Saudi Arabia due to the highly adapted showed by these cultivars to desert environment. therefore, it might be sharing the same physiological response under drought with Saudi wheat local cultivars. This up regulation of YSK2 dehydrin 3 expression could be related to its protective functions, Dehydrins protect cells against oxidative damage caused by ROS generated under water stress and contribute to cellular homeostasis (Kumar et al., 2014; Shah et al., 2017). Dehydrins might also up-regulate protective mechanisms in the cell, however, it is not clear which signalling pathways are regulated by these proteins, some research has shown that DHn3 was involved in regulation via an ABA-dependent pathway (Wang et al., 2014b, Verma et al., 2017). Other research has shown that Dhn genes up-regulate stress-responsive proteins in response to drought and other abiotic stresses through a series of “pleiotropic effects” that may be involved in ROS scavenging. (Vítámvás et al., 2015; Shah et al., 2017; Zhang et al., 2018,).

5.4.2 Expression patterns of Bidirectional sugar transporter (Sweet) in the six cultivars under study

SWEET (The Sugars Will Eventually be Exported Transporter) or Sugar transporter genes in wheat (*Triticum aestivum* L.) play an important role in plant development and stress responses (Gautam et al., 2019, Spinner et al., 2015). Bidirectional sugar transporter (SWEET) is mainly involved in carbohydrate transport mediated both via low-affinity uptake and efflux of sugars across the membrane (Uniprot, 2020). The qRT-PCR analysis showed that Sugar transporter (Sweet) gene has high expression in all studied wheat cultivars under water stress. This could be related to the high drought tolerance of the studied Saudi wheat cultivars due to adaptation to the drought conditions prevailing in Saudi Arabia, this result is in agreement with (QIN et al., 2020) which concluded that hexaploid wheat has the flexibility to adapt to ever changing environments based on sweet gene expression patterns.. Also, SWEET genes are known as susceptibility genes to plant development and stress responses (Gao et al., 2018, Gautam et al., 2019) which may explain the high expression of this gene in both resistant and sensitive wheat cultivars under drought. The expression of this gene can affect the accumulation of sugar molecules in vacuoles, which could maintain cell homoeostasis under drought stress (Chardon et al., 2013, Guo et al., 2014, Klemens et al., 2014) . In addition, sweet genes help transport sugars from their synthesizing organs such as leaves and stems to other tissues such as roots to enhance plant water absorption or searching (Griffiths et al., 2016, Li and Sheen, 2016). My results are consistent with this, high expression levels of Sweet gene in Cv1, Cv2 and Cv3 could illustrate the significant increase showed by root length. however, some of the qRT-PCR results of sweet transcript levels showed inconsistencies with transcriptomics and proteomics data; this could be due to the sensitivity of qRT-PCR which depends mainly on high quality RNA template that may be affected by extraction and storage, especially when the transcript level is low. In addition, both qRT- PCR and transcriptomics are quantitative methods that may vary, creating a slight inconsistency (Khan, 2014). In other words, in qPCR we amplify only one short specific region of cDNA. while in RNAseq the mapping and read counting strategy of transcript numbers can be biased. Gene-Count in RNAseq could be a couple of library pieces coming from the same RNA molecule, which will be mapped to the different areas/exomes, especially if the sequence is long. Which subsequently results in a couple of Gene-Count-hits coming from the same RNA molecule. However the comparison between samples is anyway valid, because they are treated in the same way.

5.4.3 Expression patterns of Phenylalanine ammonia-lyase (Pal5) in the six wheat cultivars under study.

Phenylalanine ammonia-lyase (PAL) is a key enzyme that mediates carbon flux from primary to secondary metabolism in plants (Barros and Dixon, 2020), the PAL gene is widely present in higher plants (Yan et al., 2019). Also, PAL acts as positive regulator in the phenylpropanoid pathway (Kim and Hwang, 2014) and a positive regulator of rice allelopathic potential (Fang et al., 2013). qRT-PCR analysis showed that PAL gene was down regulated at grain filling stage of all wheat cultivars under study, which can be related to the high phenolic content accumulated during this developmental stage. Noticeably, Cv2 had a down regulation of Pal gene which might have resulted in decreased conversion of l-phenylalanine into cinnamic acid (Shu et al., 2011). RNAseq and proteomics output showed an up regulation of Phenylpropanoid pathway and phenylalanine pathway in this wheat cultivar under water stress which is consistent with the role of these pathways under water-stress. This results is in agreement with (Fang et al., 2013; Li et al., 2017). As I mentioned in the third chapter, Cv2 coped with water stress by up regulating phenylpropanoid biosynthesis and lignin catabolic process which could be part of the wheat antioxidative system (Gholizadeh and Kohnehrouz, 2010). The qRT-PCR analysis showed a variety in Pal gene responses measured as fold change in transcript levels in the wheat cultivars under study. It is known that the change in Pal expression could be specific to the species, tissue, developmental stage and stress type (Munns and Tester, 2008; Arbona et al., 2009; Karowe and Grubb, 2011; Pandey et al., 2015). In conclusion, all the studied Saudi wheat cultivars have shown relatively high PAL expression levels with a change in transcript levels under water stress dependent on the Cultivar and the developmental stage. Consequently, it is expected that phenolic compounds would be high in the tissues of these plants and their level vary with the Cultivar and the developmental stage

5.4.4 Expression patterns of Serine hydroxy methyl transferase (Shmt) in the six cultivars under study.

SHMT (serine hydroxymethyltransferase; EC 2.1.2.1) catalyses reversible hydroxymethyl group transfer from serine to H4PteGlun (tetrahydrofolate) by exchange of the pro-2S proton of glycine with solvent protons, yielding glycine and 5,10-methylenetetrahydrofolate (Kim et al., 1997). Photosynthetic-inhibition is one of the primary detrimental effects of water stress due to stomatal closure (Ghotbi-Ravandi et al., 2014; Patro et al., 2014). Patro et al. (2014) and Voss et al. (2013) reported that photorespiration occur due to stomatal closure could protect photosynthesis, because it removes toxic 2-phoshoglycolate made by oxygenase activity of ribulose-1,5- bisphosphate carboxylase/oxygenase and retrieves its carbon as 3-phosphoglycerate. SHMT (serine hydroxymethyltransferase) is one of the key enzymes involved in the photorespiration pathway converting glycine to serine in mitochondria (Liu et al., 2019b). It found that reduced SHMT portions amounts and activity would impacted leaf metabolism leading to proline under accumulation and overaccumulation of polyamines, and increasing sensitivity to salt in Arabidopsis thaliana (Liu et al., 2019b). In addition SHMT was found to be highly expressed in drought tolerant plants (Moreno et al., 2005; Wang et al., 2016a; Liu et al., 2019b,). Results in current study showed that Cv2 which is considered as drought tolerant among the six Saudi wheat cultivars under study had an increased SHMT gene expression at all growth stages under water stress. Moreover, Sensitive wheat cultivar (Cv4) showed significant ($P > 0.00$) decrease SHMT gene expression in vegetative and grain filling stages, this could negatively impact the Calvin Cycle activity and CO_2 assimilation (Liu et al., 2019b) which would effect on plant growth. This was clearly shown by the sensitive wheat cultivars (Cv3, Cv4, Cv5 and Cv6). A strong correlation between RNA-seq and qRT-PCR data was observed. These findings were consistent with the physiological and molecular results recorded in Saudi wheat cultivars under current study and other different plants such as Australian wheat cultivars (Yadav et al., 2019a), transgenic wheat (*Triticum aestivum* L.) (Qin et al., 2016), wild and modern wheat (*Triticum turgidum* ssp) (Budak et al., 2013) and Indica rice genotypes (Mishra et al., 2016).

5.5.Conclusion

- Based on the collected transcriptomic and proteomic data in the third and fourth chapter, four drought related genes were determent in six wheat cultivars.
- **Dehydrins gene (YSK2 dehydrin 3)** is associated with wheat response to drought stress. it was up regulation at the vegetative stage in the six studied wheat cultivars under drought.
- **Bidirectional sugar transporter (Sweet)** belongs to SWEET proteins which play an important role in many essential developmental and physiological processes also was found to be highly up regulated under drought stress in all six wheat cultivars at the three stages.
- **Phenylalanine ammonia-lyase (Pal5)** is involved in the biosynthesis of phenylpropanoids, was down regulated at grain filling stage of all wheat cultivars under study, which can be related to the high phenolic content accumulated during this developmental stage. Noticeably, Cv2 had a down regulation of Pal gene.
- **Serine hydroxy methyl transferase (Shmt)** is involved in amino acid transport and metabolism showed that Cv2 which is considered as drought tolerant among the six Saudi wheat cultivars under study had an increased SHMT gene expression at all growth stages under water stress. Moreover, Sensitive wheat cultivar (Cv4) showed significant ($P > 0.00$) decrease SHMT gene expression in vegetative and grain filling stages.
- The results illustrated that the change in transcript levels of selected DEGs determined by qRT-PCR correlated highly to that obtained by RNA-Seq analysis. This demonstrates the reliability of the profiling data obtained by transcriptomics (see chapter 3). However, some of the qRT-PCR results have shown inconsistencies in terms of log2-fold-change in transcript with transcriptomics and proteomics data respectively. This could be due to the sensitivity of the qRT-PCR method which is dependent mainly on high quality RNA templates. RNA used in some qRT-PCRs might have been affected by extraction and storage, especially when the transcript level is low. The qRT-PCR analysis of six Saudi cultivars under drought stress showed high levels of expression of genes known to be up regulated under drought stress

Chapter 6 General Discussion and Conclusions

6.1 General discussion

Water stress is a major global issue with high impact on plant physiology and development. In wheat it affects several physiological responses and alters many biochemical pathways in leaves causing major crop loses (Kar, 2011; Osakabe et al., 2014). New wheat cultivars with higher tolerance to drought are needed to fulfil extra demand for wheat crop. Attempts to develop resistance to water stress in wheat often resulted in reduced productivity. Understanding the specific responses of wheat plants to water stress at different growth stages is needed to inform the development of wheat cultivars that meet the stress caused by drought conditions. Therefore, transcriptional and proteomic profiling of responses to water stress in the leaves of contrasting wheat cultivars could be a desirable approach to gain molecular insights into drought tolerance in wheat (Kumar et al., 2018). Although, plant responses to drought stress involve complex networks (Wang et al., 2016; Lv et al. 2019,) it would be essential to unravel the mechanisms underpinning resistance to drought in wheat by comparing the physiological responses to water-stress in cultivars having differential resistance to drought taking advantage of the released wheat genome sequence.

6.1.1 Molecular basis of tolerance to water-stress in the Najran wheat cultivar.

Plants respond variably to drought during their development, determining the effect of drought stress and plant response at each growth stage could facilitate the understanding of the molecular mechanisms of resistance to drought in wheat cultivars (Sinclair, 2011). Local plant cultivars are considered as a source of useful genetic variation that are better adapted to local environmental stress (Chorfi and Taıbi, 2011; Pradhan and Prasad, 2015). For instance, wild barley was used as a source of genes for breeding programs (Ellis et al., 2000; Pickering et al., 2005; Hübner et al., 2015). Therefore, studying local wheat cultivars adapted under sever environments such as Saudi wheat cultivars would help to unravel the genes and gene regulations associated with resistance to water stress (Al-Turki, 2002; Alghamdi et al., 2017; Al-Turki et al., 2020). Our Study showed that 193 Najran wheat cultivar which is grown in border of driest deserts in Asia (Searle, 2019) has high growth performance among six Saudi local cultivars under drought stress. This result was consistent with Albokari et al. (2016) who have studied other wheat genotypes grown Saudi Arabia under similar environment. Water-stress induced differential expression of a large number of genes (DEGs) resulting in change

in protein accumulation. These genes and proteins were involved in major physiological pathways such as Photosynthesis, Amino acid metabolism, Carbohydrate metabolism and Biosynthesis of secondary metabolites. The results indicated that the 193 Najran wheat (Cv2) maintained high yield performance under water stress which attest for its high abilities to cope with water limitation. This finding was consistent with many physiological and biochemistry studies on wheat such as those of Chaves et al., (2003); Mahajan and Tuteja; (2005); Kaya et al., (2006); Ergen et al., (2009); Guo et al., (2009); El-Nakhlawy et al., (2015); Gregorová et al., (2015); Sevik and Cetin, (2015); Sheoran et al., (2015); Ihsan et al., (2016); Swamy et al.,(2017); Nagy et al., (2018). The high adaptation to drought shown by the 193 Najran wheat cultivar could be related to the up regulation of biosynthesis of many secondary metabolites such as phenylpropanoids which could be part of the wheat antioxidative system (Gholizadeh and Kohnehrouz, 2010; Cabane et al., 2012; Tattini et al., 2015). The accumulation of these compounds could enhance the cell development and root extension under water stress (Ahmad and Wani,2013). All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia-lyase (PAL) (Dixon and Paiva, 1995). The 193 Najran wheat cultivar (Cv2) showed up regulation of phenylalanine ammonia-lyase (PAL) gene at the vegetative and grain filling stages. PAL has been shown to be one of the most important enzymes in the phenylpropanoid metabolism under stress (Wahid et al., 2007). However, the flowering stage of the Najran wheat cultivar (Cv2) showed a down regulation of PAL gene, this could be due to accumulation of tans-cinnamic acid which is a precursor in the first step of the phenylpropanoid pathway. (Heldt and Piechulla, 2011) found that PAL was inhibited by tans-cinnamic acid and the phenylalanine analogue aminoxyphenylpropionic acid This result agreed with (Ma et al., 2017a) who have shown in winter wheat decreased Pal gene expression under drought stress. Phenol compounds act as strong antioxidants in plant tissues during stress (Sgherri et al., 2000; Ahmad and Wani, 2014; Jaswanthi et al., 2019; Sharma et al., 2019. Chaichi et al. (2019) and other researchers have shown that Wheat cultivars with different mechanisms of acclimation to drought could be used as donors of appropriate genes to improve the bread wheat varieties for drought resistance. Thus, the 193 Najran wheat cultivar can be a good drought stress genetic resource in wheat breeding programs.

6.1.2 Leaf-age dependent responses are important for drought tolerance in wheat.

The findings of this study clearly show that water-stress affects differently wheat depending on the cultivar and the growth stage of the plant. This is highly relevant from the practical point of view as it can allow better management of wheat cultivation in arid areas like Saudi Arabia.

This would implicate the choice of the wheat Cv and the timing of cultures depending on predictions of dry periods. Plants respond variably to drought during their development and growth, by inducing responses via signalling pathways activated by hydraulic and non-hydraulic root signals (nHRS) and leaf signals, including hormones produced under water stress (Chaves et al., 2003; Fan et al., 2008; Lv et al., 2019; Mellacheruvu et al., 2019). These responses seem to be dependent on plant´s age and may vary between organs and cultivars. The findings of this research are in accordance with other studies, for example many researchers have found that when vegetative and flowering stages of growth are affected by drought, seed quality and performance is reduced from poor grain filling (Kaya et al., 2006; Guo et al., 2009; El-Nakhlawy et al., 2015; Sevik and Cetin, 2015; Swamy et al., 2017; Nagy et al., 2018). Crops specially wheat are sensitive to drought at booting, flowering and grain filling stages (Ihsan et al., 2016). Wheat's ability to resist water stress depends not only on the cultivar or genotype, but also on the developmental stage, tissue type and the environmental impact. The results of the current study could be considered as an example of age dependent response to water stress at physiological and molecular levels. It was found that the highest decrease of shoot dry weight and plant length happened at flowering and grain filling stages in all cultivars under water stress compared to the vegetative stage. Also, it was found that Cv3, Cv4 and Cv6 wheat cultivars have significant reduction in proline levels at the vegetative and flowering stages under water stress. Similarly, an age dependent response to water stress was apparent for soluble sugar accumulation. In transcriptomics data, the first comparison conducted between water stressed samples and well-watered samples at the same growth stage, found a highly up-regulated DEGs ratio at the vegetative stage of Cv2 and Cv4 wheat under water stress. This was consistent with the second comparison which was conducted between well-watered samples at different growth stages, there were highly up-regulated DEGs at the grain filling and flowering stages, compared with the vegetative stage in Cv2 and Cv4. The proteomics data, have shown an age dependent protein level, for instance, the expression of Phenylalanine ammonia lyase (PAL) was up regulated at vegetative and grain filling stage and down regulated at flowering stage in both cultivars under water stress. This could be evidence that changes in transcript and protein levels under water stress were dependent on the different wheat growth stages. Therefore studying wheat response to water-stress at different growth stages is essential for understanding the physiological response of this plant to drought which is in agreement with many studies such as those of Quarrie and Henson, (1981); Kong et al., (2010); Ruocco et al., (2019) and Berens et al., (2019)

6.1.3 Differential expression of the transcriptome and the proteome under water-stress in wheat.

Transcriptomics and proteomics analysis of the drought tolerant (193 Najran cultivar) and the drought sensitive cultivar 377 Rafha cultivar showed that the differentially expressed genes (DEGs) and differentially expressed proteins (DEPs) were mostly involved in amino acid metabolism (Phenylalanine metabolism), biosynthesis of secondary metabolites (Phenylpropanoid biosynthesis, Glutathione metabolism), carbohydrate metabolism and photosynthesis. All these pathways are well known to be the first line of defence under water stress in wheat (Caruso et al., 2009; Moumeni et al., 2011; Cabane et al., 2012; Purwar et al., 2012; Tattini et al., 2015; Cheng et al., 2016; Yadav et al., 2019). Water stress rapidly induces or dissuades expression of specific genes and proteins involved in critical physiological pathways in wheat. Transcriptomics and proteomics data of 193 Najran cultivar obtained at all growth stages showed consistency in response to water stress, such as Dehydrin (YSK2 Dhn 3) transcripts and proteins which are involved in water stress response were up regulated in both analyses. However, 377 Rafha cultivar has shown poor correlation between mRNA and protein expression data for dehydrin. Also, some key enzymes involved in photorespiration pathways such as SHMT (serine hydroxymethyltransferase) were found to be highly expressed by Transcriptomics data and had low expression by proteomics data in vegetative and grain filling stages of the 193 Najran cultivar under water stress. In contrast 377 Rafha cultivar showed high correlation between Transcriptomics data and proteomics data. Differential Expression of bidirectional sugar transporter (SWEET) which is involved in carbohydrate transport was detected only at transcript level, however the SWEET protein was not detected in both cultivars. This could be related to low correlation between mRNA and proteins often shown by wheat (Lan et al., 2012, Faghani et al., 2015). This might be due to the polyploidy nature of wheat, post-transcriptional events and post-translational modifications such as phosphorylation and glycosylation, differential stability of mRNA and proteins, and noise in quantitative analysis. (Yan et al., 2020).

6.2 Conclusions

In this study, six different wheat cultivars from three main wheat growing areas in Saudi Arabia were investigated in terms of their response to water stress at physiological and molecular levels at three growth stages. These wheat cultivars showed different responses to water-stress, results demonstrated age and genetic-dependent variations in terms of wheat responses to water stress created by 15% 6000 PEG. Among the investigated cultivars 193 Najran (Cv2) and 357 Sama (Cv3) were resistant to water stress, due to their high performance in terms of plant growth and productivity (table S1. 13). In contrast, 181 Jizan (Cv1), 377 Rafha (Cv4), 562 Ma'ayah (Cv5) and 981 Najd (Cv6) were sensitive to water-stress. This result is consistent with the known environmental conditions in the Saudi Arabia regions where these Cvs are grown. My research points out the importance of leaf age and the importance of plant age in terms of resistance to water stress. Transcriptomic analyses indicted three main physiological pathways to be regulated under water stress in the 193 Najran (water-stress resistant) and 377 Rafha (water-stress sensitive) Cvs at three growth stages. These pathways including Photosynthesis, Amino acid metabolism, and Secondary metabolism such as phenylpropanoid biosynthesis. In addition, Proteomic analysis of these two Saudi wheat cultivars showed tangible changes in protein levels indicating a general regulation trend of plant defence proteins such as Stress/defence/detoxification proteins, Photosynthesis proteins, Carbohydrate metabolism proteins, and Amino acid metabolism proteins. Transcriptomic together with the proteomic results indicated that the phenylpropanoid metabolic pathway was highly up-regulated in 193 Najran (water-stress resistant) under water stress at vegetative and grain filling stages and down-regulated at flowering stage. But no significant up-regulation of the pathway was seen under water stress at all growth stages in 377 Rafha (water-stress sensitive). This research illustrated that up regulation of Phenylpropanoid biosynthesis in leaves could play an important role in the resistance of wheat to water stress.

Transcript levels of four drought related genes including Dehydrin gene (YSK2 dehydrin 3), Bidirectional sugar transporter (Sweet), Phenylalanine ammonia-lyase (Pal5) and Serine hydroxy methyl transferase (Shmt) were determined in six wheat cultivars by qRT-PCR. The four genes exhibited differential regulation under water stress depending on the growth stage and cultivar. This result confirmed the grouping of the six cultivars into two groups one water-stress tolerant and one water-stress sensitive.

The 193 Najran (water stress- resistant) Cv responded to water stress at the vegetative stage by increasing different anti-stress responses including anti-oxidative stress responses and

inhibition of proteolysis. Same responses were induced in 377 Rafha (water stress sensitive) in the grain filling stage but not in the flowering and vegetative stages. This might suggest that 193 Najran (water stress- resistance) is equipped with the signalling and regulatory components allowing an early response to water-stress providing the plant with higher resistance to it. The late response in 377 Rafha (water stress sensitive) might explain the lower resistance to water-stress exhibited by this cultivar compared to 193 Najran (water stress- resistant), the vegetative and the flowering stages of 377 Rafha (water stress sensitive) were affected by water stress, which might explain the low performance under water stress compared to 193 Najran (water stress- resistance). The late response in 377 Rafha (water stress sensitive) would not compensate for the growth loss caused by water stress in the earlier growth stages resulting in a large yield reduction.

6.3 Future prospects

The following research initiatives would help to better understand the mechanisms of drought resistance in wheat

- Improving wheat genome annotation, due to the lack of full annotation of *Triticum aestivum* L. genome, the list of uncharacterized proteins was long. therefore, updated annotations could lead to interesting information in terms of facilitating the understanding of the regulation of physiological responses to water stress.
- Improving the correlation between transcriptomics and proteomics results by employing a more sensitive proteomics approach to identify potential proteins associated with wheat plant responses to water stress.
- Measuring the phenolics accumulation levels in the six Saudi wheat Culivars under current study to confirm the conclusion that Phenylpropanoid biosynthesis play important role in drought tolerance.
- Encouraging wheat breeding programs to take advantage of the genetic diversity of local wheat cultivars adapted to sever environments to enhance wheat yield and growth. This may be achieved by physiological and molecular studies on accessions of Saudi wheat germplasm held by the Ministry of Environment, Water and Agriculture under abiotic and biotic stress.

Appendix A Supporting information

Table S1. 1 Growth stages of wheat cultivars *Triticum aestivum* L.; 129 Al- Hassa (Cv1), 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) rating based on the Feeks scales (Large, 1954) and growth observation.

Cultivars/ Growth stage (days plants old		vegetative	flowering	Grain filling	Notice
Feeks scale/days plants old		20	70	90	Germinated after 3 days
CV1'	129 Al- Hassa	14	-	-	Germinated after 5 days. Show low growth performance. Removed from the study
CV1	181 Jizan	14	49	79	Germinated after 3 days
CV2	193 Najran	14	49	79	Germinated after 3 days
CV3	357 Sama	14	49	71	Germinated after 3 days
CV4	377 Rafha	14	49	79	Germinated after 3 days
CV5	562 Ma'ayah	14	49	71	Germinated After 2 days. Start show the head 7 days early than control plants.
CV6	981 Najd	14	49	79	Germinated after 3 days. Start show the head 5 days early than control plants

Table S1. 2 Shoot dry weight (g) measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	0.129 ± 0.03	0.246	5.9	0.002
		15%	0.023[b] ±0.002			
	flowering	Control	0.48 ±0.09	0.000		
		15%	0.0357[ab] ±0.009			
	filling	Control	0.61 ±0.28	0.000		
		15%	0.075[ab] ±0.01			
Cv2	Vegetative	Control	0.162 ±0.17	0.309	4.07	0.012
		15%	0.044[b] ±0.005			
	flowering	Control	0.52 ±0.15	0.016		
		15%	0.22[ab] ±0.29			
	filling	Control	0.565 ±0.08	0.003		
		15%	0.17[ab] ±0.05			
Cv3	Vegetative	Control	0.076 ±0.044	0.152	5.6	0.003
		15%	0.022[b] ±0.027			
	flowering	Control	0.174 ±0.018	0.003		
		15%	0.0514[ab] ±0.034			
	filling	Control	0.357 ±0.07	0.000		
		15%	0.074[ab] ±0.08			
Cv4	Vegetative	Control	0.109 ±0.01	0.000	2.33	0.085
		15%	0.0095[a] ±0.007			
	flowering	Control	0.175 ±0.018	0.000		
		15%	0.032[a] ±0.011			
	filling	Control	0.22 ±0.066	0.000		
		15%	0.032[a] ±0.022			
Cv5	Vegetative	Control	0.114 ±0.07	0.000	16.9	0.000
		15%	0.017[ab] ±0.004			
	flowering	Control	0.117 ±0.014	0.001		
		15%	0.016 [ab] ±0.004			
	filling	Control	0.187±0.04	0.000		
		15%	0.074 [ab] ±0.01			
Cv6	Vegetative	Control	0.49 ±0.16	0.000	4.2	0.010
		15%	0.074 [ab] ±0.02			
	Flowering	Control	0.504 ±0.17	0.000		
		15%	0.083 [ab] ±0.014			
	Filling	Control	1.008 ±0.19	0.000		
		15%	0.141 [ab] ±0.009			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.

[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 3 Shoot length (Cm) measurements in wheat cultivars *Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	27.7 ± 4.92	0.983	5.355	0.003
		15%	27.62 [b] ± 4.32			
	flowering	Control	40.62 ±8.7	0.059		
		15%	33.75 [ab] ±3.86			
	filling	Control	42±2.04	0.000		
		15%	25.37[ab]± 1.5			
Cv2	Vegetative	Control	29.05 ± 12.1	0.795	2.842	0.046
		15%	25.9 [b] ± 17.2			
	flowering	Control	49.6 ± 4.6	0.514		
		15%	41.6 [b] ±11.7			
	filling	Control	50 ±7.3	0.878		
		15%	48.12 [b] ±32.8			
Cv3	Vegetative	Control	28.22 ±5.43	0.863	4.079	0.012
		15%	26.13 [b] ±23.1			
	flowering	Control	33.43 ±4.4	0.842		
		15%	31 [b] ± 20.7			
	filling	Control	44.5 ±6.13	0.609		
		15%	38.25 [b] ± 26.1			
Cv4	Vegetative	Control	18.4 ± 8.6	0.793	3.170	0.032
		15%	16.75 [b]± 9.3			
	flowering	Control	19.57 ± 4.7	0.440		
		15%	14.75[b] ± 11.3			
	filling	Control	27.25 ± 2.4	0.292		
		15%	20.6[b] ± 13.9			
Cv5	Vegetative	Control	31.75 ± 21.6	0.251	6.073	0.002
		15%	25.87[b] ±17.3			
	flowering	Control	36.25 ± 4.5	0.177		
		15%	26.6[b] ± 1.79			
	filling	Control	44.68 ± 1.73	0.128		
		15%	33.75[b] ± 22.6			
Cv6	Vegetative	Control	31.6 ± 2.01	0.919	5.496	0.003
		15%	30.9[b] ± 3.9			
	Flowering	Control	45 ± 2.5	0.204		
		15%	35.3[b] ± 23.7			
	Filling	Control	52 ± 6.03	0.034		
		15%	35.25[ab] ± 4.11			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.
[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 4 Root dry weight (g) measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	0.185± 0.016	0.033	4.9	0.005
		15%	0.021[ab] ± 0.014			
	flowering	Control	0.378 ± 0.047	0.000		
		15%	0.027[ab] ± 0.015			
	filling	Control	0.273 ±0.24	0.005		
		15%	0.049[ab] ± 0.04			
Cv2	Vegetative	Control	0.069 ± 0.073	0.377	2.6	0.055
		15%	0.0115± 0.001			
	flowering	Control	0.227 ± 0.14	0.012		
		15%	0.049[ab] ± 0.045			
	filling	Control	0.258 ± 0.114	0.605		
		15%	0.225[b] ± 0.098			
Cv3	Vegetative	Control	0.055 ± 0.051	0.022	1.7	0.174
		15%	0.194[a] ± 0.133			
	flowering	Control	0.154 ± 0.064	0.729		
		15%	0.174 ± 0.059			
	filling	Control	0.149 ± 0.049	0.452		
		15%	0.192 ± 0.084			
Cv4	Vegetative	Control	0.133 ± 0.042	0.538	3.11	0.034
		15%	0.024[b] ± 0.020			
	flowering	Control	0.083 ± 0.08	0.865		
		15%	0.53[b] ± 0.04			
	filling	Control	0.86 ± 0.35	0.073		
		15%	0.53[b] ± 0.48			
Cv5	Vegetative	Control	0.53 ± 0.12	0.000	2.9	0.043
		15%	0.134[ab] ± 0.04			
	flowering	Control	0.102 ±0.064	0.169		
		15%	0.014[b] ± 0.013			
	filling	Control	0.326 ± 0.158	0.000		
		15%	0.028[ab] ± 0.0135			
Cv6	Vegetative	Control	0.173 ± 0.093	0.016	8.2	0.000
		15%	0.045[ab] ± 0.0135			
	Flowering	Control	0.127 ± 0.03	0.092		
		15%	0.042[b] ± 0.044			
	Filling	Control	0.312 ± 0.13	0.000		
		15%	0.028[ab] ± 0.01			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.
[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 5 Root Length (Cm) measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	8 ±1.35	0.419	2.9	0.041
		15%	10 ±1 [b]			
	flowering	Control	22.37 ±4.9	0.053		
		15%	16.12 ±2.01 [b]			
	filling	Control	14.37 ±5.28	0.009		
		15%	23.25 ±7.13 [ab]			
Cv2	Vegetative	Control	9.75 ±6.3	0.456	0.995	0.448
		15%	13.25±2.72			
	flowering	Control	16.75±4.17	0.094		
		15%	24.87±7.44			
	filling	Control	24.3±9.39	0.011		
		15%	37.25±6.76 [a]			
Cv3	Vegetative	Control	6± 2.74	0.000	3.3	0.026
		15%	49.62± 20.51 [ab]			
	flowering	Control	10.27± 0.22	0.049		
		15%	31.75± 14.8 [ab]			
	filling	Control	29.25± 17.3	0.021		
		15%	55± 17.04 [ab]			
Cv4	Vegetative	Control	14.62 ± 2.49	0.045	1.6	0.206
		15%	7.12 ±2.32 [a]			
	flowering	Control	17.42 ± 4.48	0.004		
		15%	29 ± 6.44 [a]			
	filling	Control	27.12 ± 6.04	0.364		
		15%	30.37 ± 6.01			
Cv5	Vegetative	Control	11.2 ±1.4	0.035	2.6	0.056
		15%	25.75 ±9.17 [a]			
	flowering	Control	13.37 ±5.7	0.099		
		15%	21.25 ±5.4			
	filling	Control	16.5 ±3.39	0.090		
		15%	24.62 ±3.68			
Cv6	Vegetative	Control	7.75 ±1.65	0.962	5.7	0.002
		15%	7.5 ±5.11 [b]			
	Flowering	Control	21.75 ±10.7	0.057		
		15%	11.25 ±3.20 [b]			
	Filling	Control	33.5 ±12.5	0.002		
		15%	15.12 ±3.06 [ab]			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.
[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 6 Root: shoot ratio biomass ratio measurements in wheat cultivars *Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	1.49 ± 0.355	0.041	1.5	0.212
		15%	0.883[a] ± 0.627			
	flowering	Control	0.798 ± 0.12	0.734		
		15%	0.704 ± 0.29			
	filling	Control	0.42 ± 0.22	0.492		
		15%	0.611 ± 0.489			
Cv2	Vegetative	Control	0.902 ± 0.26	0.547	3.3	0.027
		15%	0.264[b] ± 0.054			
	flowering	Control	0.475 ± 0.28	0.469		
		15%	0.81[b] ± 0.99			
	filling	Control	0.49 ± 0.29	0.040		
		15%	1.49[ab] ± 1.1			
Cv3	Vegetative	Control	0.94 ± 0.93	0.221	7.6	0.001
		15%	3.45[b] ± 4.01			
	flowering	Control	0.89 ± 0.41	0.551		
		15%	2.1[b] ± 1.6			
	filling	Control	0.45 ± 0.23	0.109		
		15%	3.79[b] ± 5.2			
Cv4	Vegetative	Control	1.25 ± 0.46	0.351	3.7	0.017
		15%	4.42[b] ± 4.6			
	flowering	Control	0.502 ± 1.25	0.759		
		15%	1.53[b] ± 0.847			
	filling	Control	4 ± 1.12	0.021		
		15%	12.4[ab] ± 10.3			
Cv5	Vegetative	Control	2.02 ± 0.6	0.000	1.4	0.258
		15%	4.15[a] ± 0.67			
	flowering	Control	0.891 ± 0.62	0.938		
		15%	0.92 ± 0.83			
	filling	Control	1.76 ± 0.88	0.034		
		15%	0.68[ab] ± 0.17			
Cv6	Vegetative	Control	0.38 ± 0.21	0.217	36.8	0.000
		15%	0.61[b] ± 0.07			
	Flowering	Control	0.29 ± 0.13	0.186		
		15%	0.54[b] ± 0.57			
	Filling	Control	0.31 ± 0.12	0.557		
		15%	0.198[b] ± 0.06			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.

.[b]The mean difference between (stages * water_stress) is significant by F-test at the .05 level

Table S1. 7 Relative water content R.W.C (%) measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	90.55 ±4.33	0.622	2.5	0.068
		15%	94.05 ±3.9			
	flowering	Control	77.5 ±10.6	0.743		
		15%	79.82 ±5.42			
	filling	Control	72.85 ±4.6	0.325		
		15%	65.8 ±19.7			
Cv2	Vegetative	Control	86.5 ±5.47	0.781	4.9	0.005
		15%	91.8[b] ±2.28			
	flowering	Control	63.01 ±4.26	0.226		
		15%	73.5[b] ±27.06			
	filling	Control	66.1 ±8.24	0.297		
		15%	75.15[b] ±5.14			
Cv3	Vegetative	Control	88.55 ±6.17	0.862	2.8	0.046
		15%	90.05[b] ±3.64			
	flowering	Control	67.87 ±11.87	0.123		
		15%	81.65[b] ±14.8			
	filling	Control	77.57 ±12.6	0.055		
		15%	60.07[b] ±17.3			
Cv4	Vegetative	Control	85.25 ± 7.36	0.708	4.3	0.009
		15%	83 [b] ± 9.27			
	flowering	Control	83.25 ± 7.08	0.648		
		15%	80.5[b] ± 12.2			
	filling	Control	82.55 ± 2.7	0.023		
		15%	67.85 [ab] ± 8.65			
Cv5	Vegetative	Control	93.1 ±2.49	0.000	4.07	0.012
		15%	67.05 [ab] ±10.7			
	flowering	Control	49.3 ±15.3	0.002		
		15%	77.15 [ab] ±11.4			
	filling	Control	93.6 ±1.97	0.000		
		15%	58.42 [ab] ±1.98			
Cv6	Vegetative	Control	80 ±4.08	0.145	2.7	0.052
		15%	74.15 [b] ±7.4			
	Flowering	Control	71.5 ±1.96	0.495		
		15%	68.82 [b] ±7.12			
	Filling	Control	71.62 ±6.94	0.026		
		15%	62.3 [ab] ±1.66			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.
[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 8 Proline content (mg/g FWt) measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	81 ±8.54	0.644	3.075	0.051
		15%	99.67[b] ±10.01			
	flowering	Control	118.67 ±24.7	0.096		
		15%	189.67[b]±80.68			
	filling	Control	229.67±20.52	0.000		
		15%	455.33[ab]±78.8			
Cv2	Vegetative	Control	438.3 ± 10.06	0.000	2.81	0.066
		15%	294.3[a] ± 10.7			
	flowering	Control	415 ± 55.4	0.003		
		15%	3094[a] ± 15.52			
	filling	Control	230.6 ± 24.2	0.150		
		15%	275.6 ± 60.01			
Cv3	Vegetative	Control	798.33 ±127.6	0.000	6.4	0.004
		15%	188.33[ab] ±31.72			
	flowering	Control	136.33 ±25.14	0.731		
		15%	152.33[b] ±7.37			
	filling	Control	125 ±12.12	0.001		
		15%	313.33[ab] ±21.4			
Cv4	Vegetative	Control	562 ± 242.5	0.015	2.04	0.145
		15%	1133.3[a] ± 246.6			
	flowering	Control	703.3 ± 34.01	0.780		
		15%	761 ± 421			
	filling	Control	877.6 ±196.8	0.343		
		15%	678 ± 177.8			
Cv5	Vegetative	Control	252.33 ±225.7	0.956	1.29	0.331
		15%	242.67 ±75.9			
	flowering	Control	566.67 ±65.6	0.392		
		15%	414.33 ±234.5			
	filling	Control	767.33 ±257.1	0.044		
		15%	381.33[a] ±288			
Cv6	Vegetative	Control	493.6 ±104.2	0.142	4.25	0.019
		15%	257[b]± 223.2			
	Flowering	Control	386.3 ±337.3	0.007		
		15%	866[ab] ±102			
	Filling	Control	651 ±123.5	0.074		
		15%	945.6[b] ±33.7			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.

[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 9 Soluble Sugar content (mg/g DWt) measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	37.03 ±11.13	0.049	3.55	0.033
		15%	61.77[ab] ±9.9			
	flowering	Control	50.51 ±4.11	0.929		
		15%	49.49[b] ±5.6			
	filling	Control	71.78 ±23.7	0.001		
		15%	119.49[ab] ±17.6			
Cv2	Vegetative	Control	29.14 ±10.8	0.977	3.62	0.031
		15%	28.8[b] ±5.22			
	flowering	Control	75.5 ±24.7	0.011		
		15%	41.39[ab] ±7.68			
	filling	Control	65.41 ±7.6	0.597		
		15%	59.28[b] ±17.02			
Cv3	Vegetative	Control	76.16 ± 23.13	0.000	3.72	0.029
		15%	11.98[ab] ± 8.8			
	flowering	Control	64.36 ± 7.1	0.258		
		15%	75.65[b] ± 8.5			
	filling	Control	77.6 ± 7.6	0.061		
		15%	97.2[b] ± 4.09			
Cv4	Vegetative	Control	72.66 ±17.91	0.707	3.69	0.03
		15%	67.83[b] ± 19.47			
	flowering	Control	85.38 ± 3.31	0.157		
		15%	66.4[b] ± 24.24			
	filling	Control	54.61 ± 1.42	0.736		
		15%	58.95[b] ± 10.8			
Cv5	Vegetative	Control	56.52 ±18.08	0.170	2.9	0.059
		15%	38.29 ±4.11			
	flowering	Control	52.47 ±26.34	0.355		
		15%	64.48 ±4.5			
	filling	Control	59.19 ±14.87	0.944		
		15%	60.1 ±11.13			
Cv6	Vegetative	Control	87.3 ±11.75	0.402	2.455	0.094
		15%	96.95 ±5.3			
	Flowering	Control	91.11 ±7.4	0.789		
		15%	88.08 ±22.5			
	Filling	Control	103.01 ±18.7	0.161		
		15%	86.43 ±5.4			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.
[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 10 Protein content (mg/g DWt) measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Stages	Water stress	Mean ±Std. Deviation	L.S.D at 0.05	F-test	
					F	sig
Cv1	Vegetative	Control	8.13± 1.13	0.021	2.07	0.139
		15%	4.55[a]± 1.59			
	flowering	Control	6.37± 2.09	0.001		
		15%	0.52[a] ± 0.37 [ab]			
	filling	Control	5.59± 0.79	0.083		
		15%	3.04± 2.27 [b]			
Cv2	Vegetative	Control	5.58 ±1.29	0.182	2.13	0.131
		15%	7.37 ±0.73 [ab]			
	flowering	Control	2.78 ±2.53	0.756		
		15%	3.18 ±0.44 [ab]			
	filling	Control	2.7 ±1.95	0.225		
		15%	4.33 ±1.36 [ab]			
Cv3	Vegetative	Control	5.72 ±1.51	0.621	2.83	0.065
		15%	4.97 ±1.4			
	flowering	Control	5.02 ±3.2	0.956		
		15%	5.11 ±1.7			
	filling	Control	7.45 ±0.56	0.977		
		15%	7.41 ±1.46			
Cv4	Vegetative	Control	6.23 ± 5.3	0.349	2.05	0.143
		15%	3.13 ± 5.4			
	flowering	Control	4.7 ± 1.4	0.755		
		15%	3.7 ± 3.4			
	filling	Control	5.8 ± 2.3	0.521		
		15%	3.7 ± 3.5			
Cv5	Vegetative	Control	9.5 ± 1.5	0.429	1.232	0.353
		15%	10.7 ± 1.5			
	flowering	Control	9.6 ± 1.5	0.032		
		15%	6.13[a] ± 2.5			
	filling	Control	5.03 ± 2.16	0.059		
		15%	8.03[a]±0.42			
Cv6	Vegetative	Control	11.9 ± 0.39	0.907	6.3	0.004
		15%	11.77 ± 0.34			
	Flowering	Control	2.5 ± 2.03	0.000		
		15%	10.08[ab] ± 0.88			
	Filling	Control	7.16 ± 2.06	0.002		
		15%	11.34[ab] ± 0.47			

[a]. The mean difference between water stress is significant by L.S.D at the .05 level, Based on estimated marginal means.

[b]. The mean difference between (stages * water_stress) is significant by F-test at the .05 level.

Table S1. 11 grain filling measurements in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia subjected to 15% PEG6000.. Mean of four replicates ± Std. Deviation. n=4

Cultivars	Grain filed measurements	Water stress	Mean ±Std. Deviation	F-test	
				F	sig
Cv1	Weight seeds(g)	Control	0.48±0.076	5.9	0.051
		15%	0.28±0.145[b]		
	Seeds number/spike	Control	10±0	57	0.000
		15%	5.25±1.25[b]		
	Germination (%)	Control	66.42±0.28	81.81	0.000
		15%	53.20±0.24[b]		
Cv2	Weight seeds(g)	Control	0.86±0.75	0.07	0.801
		15%	0.85± 0.05		
	Seeds number/spike	Control	10±0.00	1.00	0.356
		15%	9.75±0.50		
	Germination (%)	Control	98.25±3.5	9.065	0.024
		15%	92.9±0.61[b]		
Cv3	Weight seeds(g)	Control	1.03±0.11	0.377	0.000
		15%	0.97±0.17[b]		
	Seeds number/spike	Control	14.75±0.50	0.857	0.390
		15%	14.25±0.95		
	Germination (%)	Control	71.2±0.84	345.7	0.000
		15%	51±2[b]		
Cv4	Weight seeds(g)	Control	0.475±0.288	10.3	0.018
		15%	0.220±0.155[b]		
	Seeds number/spike	Control	7.75±0.5	98.45	0.000
		15%	3±0.81[b]		
	Germination (%)	Control	66.65±0.47	534.26	0.000
		15%	60.25±0.28[b]		
Cv5	Weight seeds(g)	Control	0.83±0.085	24.7	0.003
		15%	0.56±0.069[b]		
	Seeds number/spike	Control	9.75±0.50	57.6	0.000
		15%	3.75±1.50[b]		
	Germination (%)	Control	46.2±0.80	966.8	0.000
		15%	6.3±2.4[b]		
Cv6	Weight seeds(g)	Control	1.85±0.092	7.57	0.033
		15%	1.61±0.145[b]		
	Seeds number/spike	Control	22.5±1.73	0.667	0.445
		15%	21.5±1.73		
	Germination (%)	Control	73.65±0.91	79.48	0.000
		15%	62±2.44[b]		

[b]. The mean difference is significant by F-test at the .05 level.

Table S1. 12 Scores among wheat cultivars (*Triticum aestivum* l.; 181 Jizan (cv1) ; 193najran (cv2); 357 sama(cv3); 377 rafha (cv4); 562 ma'ayah (cv5) and 981 najd (cv6)) for their relative water stress tolerance on root length, shoot length, root weight, shoot weight, root/shoot ratio, rwc, proline, sugar, protein, weight of seeds, number of seeds and germination of seeds at three growth stages. classified into four classes based on wst value for each measurement.

Cultivars	Stages	Ro. length	Sh. length	Ro. weight	Sh. weight	Ro/Sh.ratio	RWC	proline	sugar	protein	W. seeds	NO. seeds	germination
Cv1	Veg	125	99.7	11.5	18.39	62.82	1.0387	1.230	1.668	0.560			
	flow	72.06	60.2	7.01	7.42	94.45	1.0299	1.598	0.980	0.082			
	fill	161.7	83.1	17.87	12.39	144.18	0.9032	1.983	1.665	0.544	0.583	0.525	0.801
Cv2	Veg	135.8	89.1	16.61	27.17	61.12	1.0613	1.489	0.988	1.321			
	flow	148.47	83.8	21.68	42.12	51.47	1.1665	1.365	0.548	1.144			
	fill	153.2	96.25	87.01	30.81	282.39	1.1369	1.195	0.906	1.604	0.988	0.975	0.946
Cv3	Veg	827	92.55	355.1	28.36	1252.1	1.0169	0.236	0.367	0.869			
	flow	309.2	92.74	112.7	29.41	383.22	1.2030	1.117	1.622	1.018			
	fill	188.03	85.95	128.8	20.97	614.36	0.7744	2.507	1.235	0.995	0.942	0.966	0.716
Cv4	Veg	48.7	91.15	18.2	8.74	208.2	0.9736	2.017	0.934	0.502			
	flow	166.5	75.35	63.9	18.46	346.1	0.9670	0.773	0.778	0.788			
	fill	111.9	75.68	61.55	14.43	426.5	0.8219	1.082	1.079	0.639	0.463	0.387	0.904
Cv5	Veg	74.6	80.88	25.1	11.57	216.8	0.7202	0.962	0.677	1.123			
	flow	158.9	73.44	13.8	13.54	101.9	1.5649	0.731	1.229	0.637			
	fill	149.2	75.54	8.7	21.34	40.6	0.6241	0.497	1.015	1.598	0.675	0.385	0.136
Cv6	Veg	96.8	97.62	26.25	15.28	171.8	0.9269	0.521	1.111	0.869			
	flow	51.7	67.78	33.02	16.49	200.14	0.9625	2.242	0.967	0.434			
	fill	45.13	78.61	8.9	13.95	64.4	0.8699	1.453	0.839	0.230	0.870	0.956	0.842
score	4 (highly sensitive)	<60	<60	<60	<60	<60	<60	<60	<60	<60	<60		
	3 (sensitive)	60-80	60-80	60-80	60-80	60-80	60-80	60-80	60-80	60-80	60-80		
	2 (moderate tolerant)	80-100	80-100	80-100	80-100	80-100	80-100	80-100	80-100	80-100	80-100		
	1 (highly tolerant)	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		

Table S1. 13 Ranking among wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) for their relative water stress tolerance on root length (Ro.length), Shoot length (Sh.length), Root weight (Ro.weight), Shoot weight (Sh.weight), Root/Shoot ratio (Ro/Sh.ratio), RWC, Proline, soluble Sugar, Protein, Seeds weight (W.Seeds), Seeds Number (NO.seeds) and Seeds germination of at Vegetative stage (Veg), Flowering stage (flow) and Grain filling stage (fill).

Cultivars	Stages	Ro.length	Sh.length	Ro.weight	Sh.weight	Ro/Sh.ratio	RWC	proline	sugar	protein	W.seeds	NO.seeds	Germination
Cv1	Veg	1	2	4	4	2	1	1	1	1			
	flow	3	3	4	4	3	1	1	2	4			
	fill	1	2	4	4	4	2	1	1	4	4	4	3
Cv2	Veg	1	2	4	4	2	1	1	2	1			
	flow	1	2	4	4	1	1	1	4	1			
	fill	1	2	2	4	4	1	1	2	1	2	1	1
Cv3	Veg	1	2	1	4	4	1	4	4	2			
	flow	1	2	1	4	4	1	1	1	1			
	fill	1	2	1	4	4	3	1	1	2	2	1	3
Cv4	Veg	4	2	4	4	4	2	1	2	4			
	flow	1	3	3	4	4	2	3	3	3			
	fill	1	3	3	4	4	2	1	1	3	4	4	2
Cv5	Veg	3	2	4	4	4	3	2	3	1			
	flow	1	3	4	4	4	1	3	1	3			
	fill	1	3	4	4	1	3	4	1	1	3	4	4
Cv6	Veg	2	2	2	4	4	2	4	1	2			
	flow	4	2	2	4	4	2	1	2	4			
	fill	4	2	4	4	2	2	1	2	4	2	2	2

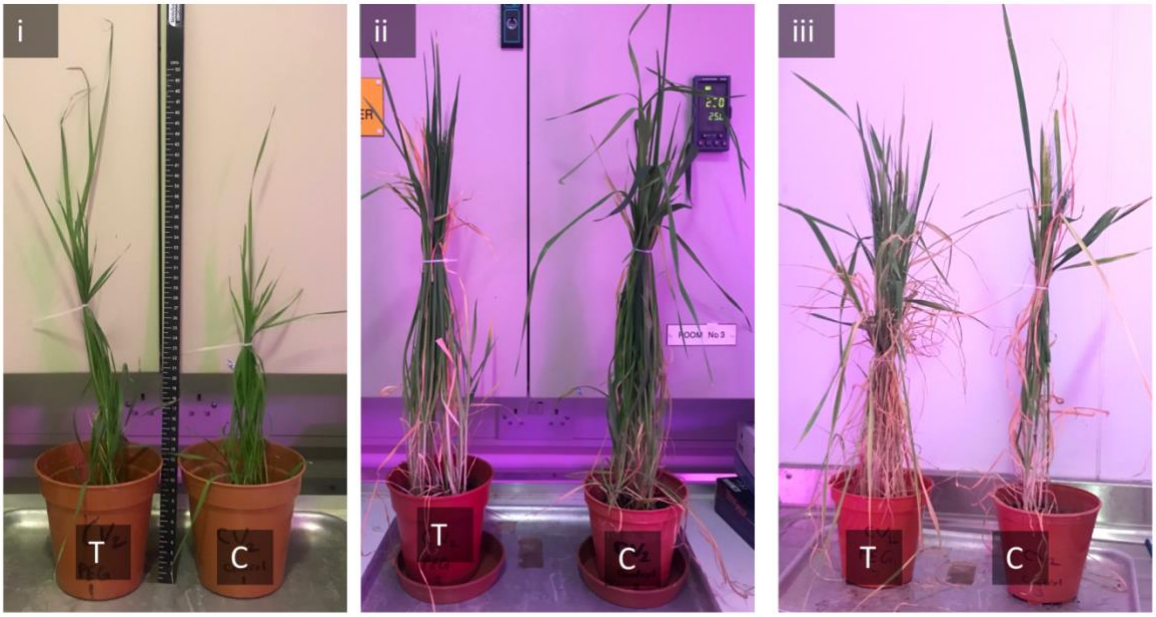


Figure S1. 1 Morphological measurements photo of wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) at three growth stages. i.at vegetative stage, ii.at flowering stage. iii.at grain filling stage. C. plants under control conations, T. plants subjected to 15%PEG6000.

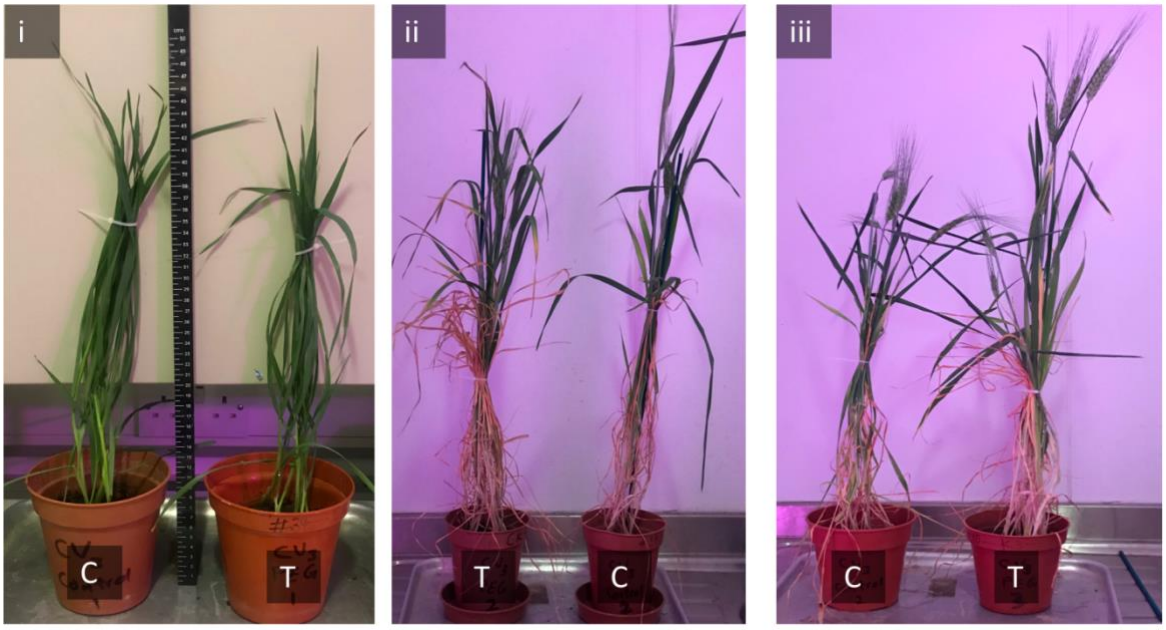


Figure S1. 2 Morphological measurements photo of wheat cultivars (*Triticum aestivum* L.; 193Najran (Cv2) at three growth stages. i. at vegetative stage, ii. at flowering stage. iii. at grain filling stage. C. plants under control conations, T. plants subjected to 15%PEG6000.

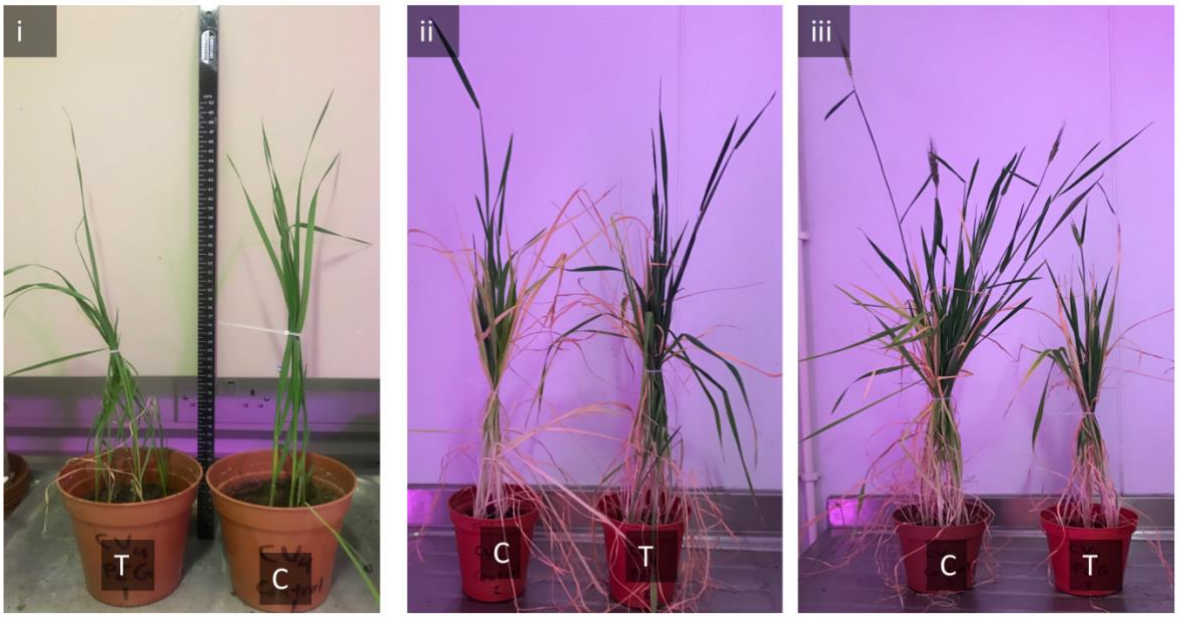


Figure S1. 3 Morphological measurements photo of wheat cultivars (*Triticum aestivum* L.; 357 Sama (Cv3) at three growth stages. i. at vegetative stage, ii. at flowering stage. iii. at grain filling stage. C. plants under control conations, T. plants subjected to 15%PEG6000.

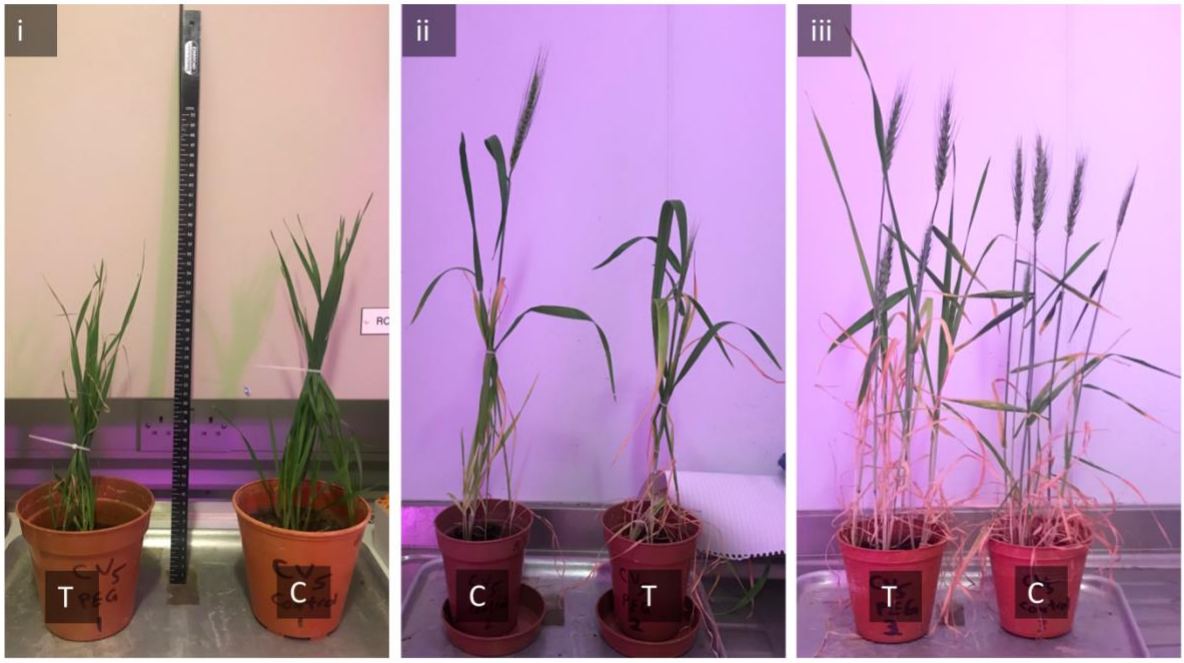


Figure S1. 4 Morphological measurements photo of wheat cultivars (*Triticum aestivum* L.; 377 Rafha (Cv4) at three growth stages. i. at vegetative stage, ii. at flowering stage. iii. at grain filling stage. C. plants under control conations, T. plants subjected to 15%PEG6000.

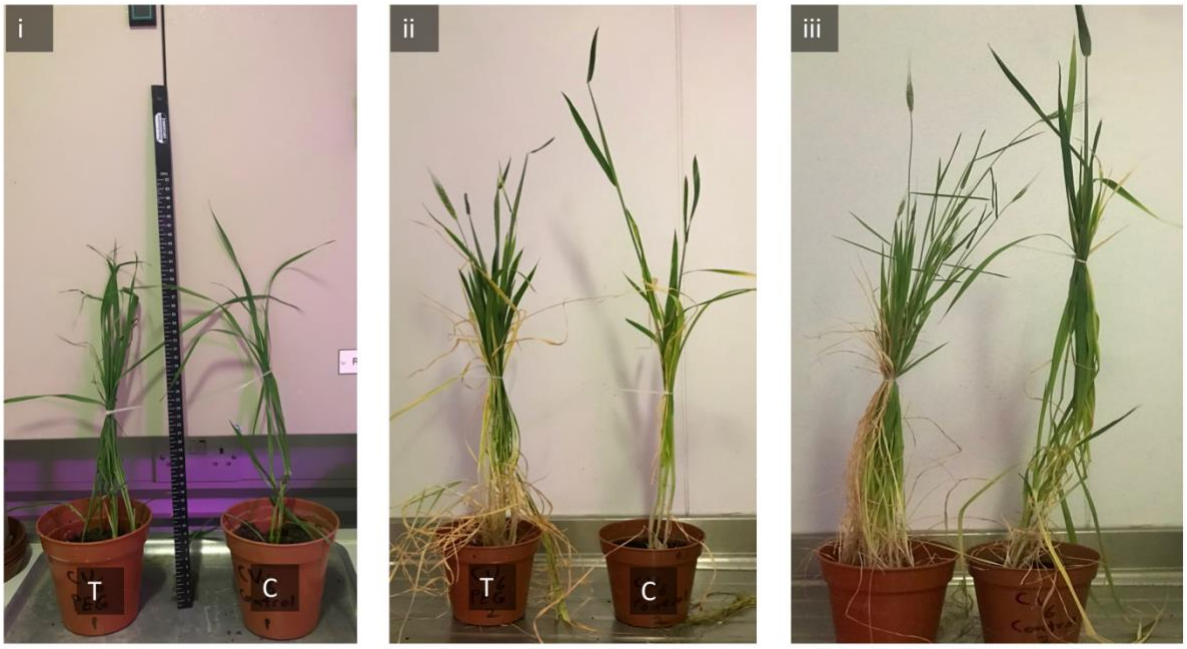


Figure S1. 5 Morphological measurements photo of wheat cultivars (*Triticum aestivum* L.; 562 Ma'ayah (Cv5) at three growth stages. i. at vegetative stage, ii. at flowering stage. iii. at grain filling stage. C. plants under control conations, T. plants subjected to 15%PEG6000.

Figure S1. 6 Morphological measurements photo of wheat cultivars (*Triticum aestivum* L.; 981 Najd (Cv6) at three growth stages. i. at vegetative stage, ii. at flowering stage. iii. at grain filling stage. C. plants under control conations, T. plants subjected to 15%PEG6000.

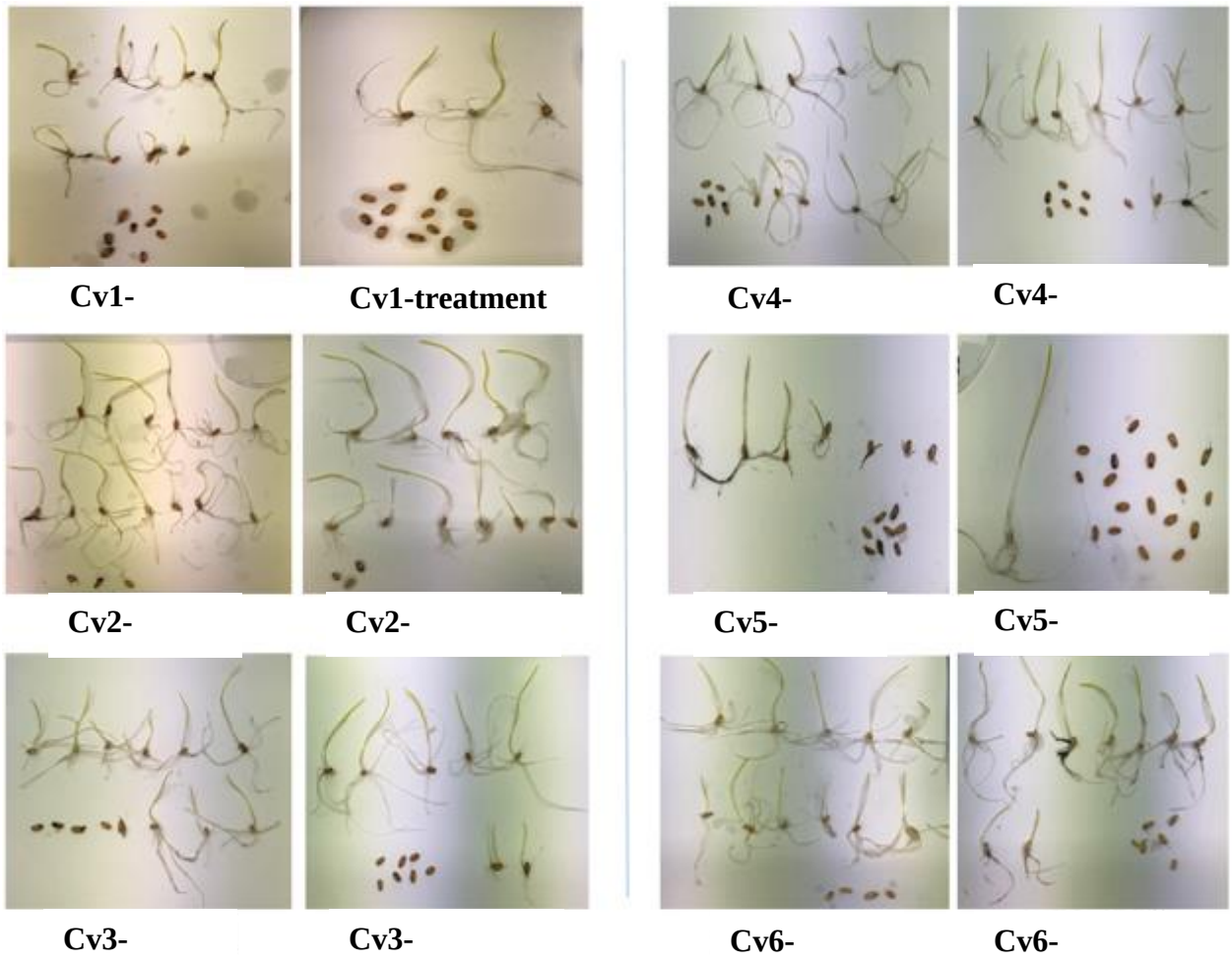


Figure S1. 7 Seeds germination in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1); 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)). control: plants under normal conations. treatment: plants subjected to 15% PEG6000.

Appendix B Supporting information

Table S2. 1 Morphological measurements in wheat cultivars *Triticum aestivum* L.; 193Najran (Cv2) (Drought-resistant) and 377 Rafha (Cv4) (Drought-sensitive) at three growth stages: veg. vegetative, flow. flowering and fill. grain filling stages subjected to 15% PEG6000. C. control sample, T. treatment sample. Mean of nine replicates ± Std. Deviation. n=9

cultivars	stages	root dry weight (g)		root length		shoot dry weight		shoot length		Shoot/Root ratio		Proline content (mg/gFWt)		Soluble sugar(mg/gDWt)	
		C	T	C	T	C	T	C	T	C	T	C	T	C	T
Cv2	veg	0.533 ±0.034	0.033 ±0.005	26.5±0.57	32.25 ±2.88	0.66 ±0.10	0.094 ±0.005	44.33 ±1.73	37.5 ±25.6	0.81 ±0.1	0.35 ±0.05	525 ±491.8	766.66 ±76.3	99.35 ±19.5	95.09 ±42.4
	flow	1.066 ±0.01	0.93 ±0.057	31.75±3.8	37.25 ±0.57	0.57 ±0.28	0.24 ±0.011	84.83 ±3.46	67 ±1.7	1.86 ±0.12	3.78 ±0.2	361.7 ±189.4	746.33 ±134.5	201.18 ±6.2	117.39 ±16.6
	fill	1.1 ±0.01	0.88 ±0.028	35.75±1.7	40.37 ±0.57	1.02 ±0.202	0.53 ±0.005	74.33 ±0.57	73.5 ±2.3	1.09 ±0.01	1.67 ±0.057	173.33 ±42.1	606.67 ±75.05	147.84 ±53.81	128.12 ±33.8
Cv4	veg	0.78 ±0.005	0.09 ±0.001	16.25±0.0	15 ±5.77	0.13 ±0.006	0.02 ±0.01	35.5 ±1	23.5 ±0.57	5.83 ±0.22	4.3 ±0.29	276.67 ±248.2	2153.3 ±1651	121.18 ±115	117.01 ±114.36
	flow	0.93 ±0.06	0.25 ±0.22	17.375±0.28	26.12 ±4.3	0.2 ±0.010	0.05 ±0.0057	64.25 ±0.28	51.5 ±1.15	4.42 ±0.13	4.44 ±3.4	640 ±105.8	134.67 ±7.7	248.79 ±17.16	150.63 ±19.9
	fill	1.01 +0.005	0.213 ±0.055	20.37±0.57	33.75 ±2.8	0.56 ±0.005	0.06 ±0.0057	74 ±0.57	54.5 ±0.57	1.8 ±0.015	3.23 ±0.97	383.33 ±275.3	1783.3 ±778.5	163.76 ±43.25	236.22 ±70.9
F- test		55.4		17.56		115.2		11.28		186.3		3.783		55.4	
P value		0.000		0.000		0.000		0.000		0.000		0.027		0.000	

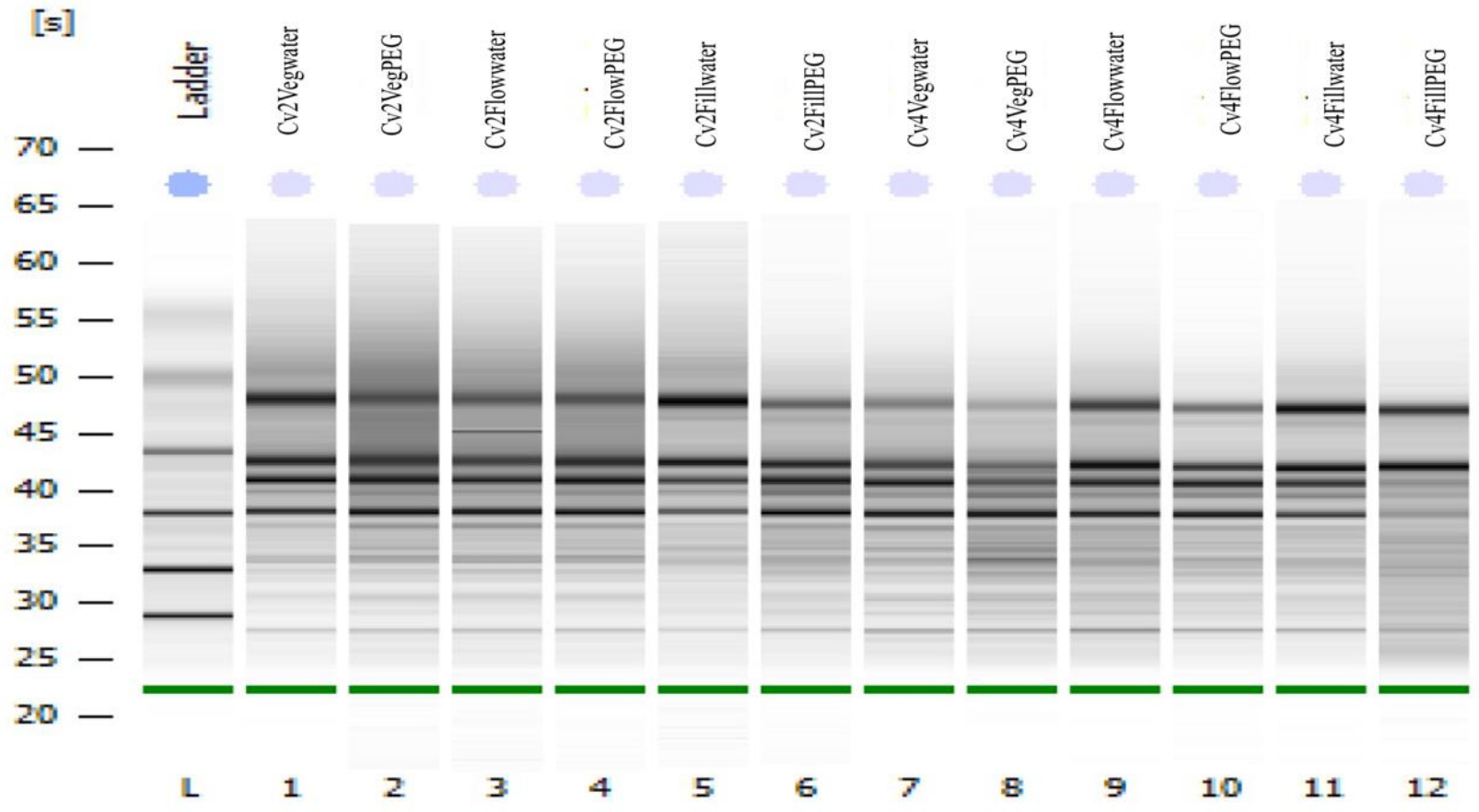


Figure S2. 1 Agilent 2100 Bioanalyzer gel images from high Sensitivity 12 RNA samples.

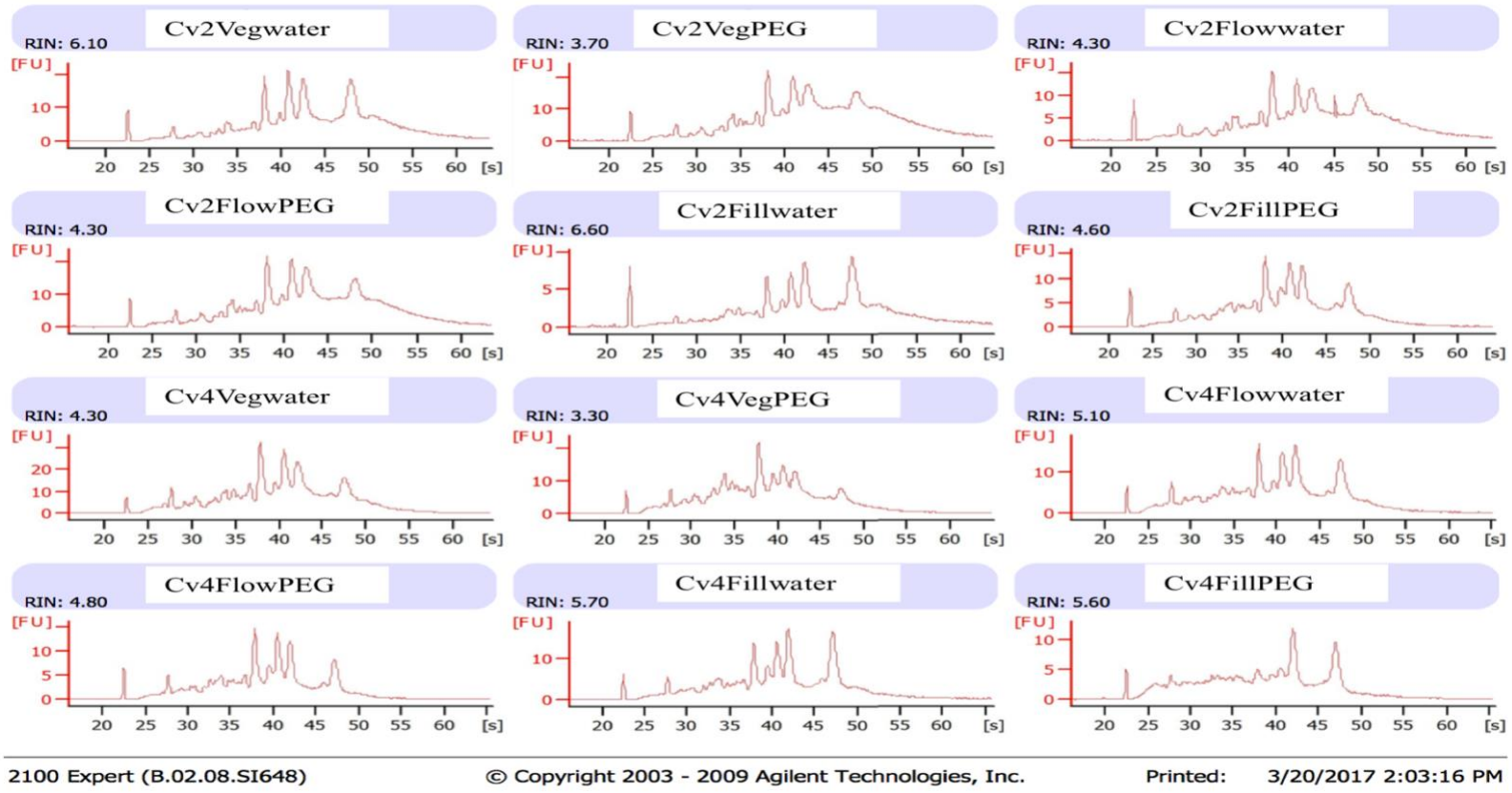
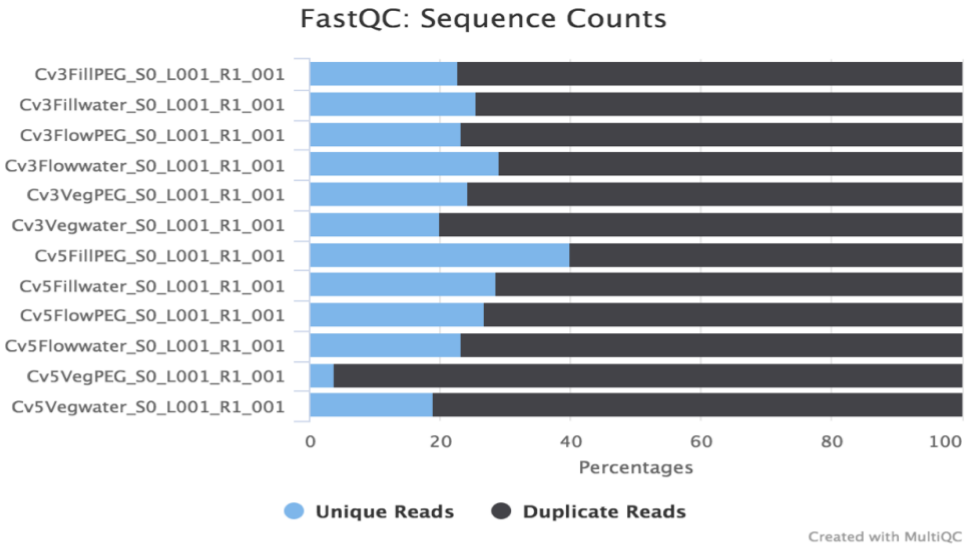
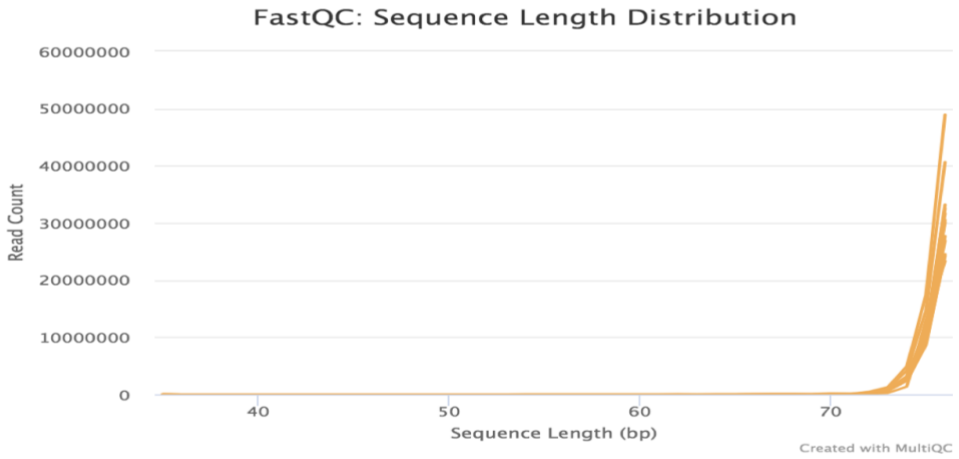


Figure S2. 2 Agilent 2100 Bioanalyzer chromatogram representation of bands in bioanalyzer gel images showing high quality RNA samples.

a.



b.



c.

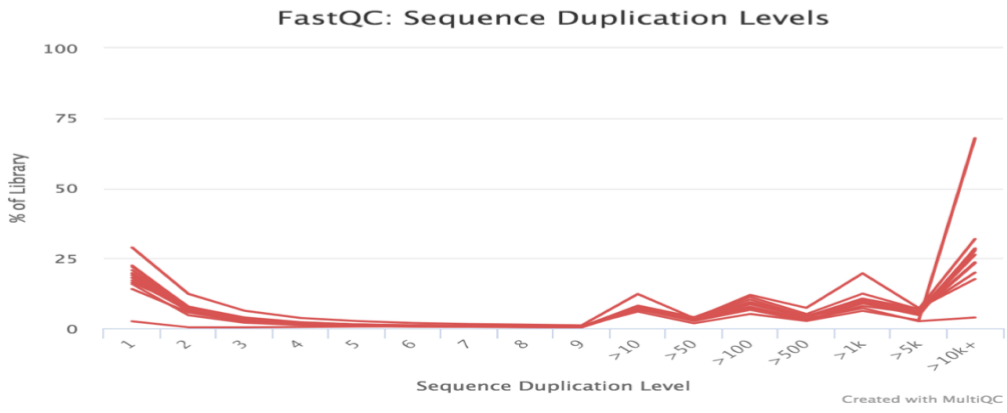
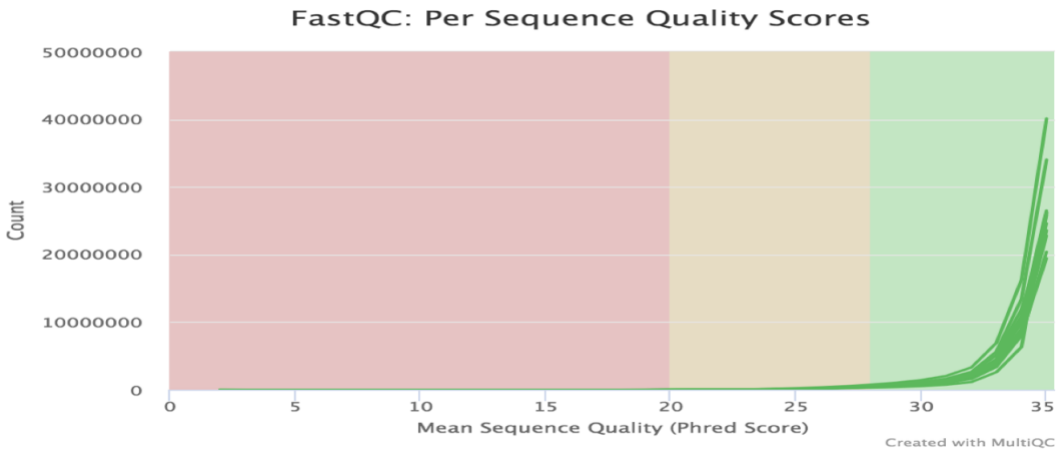
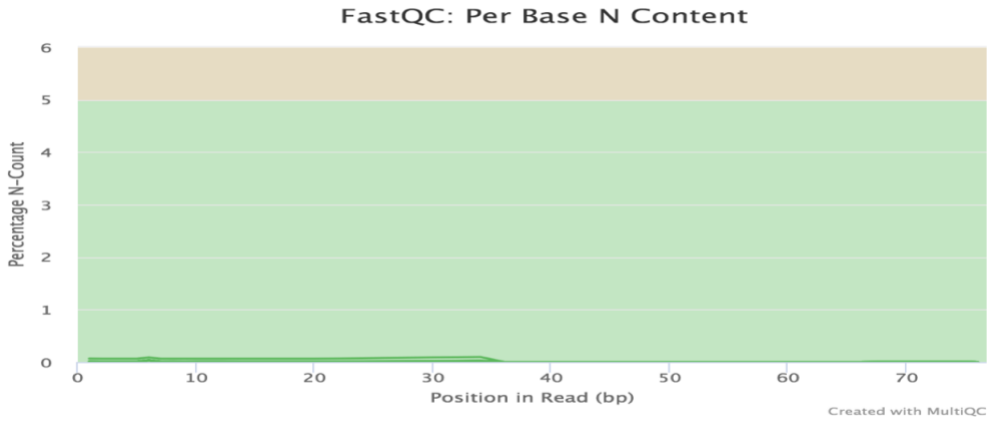


Figure S2. 3 The FastQC results. a. Sequence counts for each sample. b. The percentage of base calls at each position for which an N was called. c. The relative level of duplication found for every sequence. (Ewels et al., 2016)

a.



b.



c.

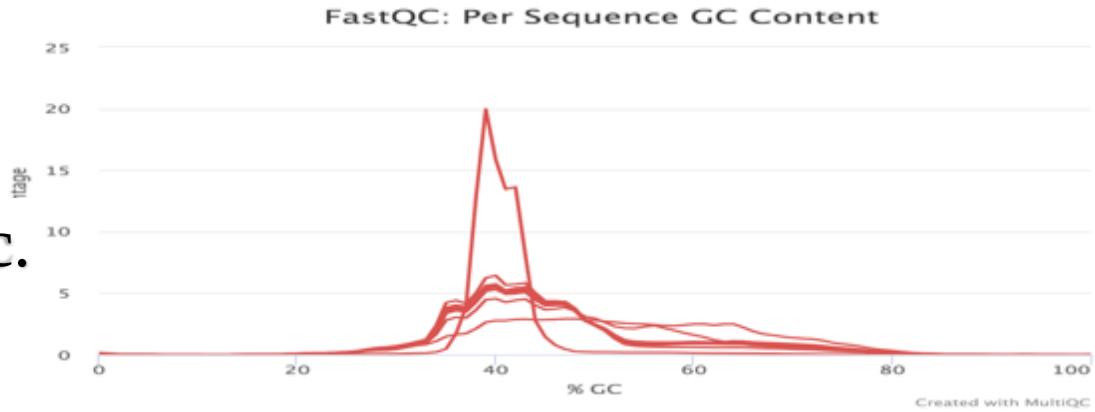


Figure S2. 4 The FastQC results a. The number of reads with average quality scores. Shows if a subset of reads has poor quality. b. The mean quality value across each base position in the read. c. The average GC content of reads. Normal random library typically have a roughly normal distribution of GC content (Ewels et al., 2016)

Table S2. 2 KEGG pathways significantly enriched for DEGs at 193Najran (Cv2) (Drought-resistant) under water stress at the flowering stage and grain filling stage.

Samples	Main pathway	Name	KEGG_pathway
Cv2Flow (PEG_vs_water)	Amino acid metabolism	Tyrosine metabolism	path:osa00350
		Phenylalanine metabolism	path:osa00360
		Tryptophan metabolism	path:osa00380
		Glutathione metabolism	path:osa00480
	Carbohydrate metabolism	Ascorbate and aldarate metabolism	path:osa00053
		Starch and sucrose metabolism	path:osa00500
		Amino sugar and nucleotide sugar metabolism	path:osa00520
	Lipid metabolism	Linoleic acid metabolism	path:osa00591
		alpha-Linolenic acid metabolism	path:osa00592
	Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis	path:osa00940
		Stilbenoid, diarylheptanoid and gingerol biosynthesis	path:osa00945
		Isoquinoline alkaloid biosynthesis	path:osa00950
		Betalain biosynthesis	path:osa00965
	Metabolism of cofactors and vitamins	Ubiquinone and other terpenoid-quinone biosynthesis	path:osa00130
		Thiamine metabolism	path:osa00730
	Environmental information processing	MAPK signalling pathway - plant	path:osa04016

	Environmental adaptation	Plant-pathogen interaction	path:osa04626
	Metabolism of terpenoids and polyketides	Terpenoid backbone biosynthesis	path:osa00900
		Monoterpenoid biosynthesis	path:osa00902
		Diterpenoid biosynthesis	path:osa00904
		Metabolic pathways	path:osa01100
		Biosynthesis of secondary metabolites	path:osa01110
Cv2Fill (PEG_vs_water)	Amino acid metabolism	Phenylalanine metabolism	path:osa00360
	Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis	path:osa00940
	-	Metabolic pathways	path:osa01100
	-	Biosynthesis of secondary metabolites	path:osa01110
Cv2Flow_Cv2Veg (water)	Amino acid metabolism	Cysteine and methionine metabolism	path:osa00270
		Arginine and proline metabolism	path:osa00330
		Phenylalanine metabolism	path:osa00360
		Glutathione metabolism	path:osa00480
	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism	path:osa00520
		Pyruvate metabolism	path:osa00620
		Glyoxylate and dicarboxylate metabolism	path:osa00630
	Carbon metabolism	Carbon fixation in photosynthetic organisms	path:osa00710
	-	Metabolic pathways	path:osa01100

	-	Biosynthesis of secondary metabolites	path:osa01110
	-	Carbon metabolism	path:osa01200
	Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis	path:osa00940
	Environmental information processing	RNA polymerase	path:osa03020
	Environmental information processing	MAPK signalling pathway - plant	path:osa04016
	Environmental adaptation	Plant-pathogen interaction	path:osa04626
Cv2Fill_vs_Cv2Veg (water)	Amino acid metabolism	Phenylalanine metabolism	path:osa00360
		Glutathione metabolism	path:osa00480
	Carbohydrate metabolism	Pyruvate metabolism	path:osa00620
	Energy metabolism	Carbon fixation in photosynthetic organisms	path:osa00710
	Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis	path:osa00940
	-	Metabolic pathways	path:osa01100
	-	Biosynthesis of secondary metabolites	path:osa01110
	-	Carbon metabolism	path:osa01200

	Genetic information Processing, transcription	RNA polymerase	path:osa03020
	Environmental adaptation	Plant-pathogen interaction	path:osa04626

Table S2. 3 KEGG pathways significantly enriched for DEGs at 377 Rafha (Cv4) (Drought-sensitive) under water stress at the flowering stage and grain filling stage.

Samples	Main pathway	Name	KEGG_pathway
Cv4flowPEG_Cv4Flowwater	Biosynthesis of other secondary metabolites	Flavonoid biosynthesis	path:osa00941
		Metabolic pathways	path:osa01100
		Biosynthesis of secondary metabolites	path:osa01110
	Environmental information processing, signal transduction	Plant hormone signal transduction	path:osa04075
	Genetic information processing, folding, sorting and degradation	Protein processing in endoplasmic reticulum	path:osa04141
	Environmental adaptation	Plant-pathogen interaction	path:osa04626
		Circadian rhythm - plant	path:osa04712
Cv4FillPEG_vs_Cv4Fillwater	Carbohydrate metabolism	Glycolysis / Gluconeogenesis	path:osa00010
		Pentose phosphate pathway	path:osa00030
		Fructose and mannose metabolism	path:osa00051
		Pyruvate metabolism	path:osa00620
		Glyoxylate and dicarboxylate metabolism	path:osa00630
	Energy metabolism	Oxidative phosphorylation	path:osa00190
		Photosynthesis	path:osa00195
		Photosynthesis - antenna proteins	path:osa00196
		Carbon fixation in photosynthetic organisms	path:osa00710
	Amino acid metabolism	Tyrosine metabolism	path:osa00350
		Phenylalanine metabolism	path:osa00360
		Tryptophan metabolism	path:osa00380
	Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis	path:osa00940
		Isoquinoline alkaloid biosynthesis	path:osa00950
		Betalain biosynthesis	path:osa00965
	Nucleotide metabolism	Purine metabolism	path:osa00230

	Lipid metabolism	Linoleic acid metabolism	path:osa00591
	Environmental information processing	MAPK signalling pathway - plant	path:osa04016
		Plant hormone signal transduction	path:osa04075
		Metabolic pathways	path:osa01100
		Biosynthesis of secondary metabolites	path:osa01110
		Carbon metabolism	path:osa01200
		Biosynthesis of amino acids	path:osa01230
Cv4Flowwater_vs_Cv4Vegwater			
Cv4Fillwater_vs_Cv4Vegwater	Photosynthesis	Photosynthesis - antenna proteins	path:osa00196
	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	path:osa00630
	Energy metabolism	Carbon fixation in photosynthetic organisms	path:osa00710
	-	Metabolic pathways	path:osa01100
	-	Carbon metabolism	path:osa01200
	Genetic Information Processing,Transcription	RNA polymerase	path:osa03020

Table S2. 4 KEGG pathways significantly enriched for DEGs between 193 Najran (Cv2) (Drought-resistant) and 377 Rafha (Cv4) (Drought-sensitive) under water stress at three growth stages.

Samples	Main pathway	Name	KEGG_pathway
Cv2Vegwater_vs_Cv4Vegwater	Amino acid metabolism	Cysteine and methionine metabolism	path:osa00270
		Arginine and proline metabolism	path:osa00330
		Phenylalanine metabolism	path:osa00360
	Carbohydrate metabolism	Starch and sucrose metabolism	path:osa00500
		Amino sugar and nucleotide sugar metabolism	path:osa00520
	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism	path:osa00860
	Metabolism of terpenoids and polyketides	Diterpenoid biosynthesis	path:osa00904
		Carotenoid biosynthesis	path:osa00906
	Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis	path:osa00940
	-	Metabolic pathways	path:osa01100
	-	Biosynthesis of secondary metabolites	path:osa01110
	-	Biosynthesis of amino acids	path:osa01230
	Environmental Information Processing, Signal transduction	MAPK signalling pathway - plant	path:osa04016
	Environmental adaptation	Plant-pathogen interaction	path:osa04626

Cv2Flowwater_vs_Cv4Flowwater	Amino acid metabolism	Tyrosine metabolism	path:osa00350
		Phenylalanine metabolism	path:osa00360
		Tryptophan metabolism	path:osa00380
	Metabolism of other amino acids	Glutathione metabolism	path:osa00480
	Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis	path:osa00940
		Isoquinoline alkaloid biosynthesis	path:osa00950
		Betalain biosynthesis	path:osa00965
		Metabolic pathways	path:osa01100
		Biosynthesis of secondary metabolites	path:osa01110
Cv2Fillwater_vs_Cv4Fillwater	Metabolism of terpenoids and polyketides	Diterpenoid biosynthesis	path:osa00904
		Photosynthesis - antenna proteins	path:osa00196
		Metabolic pathways	path:osa01100
		Biosynthesis of secondary metabolites	path:osa01110

Table S2. 5 Most frequently detected KEGG pathways in different compressions.

Most frequently detected KEGG pathways under water stress in shoot transcriptome analysis.	Pathways may relate to water stress		Pathways relate to water stress and growth stage	Pathways relate to drought tolerance of cultivar
	Cv2 Flow (PEG vs water)	Cv4 Fill (PEG vs water)	Cv4flowPEG_Cv4Flowwater	Cv2Vegwater_vs_Cv4Vegwater
Phenylalanine metabolism.	Ascorbate and aldarate metabolism	Pentose phosphate pathway	Flavonoid biosynthesis	Porphyrin and chlorophyll metabolism
Glutathione metabolism.	Ubiquinone and other terpenoid-quinone biosynthesis	Fructose and mannose metabolism	Circadian rhythm - plant	Carotenoid biosynthesis
Phenylpropanoid biosynthesis.	alpha-Linolenic acid metabolism	Oxidative phosphorylation		
MAPK signalling pathway - plant.	Thiamine metabolism	Photosynthesis		
Plant-pathogen interaction.	Terpenoid backbone biosynthesis	Photosynthesis - antenna proteins		
	Monoterpenoid biosynthesis	Purine metabolism		
	Stilbenoid, diarylheptanoid and gingerol biosynthesis	Biosynthesis of amino acids		

Table S2. 6 Most frequently detected KEGG pathways in different compressions.

	Pathways	Cv2FlowPEG_vs_C	Cv2FillPEG_vs_C	Cv2Flow_vs_Veg	Cv2Fill_vs_Veg	Cv4flowPEG_vs_W	Cv4FillPEG_vs_w	Cv4Fill_vs_Veg	Cv2Veg_vs_Cv4Veg	Cv2Flow_vs_Cv4Flow	Cv2Fill_vs_Cv4Fill
path:osa00053	Ascorbate and aldarate metabolism	✓									
path:osa00130	Ubiquinone and other terpenoid-quinone biosynthesis	✓									
path:osa00350	Tyrosine metabolism	✓					✓			✓	
path:osa00360	Phenylalanine metabolism	✓	✓	✓	✓		✓		✓	✓	
path:osa00380	Tryptophan metabolism	✓					✓			✓	
path:osa00480	Glutathione metabolism	✓		✓	✓					✓	
path:osa00500	Starch and sucrose metabolism	✓							✓		
path:osa00520	Amino sugar and nucleotide sugar metabolism	✓		✓					✓		
path:osa00591	Linoleic acid metabolism	✓					✓				
path:osa00592	alpha-Linolenic acid metabolism	✓									
path:osa00730	Thiamine metabolism	✓									
path:osa00900	Terpenoid backbone biosynthesis	✓									
path:osa00902	Monoterpenoid biosynthesis	✓									
path:osa00904	Diterpenoid biosynthesis	✓							✓		✓
path:osa00940	Phenylpropanoid biosynthesis	✓	✓	✓	✓		✓		✓	✓	

path:osa00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	✓									
path:osa00950	Isoquinoline alkaloid biosynthesis	✓					✓			✓	
path:osa00965	Betalain biosynthesis	✓					✓			✓	
path:osa01100	Metabolic pathways	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
path:osa01110	Biosynthesis of secondary metabolites	✓	✓		✓	✓	✓		✓	✓	✓
path:osa04016	MAPK signalling pathway - plant	✓		✓			✓		✓		
path:osa04626	Plant-pathogen interaction	✓		✓	✓	✓			✓		
path:osa00270	Cysteine and methionine metabolism			✓					✓		
path:osa00330	Arginine and proline metabolism			✓					✓		
path:osa00620	Pyruvate metabolism			✓	✓		✓				
path:osa00630	Glyoxylate and dicarboxylate metabolism			✓			✓				
path:osa00710	Carbon fixation in photosynthetic organisms			✓	✓		✓	✓			
path:osa01200	Carbon metabolism			✓	✓		✓	✓			
path:osa03020	RNA polymerase			✓	✓			✓			
path:osa00941	Flavonoid biosynthesis					✓					
path:osa04075	Plant hormone signal transduction					✓	✓				
path:osa04141	Protein processing in endoplasmic reticulum										

path:osa04712	Circadian rhythm - plant					✓					
path:osa00010	Glycolysis / Gluconeogenesis						✓	✓			
path:osa00030	Pentose phosphate pathway						✓				
path:osa00051	Fructose and mannose metabolism						✓				
path:osa00190	Oxidative phosphorylation						✓				
path:osa00195	Photosynthesis						✓				
path:osa00196	Photosynthesis - antenna proteins						✓	✓			✓
path:osa00230	Purine metabolism						✓				
path:osa01230	Biosynthesis of amino acids						✓				
path:osa00860	Porphyrin and chlorophyll metabolism								✓		
path:osa00906	Carotenoid biosynthesis								✓		

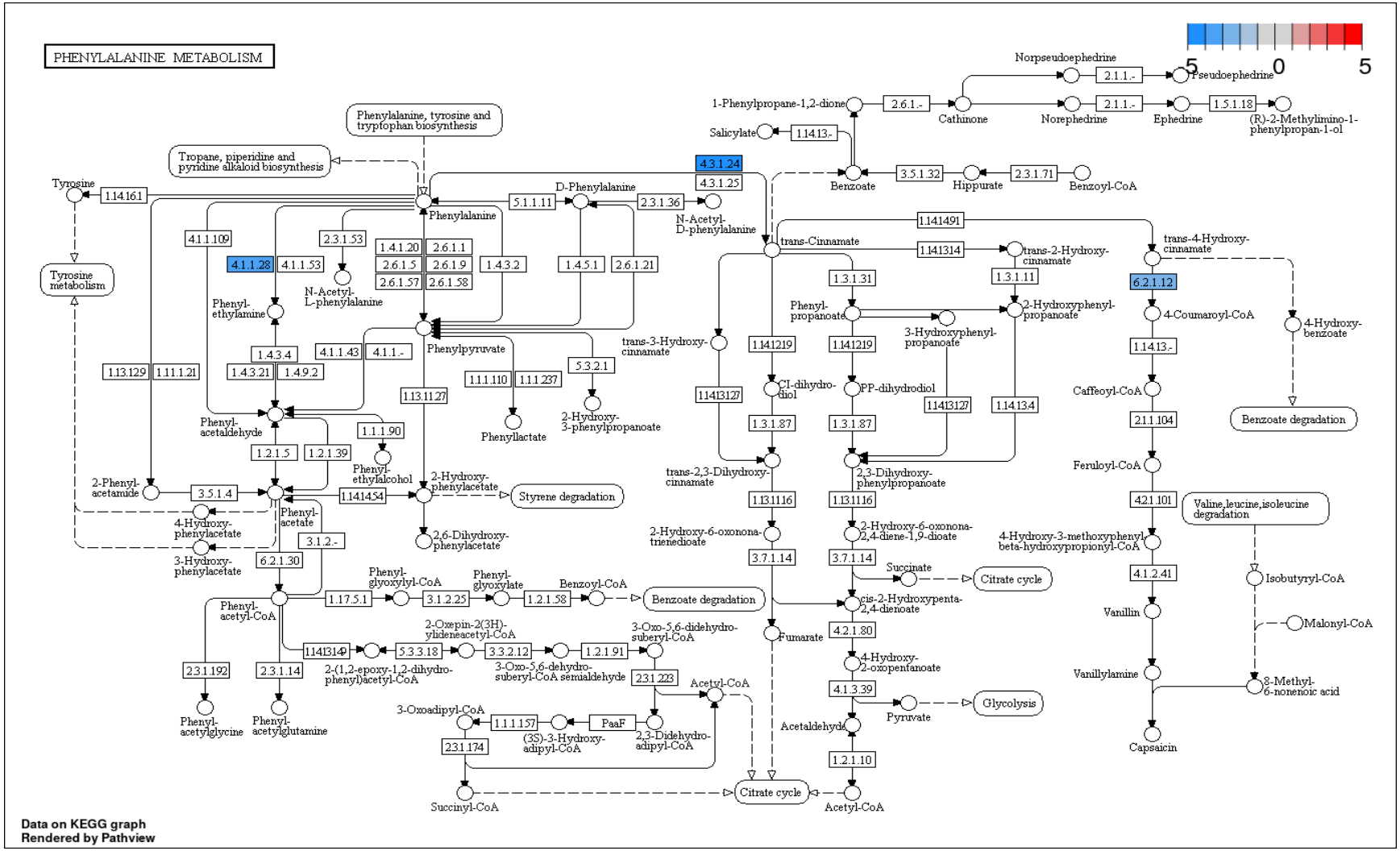


Figure S2. 5 KEGG map of physiological pathways regulated under water stress in 193 Najran (Cv2) wheat at flowering stage. DEGs involved in Phenylalanine metabolism. Genes down-regulated by water stress are 4.1.1.28 aromatic-L-amino-acid decarboxylase, 4.3.1.24 phenylalanine ammonia-lyase and (6.2.1.12) 4-coumarate---CoA ligase. p-value cut-off of 0.05, The colouring of the pathways is based on the mean log2 fold changes (-5 > p < 5).

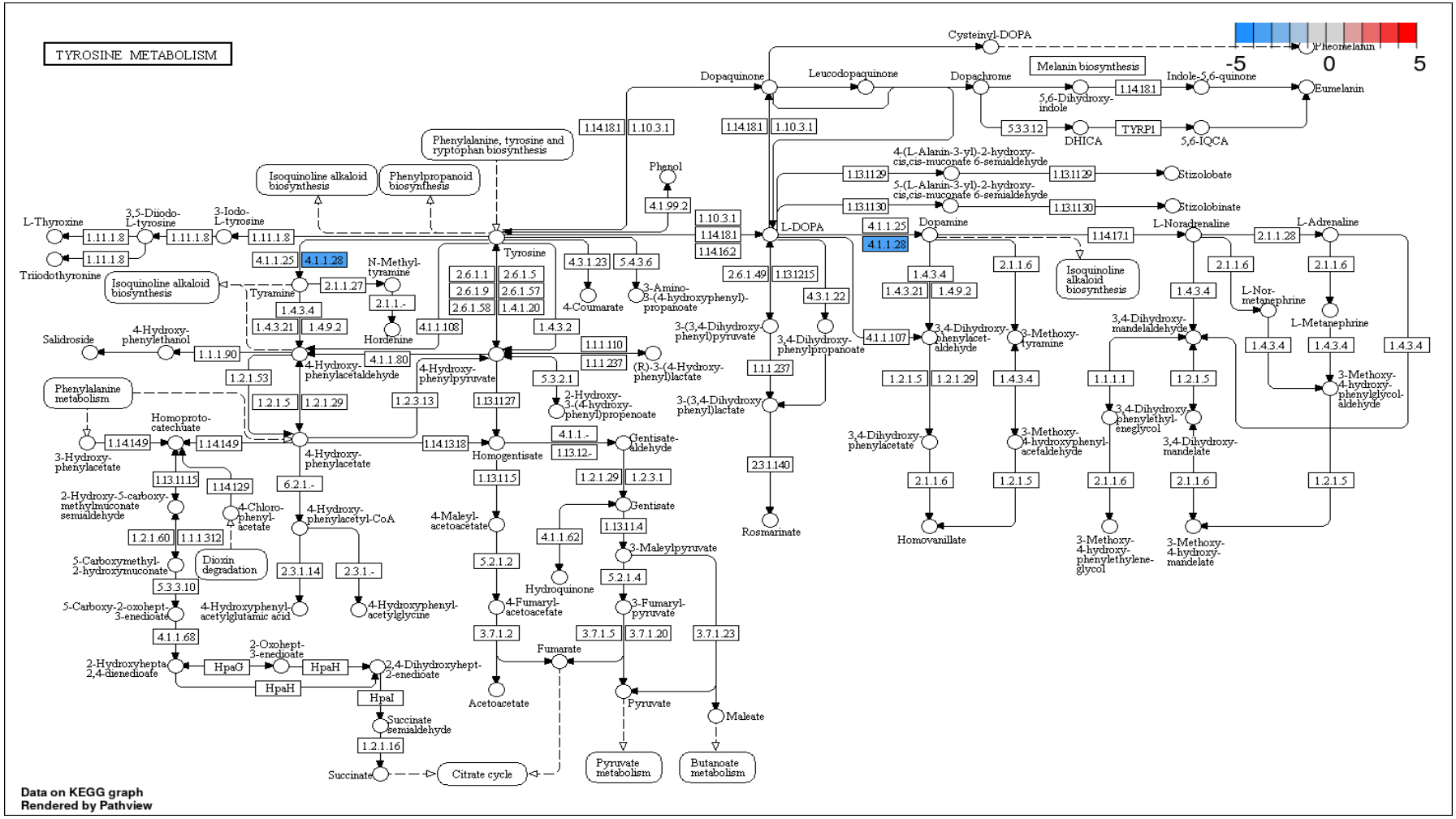


Figure S2. 6 KEGG map of physiological pathways regulated under water stress in 193 Najran (Cv2) wheat at flowering stage. DEGs involved in Tyrosine metabolism. Genes down-regulated by water stress in blue box are 4.1.1.28 aromatic-L-amino-acid decarboxylase, *p*-value cut-off of 0.05, The colouring of the pathways is based on the mean log2 fold changes ($-5 > p < 5$).

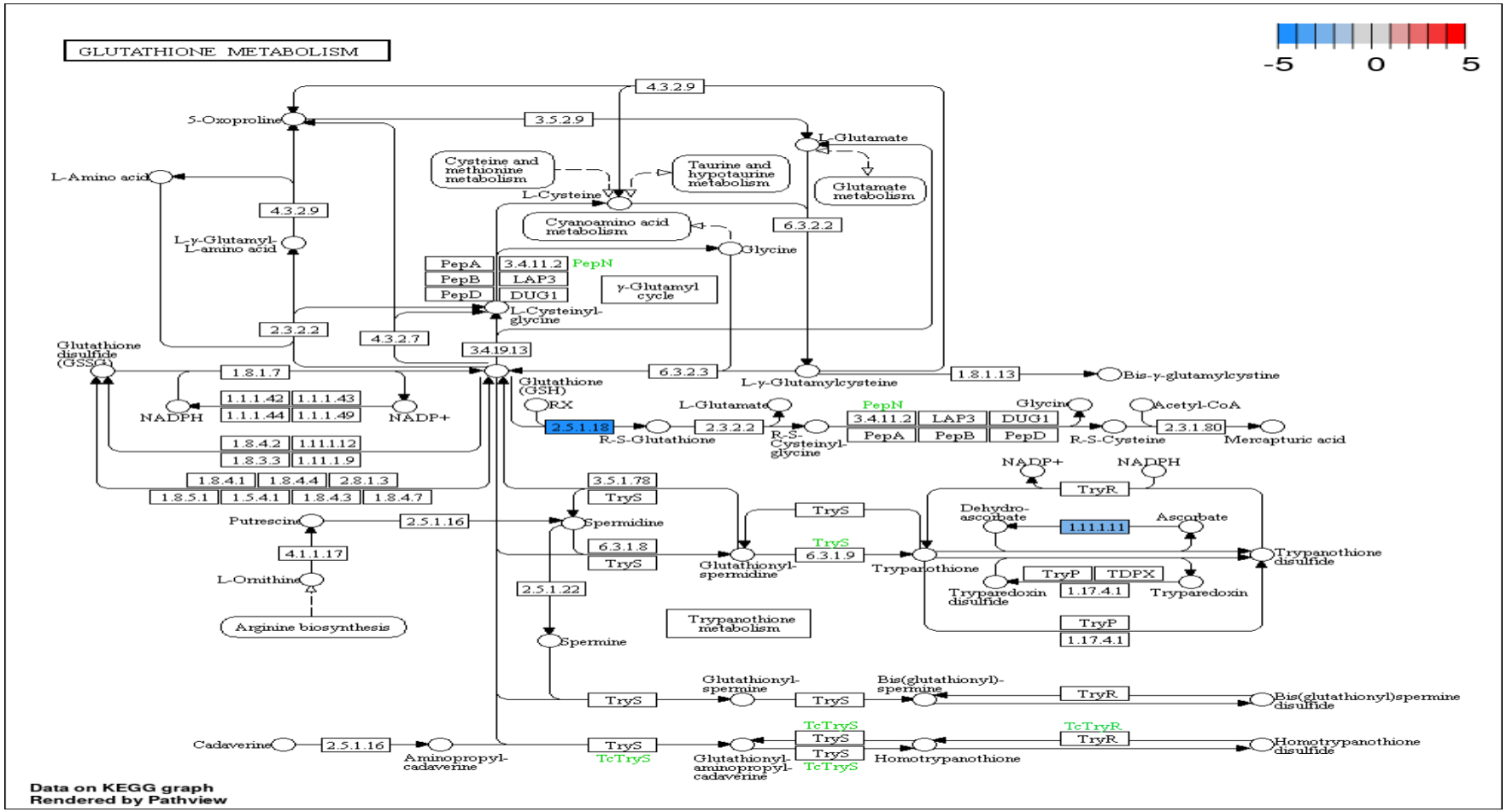


Figure S2. 7 KEGG map of physiological pathways regulated under water stress in 193 Najran (Cv2) wheat at flowering stage. DEGs involved in Glutathione metabolism, Genes down-regulated by water stress in blue box are 2.5.1.18 glutathione transferase and 1.11.1.11 L-ascorbate peroxidase. *p*-value cut-off of 0.05, The colouring of the pathways is based on the mean log2 fold changes (-5 > p < 5).

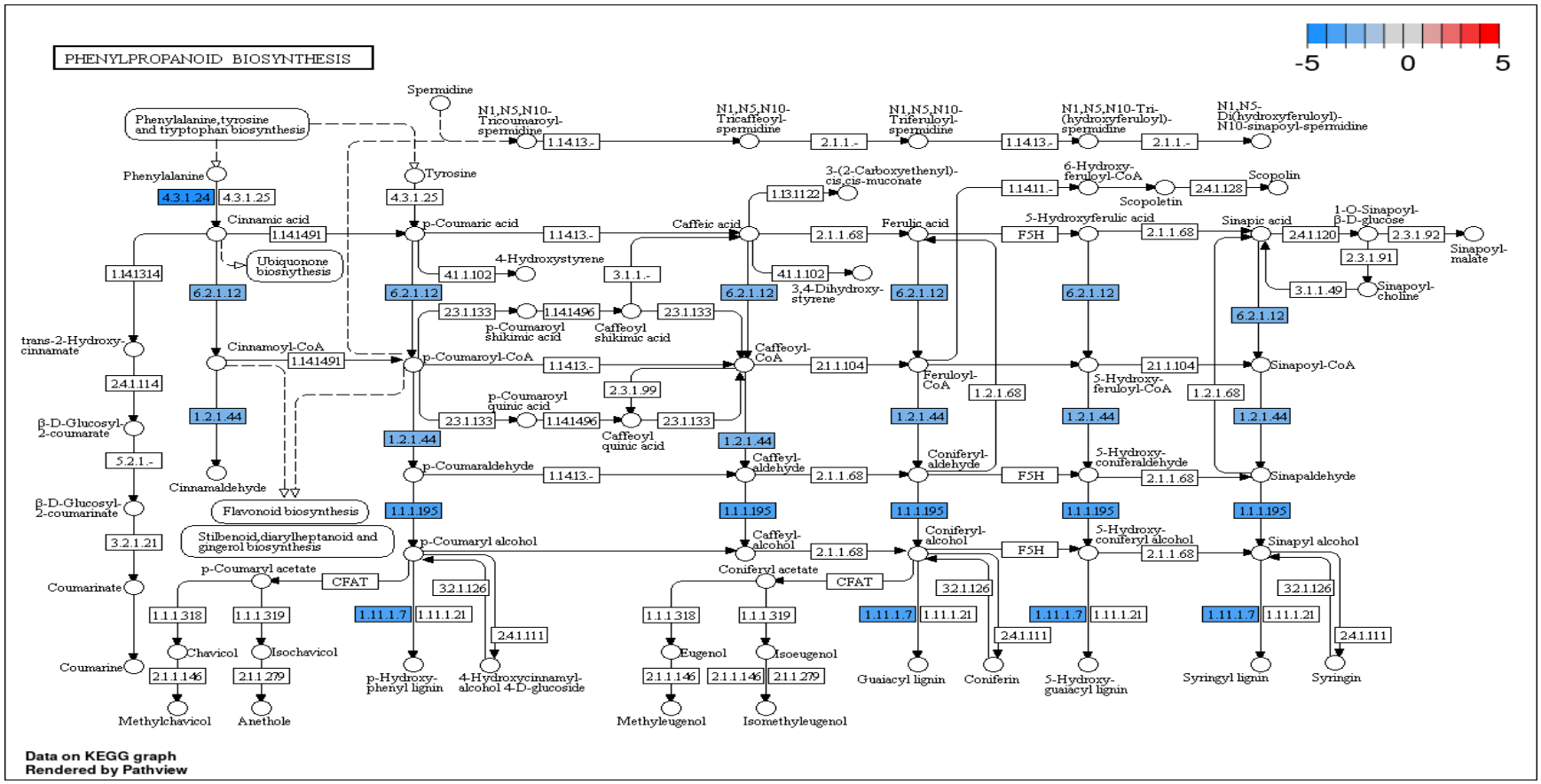


Figure S2. 8 KEGG map of physiological pathways regulated under water stress in 193 Najran (Cv2) wheat at flowering stage. DEGs involved in Phenylpropanoid biosynthesis. Genes down-regulated by water stress in blue box are 4.3.1.24 phenylalanine ammonia-lyase, 6.2.1.12 4-coumarate--CoA ligase, 1.2.1.44 cinnamoyl-CoA reductase, 1.11.1.7 peroxidase and 1.1.1.195 cinnamyl-alcohol dehydrogenase. p-value cut-off of 0.05, The colouring of the pathways is based on the mean log2 fold changes ($-5 > p < 5$).

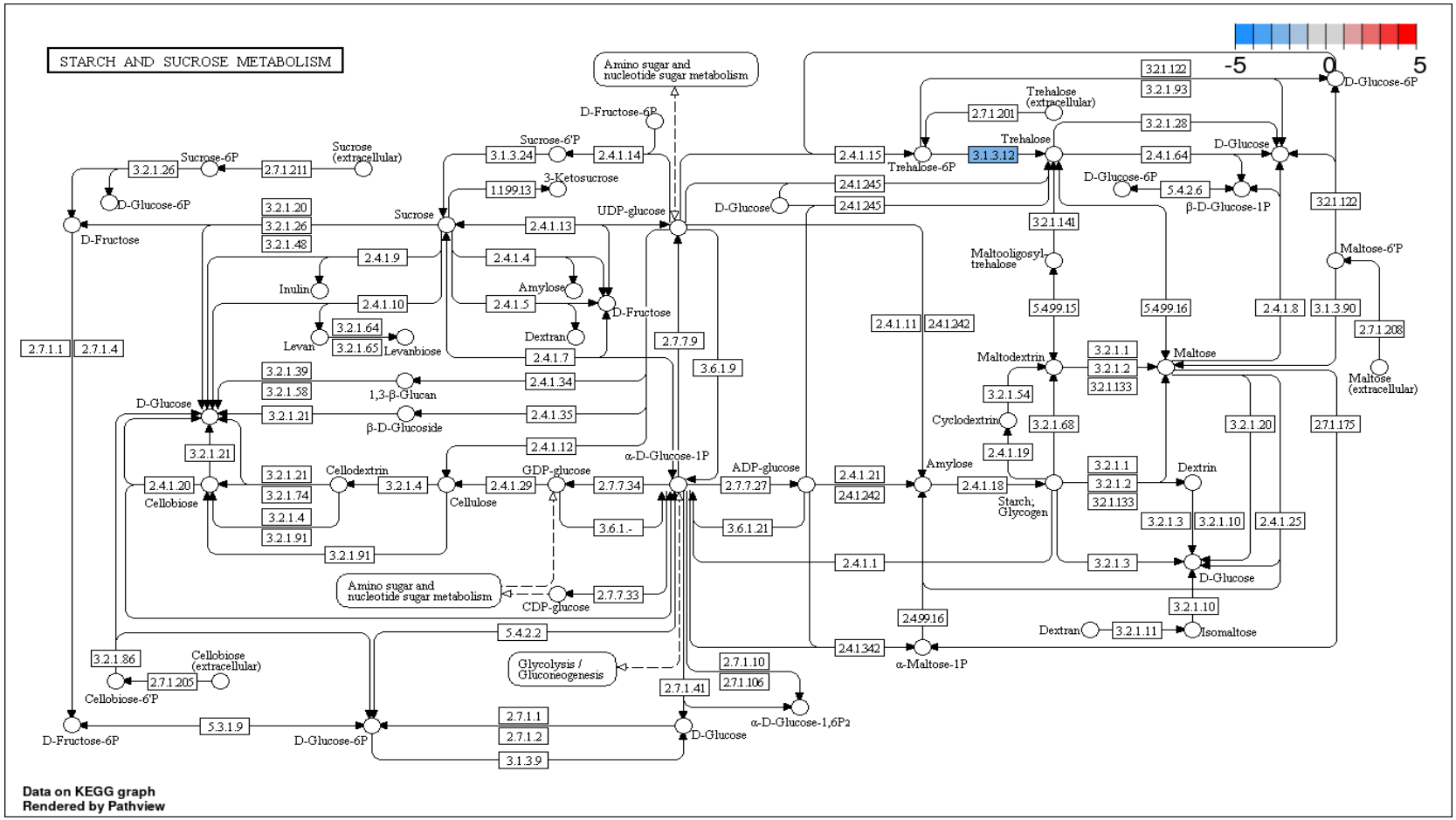


Figure S2. 9 KEGG map of physiological pathways regulated under water stress in 193 Najran (Cv2) wheat at flowering stage. DEGs involved in Starch and sucrose metabolism, Genes down-regulated by water stress in blue box are 3.1.3.12 trehalose 6-phosphate phosphatase. *p*-value cut-off of 0.05, The coloring of the pathways is based on the mean log2 fold changes (-5 > p < 5).

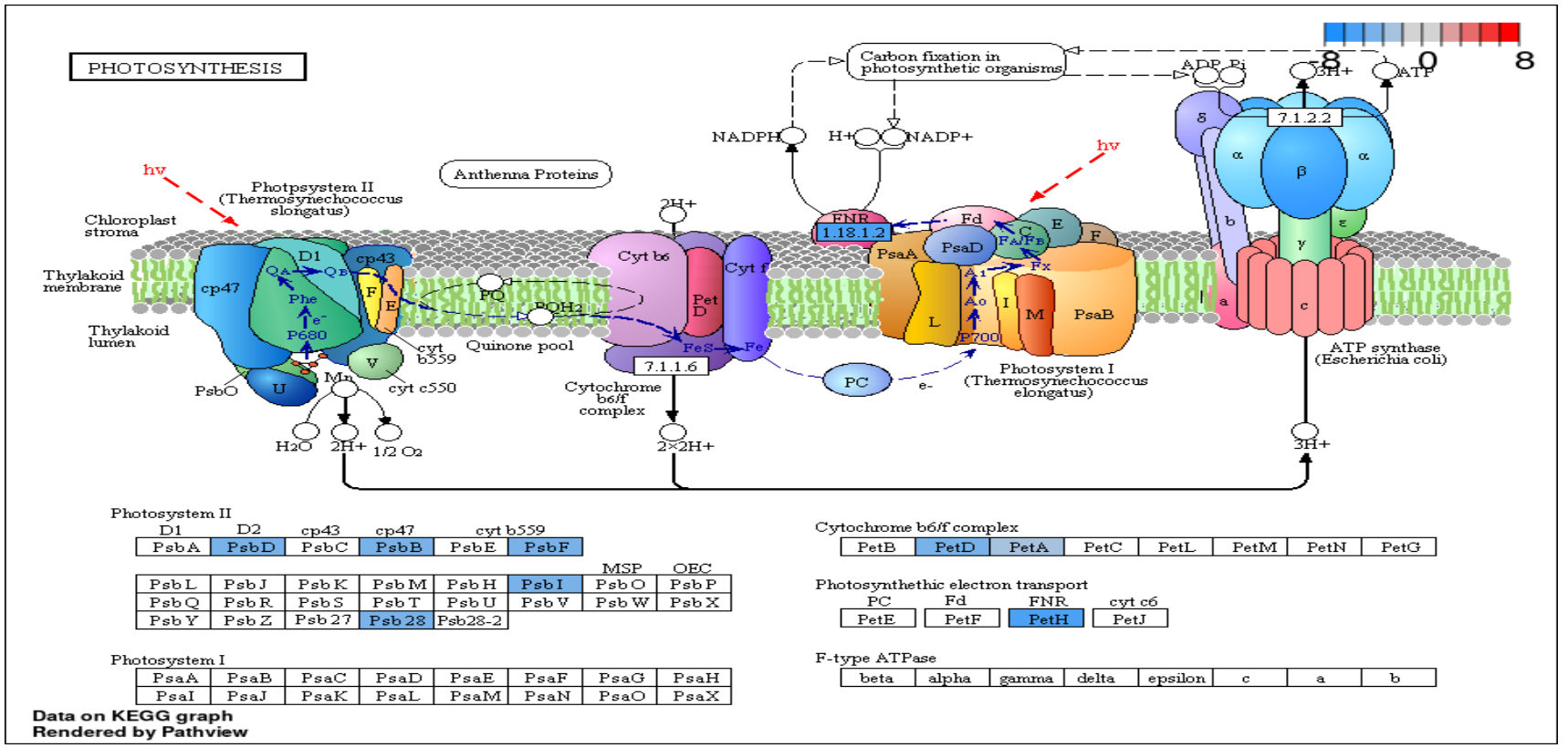


Figure S2. 10 KEGG map of physiological pathways regulated under water stress in 377 Rafha wheat cultivar (Cv4) at grain filling stage. DEGs involved in Photosynthesis, Genes down-regulated by water stress in blue box are psbD; photosystem II P680 reaction center D2 protein, psbB; photosystem II CP47 chlorophyll apoprotein, psbF; photosystem II cytochrome b559 subunit beta, psbI; photosystem II PsbI protein, psb28; photosystem II 13kDa protein, petD; cytochrome b6-f complex subunit 4, petA; apocytochrome f, petH; ferredoxin--NADP+ reductase. *p*-value cut-off of 0.05, The colouring of the pathways is based on the mean log2 fold changes ($-5 > p < 5$).

Appendix C Supporting information

Table S3. 1 list of common differentially expressed proteins in wheat cultivars *Triticum aestivum* L.; 193Najran (Cv2) (Drought-resistant) and 377 Rafha (Cv4) (Drought-sensitive) at three growth stages: a. vegetative, b. flowering and c. grain filling, subjected to 15% PEG6000. Up-regulated and down-regulated proteins (GO terms) are indicated in red and blue, respectively, with colour gradients indicating different levels of significance.

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A096UJV2	Chlorophyll a-b binding protein, chloroplastic	0.263	-0.536	0.128	-0.345	0.71	1.39	1.207	-1.623	0.395	0.769	0.883	1.017
A0A0C4BJ37	Chlorophyll a-b binding protein, chloroplastic	0.67	0.94	0.512	1.172	0.136	-0.219	1.922	-1.834	0.874	-0.808	1.975	-1.947
A0A0C4BK97	Glycine cleavage system H protein; mitochondrial	0.333	-0.976	0.477	-0.845	0.138	0.289	0.155	0.301	0.952	-0.612	0.627	1.253
A0A0C4BKF6	40S ribosomal protein S14	0.901	-1.346	0.086	-0.295	0.02	0.058	0.36	0.556	0.41	-0.229	0.148	0.361
A0A1D5RY74	Malate dehydrogenase	0.102	-0.355	0.014	0.04	1.239	-1.804	0.127	0.169	0.315	0.685	0.761	-0.902
A0A1D5S7U8	Pyruvate kinase	0.483	-0.856	0.02	-0.055	2.222	1.621	0.302	-0.702	0.031	0.084	0.743	1.31
A0A1D5SCA9	NADPH-protochlorophyllide oxidoreductase	0.927	1.608	1.087	1.052	1.308	-2.06	1.004	1.045	2.467	2.066	0.116	0.135
A0A1D5SFV9	Histone H4	0.084	0.233	0.215	0.648	0.246	0.535	1.554	-1.087	0.535	-0.608	0.693	-0.819
A0A1D5SI65	Glycosyltransferase	0.252	-0.623	0.35	-0.69	0.636	1.268	0.361	0.623	1.31	1.553	0.054	-0.116
A0A1D5SLG9	Glucose-6-phosphate isomerase	0.306	0.723	0.073	-0.212	0.105	-0.269	1.364	1.421	1.213	-1.397	0.36	-0.779
A0A1D5SPJ2	uncharacterized oxidoreductase At4g09670	0.364	-0.42	0.231	0.45	0.123	-0.297	0.609	1.316	0.716	1.289	2.14	1.704
A0A1D5SQ95	UPF0603 protein Os05g0401100, chloroplastic	0.18	0.37	0.001	0.003	0.744	-0.503	0.962	-1.483	1.406	-1.591	0.61	-1.415
A0A1D5SSC8	mitochondrial outer membrane protein porin 2-like	0.466	0.836	0.017	-0.029	1.398	-0.721	0.117	0.195	0.14	0.454	0.17	0.495
A0A1D5ST23	Ribosomal protein;50S ribosomal protein L1, chloroplast	0.056	-0.155	0.272	-0.747	0.018	-0.052	1.327	-1.646	0.237	-0.286	0.088	-0.214
A0A1D5STK8	Glycosyltransferase	0.43	-1.283	0.095	-0.279	0.899	-1.295	0.22	-0.345	0.06	-0.131	2.037	-1.784

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D5SU87	chitinase 8-like	0.386	0.485	0.502	-1.007	1.147	1.095	0.216	-0.346	0.482	-0.808	0.02	-0.055
A0A1D5SV97	ATP synthase subunit beta, mitochondrial-like;	0.233	0.623	0.147	-0.449	0.628	1.242	1.602	-1.173	1.227	-0.965	0.048	0.074
A0A1D5SZR3	Alpha-galactosidase	0.62	1.511	1.368	1.576	0.458	-0.667	0.116	-0.154	0.524	0.856	0.022	0.076
A0A1D5T057	Histone H2B	1.071	1.601	0.61	-1.117	0.808	1.44	0.445	0.816	0.384	-0.57	0.669	0.827
A0A1D5T261	isovaleryl-CoA dehydrogenase, mitochondrial	0.692	-1.552	0.451	1.052	0.546	0.651	1.298	1.271	0.016	-0.023	0.57	-1.294
A0A1D5T356	alanine aminotransferase 2 isoform X2	0.21	-0.863	0.113	-0.361	0.243	-0.469	0.103	0.075	0.225	-0.368	1.021	-1.278
A0A1D5T8H3	Aminomethyltransferase	0.177	0.55	0.522	1.108	0.6	0.923	0.031	-0.022	0.557	-0.835	0.182	0.346
A0A1D5T993	Elongation factor G, chloroplastic;	0.059	0.187	0.475	-1.081	0.539	0.783	0.346	-0.548	0.622	-0.857	0.183	-0.397
A0A1D5TCN3	Cytochrome b-c1 complex subunit Rieske, mitochondrial;	0.231	-0.559	0.463	1.017	0.003	0.008	0.424	-0.617	0.149	0.316	0.08	0.231
A0A1D5TCT8	Glutamyl-tRNA(Gln) amidotransferase subunit A, chloroplastic/mitochondrial	0.062	-0.126	0.136	0.359	1.196	-1.886	0.618	1.28	0.004	0.001	0.698	-0.753
A0A1D5TM38	Chlorophyll a-b binding protein, chloroplastic	0.055	0.086	0.229	-0.244	1.688	1.509	2.201	-2.239	0.696	-1.226	0.989	-1.194
A0A1D5TP30	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	0.089	-0.291	0.183	0.549	0.133	-0.444	0.174	0.257	0.445	-0.255	0.15	0.202
A0A1D5TQL3	Superoxide dismutase [Cu-Zn];	0.467	-1.307	0.845	-1.243	0.636	-0.852	0.007	0.014	0.231	0.546	1.04	-1.683
A0A1D5TUK7	40S ribosomal protein S8;	0.635	-1.379	0.009	-0.027	2.198	1.511	2.93	-1.695	1.107	-1.226	0.444	-0.997
A0A1D5U1Z0	Malate synthase;	0.087	0.248	0.339	0.757	0.114	-0.326	0.064	-0.135	0.202	0.465	0.182	-0.341
A0A1D5U6J5	Alpha-galactosidase	0.379	-0.288	1.348	1.958	0.153	0.324	0.018	0.062	0.004	-0.007	0.695	-1.124

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D5UKT1	36.4 kDa proline-rich protein-like	0.314	-0.581	0.295	-0.67	0.159	0.499	0.73	-1.279	0.169	-0.367	0.444	-0.569
A0A1D5UN48	putative alpha-L-fucosidase 1	1.049	1.988	0.239	0.683	0.141	0.338	0.31	0.372	0.088	0.155	0.267	0.481
A0A1D5UP29	Lipoxygenaseseed linoleate 9S-lipoxygenase-3-like isoform X1	0.085	0.253	0.307	-0.796	0.475	0.558	0.664	-1.076	1.307	1.126	0.632	1.236
A0A1D5UPW6	Serine hydroxymethyltransferase	2.092	-0.977	0.717	1.234	0.285	-0.283	0.005	-0.018	0.69	1.103	0.015	0.053
A0A1D5UVA0	probable glutathione S-transferase GSTU6;hypothetical protein	0.305	0.636	0.191	-0.589	0.134	-0.285	0.385	-0.901	0.027	-0.044	0.129	0.331
A0A1D5UXQ3	protein curvature thylakoid 1B, chloroplastic like isoform X2	0.593	1.52	0.168	0.47	0.021	0.061	0.08	-0.142	0.691	0.366	0.214	-0.395
A0A1D5V165	probable ribose-5-phosphate isomerase 3, chloroplastic	0.454	-0.766	0.36	-0.584	0.927	1.395	0.362	0.778	0.461	-0.895	0.045	0.122
A0A1D5V1U8	Cytochrome b6-f complex iron-sulfur subunit	0.027	-0.094	0.831	1.403	0.024	0.081	0.26	0.185	0.722	1.032	0.1	0.199
A0A1D5V409	Alpha-1,4 glucan phosphorylase	0.336	-0.957	0.645	1.035	1.479	2.089	0.487	-0.805	1.152	-1.566	0.539	-0.572
A0A1D5V696	Glycine cleavage system P protein	0.109	0.349	0.119	0.397	0.079	0.176	1.708	-1.137	1.314	-0.884	0.498	-0.805
A0A1D5V6B7	Wheatwin-1	0.387	-0.89	1.374	2.002	0.672	1.255	0.292	0.535	0.098	-0.15	0.673	-0.755
A0A1D5V831	S-formylglutathione hydrolase	0.152	0.314	0.677	-1.035	1.071	1.068	0.016	0.052	0.064	0.044	0.681	0.976
A0A1D5V9V3	Peroxidase	0.151	-0.173	0.267	0.581	0.814	-1.391	0.549	1.297	0.296	0.494	0.705	-0.84
A0A1D5VDW6	Aspartate aminotransferase;	1.008	1.796	0.605	1.3	0.722	-1.377	0.078	0.042	0.272	0.444	1.606	0.563
A0A1D5VFL6	40S ribosomal protein S10-1-like	0.046	-0.147	0.062	-0.181	0.052	0.137	0.854	1.083	0.286	0.597	0.075	-0.211
A0A1D5W045	NADH-cytochrome b5 reductase	1.298	-1.524	0.214	0.474	0.526	-1.222	0.048	0.136	0.196	-0.504	0.553	-0.654

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D5W4C8	Carboxypeptidase	0.065	0.175	0.079	0.089	0.151	-0.26	0.048	-0.136	0.343	0.977	0.113	0.357
A0A1D5W4K9	Non-specific lipid-transfer protein	0.173	-0.38	0.154	-0.374	0.817	-1.136	0.705	0.74	0.762	1.186	0.051	0.114
A0A1D5W9E0	Triosephosphate isomerase	0.037	-0.16	0.824	1.532	0.499	0.914	0.402	-0.281	0.048	0.063	0.066	0.184
A0A1D5WBY7	Non-specific lipid-transfer protein	0.58	-0.993	0.235	0.69	1.136	1.542	0.017	-0.038	0.147	0.241	0.062	-0.157
A0A1D5WER2	Isocitrate dehydrogenase [NADP]	0.077	0.181	0.562	-1.162	0.645	-0.808	0.379	0.652	0.042	-0.092	0.168	0.501
A0A1D5WFV2	Histone H2A	0.158	-0.402	0.011	0.032	0.03	-0.085	0.527	-0.933	0.035	-0.1	0.005	-0.015
A0A1D5WFY5	D-glycerate 3-kinase, chloroplastic	0.249	0.619	0.455	-1.001	1.145	1.24	0.863	-1.093	0.321	0.373	0.021	-0.058
A0A1D5WGJ4	Amidophosphoribosyltransferase;	1.446	-1.989	0.143	-0.482	0.311	0.637	0.643	-0.904	1.004	-0.624	0.956	-1.084
A0A1D5WIJ6	psbP domain-containing protein 6, chloroplastic	0.732	-1.743	0.237	0.509	0.393	-0.989	1.143	0.928	0.213	0.285	0.315	-0.466
A0A1D5WKD6	Malate dehydrogenase; mitochondrial-like	0.663	-1.518	0.211	-0.358	0.027	0.074	0.142	-0.299	1.024	-1.631	0.526	-0.959
A0A1D5WKK3	50S ribosomal protein L12-2, chloroplastic	1.753	-1.763	0.332	-0.841	0.058	0.156	1.058	-1.348	1.219	1.035	0.18	-0.447
A0A1D5WL54	Peroxiredoxin-2C	0.281	0.642	0.335	-0.788	0.197	0.437	1.686	-1.501	0.117	0.272	0.082	0.171
A0A1D5WLR3	thiol protease SEN102-like	0.303	-0.558	1.944	1.89	0.993	1.416	1.647	1.873	0.604	0.893	0.096	0.243
A0A1D5WNQ6	Uncharacterized protein, protein chloroplast enhancing stress tolerance, chloroplastic	0.29	0.592	0.174	-0.514	0.152	-0.305	0.38	0.881	0.549	-0.715	1.616	-1.848
A0A1D5WP16	flavonoid O-methyltransferase-like protein Os11g0303600	0.521	1.03	0.897	0.567	0.308	-0.692	0.786	1.045	0.127	0.336	0.096	-0.189
A0A1D5WP28	glucan endo-1,3-beta-glucosidase GI-like	0.66	0.979	0.941	-1.118	0.41	0.876	0.119	0.161	0.642	-1.094	1.115	-1.171

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D5WRI3	Peptidyl-prolyl cis-trans isomerase	1.193	1.611	0.272	0.681	0.617	0.834	0.225	0.534	0.456	-0.762	0.221	-0.617
A0A1D5WU82	mitochondrial dicarboxylate/tricarboxylate transporter DTC-like	0.27	0.615	0.22	0.633	0.358	-0.953	0.438	-0.847	0.418	-0.55	0.087	0.085
A0A1D5WU85	Cysteine synthase	0.045	0.17	0.273	0.729	0.418	-0.881	0.009	-0.02	0.356	-0.583	0.438	-0.83
A0A1D5WVE3	Non-specific lipid-transfer protein	2.568	1.272	0.869	1.162	0.24	0.624	0.076	0.228	1.342	1.054	0.989	-1.192
A0A1D5WVK2	inner membrane protein ALBINO3, chloroplastic isoform X2	0.077	-0.161	0.05	-0.081	0.388	0.738	0.351	0.906	0.147	0.406	0.484	-1
A0A1D5X269	Ferredoxin--NADP reductase, chloroplastic;	0.692	-1.056	1.537	-1.965	0.045	-0.129	0.652	1.214	0.222	0.333	0.405	0.579
A0A1D5XG45	Purple acid phosphatase	0.039	-0.125	0.065	0.193	0.509	-0.985	0.483	0.722	0.478	0.774	0.042	-0.112
A0A1D5XY50	Glyceraldehyde-3-phosphate dehydrogenase; chloroplastic	0.19	-0.531	0.001	0.004	0.833	0.964	1.177	-1.214	0.902	-0.86	0.338	-0.493
A0A1D5XYP8	aquaporin TIP1-1	0.309	-0.845	0.531	1.212	0.024	-0.041	0.73	0.794	0.564	-0.583	1.16	1.657
A0A1D5XZK3	Catalase	1.429	-1.561	0.248	-0.489	0.269	0.641	0.157	0.423	0.808	-1.398	0.098	-0.22
A0A1D5XZP7	ATP synthase subunit b', chloroplastic	0.289	0.482	0.241	0.255	0.468	1.092	1.705	-1.402	1.222	-1.028	0.202	0.323
A0A1D5Y3Q3	cell division control protein 48 homolog E-like	0.042	-0.114	0.014	0.044	0.19	-0.577	0.093	-0.216	0.266	-0.437	0.052	-0.092
A0A1D5Y3S2	Histone H2A	0.384	0.435	0.65	1.261	1.098	-1.086	0.13	-0.503	0.045	0.106	0.235	-0.609
A0A1D5Y3V8	photosynthetic NDH subunit of lumenal location 1, chloroplastic	0.336	-0.964	0.076	-0.236	0.079	-0.182	0.342	-0.63	1.119	-1.083	0.035	0.088
A0A1D5Y5T3	phosphoglucomutase, cytoplasmic	0.3	-1.088	0.034	0.109	0.483	1.13	0.522	-0.466	1.815	-1.411	0.163	0.282
A0A1D5YFX0	Coatomer subunit alpha	0.039	-0.133	0.143	0.283	0.279	-0.434	0.19	0.438	0.569	0.855	0.029	0.089

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D5YIA9	Putative glutathione S-transferase GSTU1	0.145	0.324	0.541	0.808	0.06	0.141	1.003	-1.429	1.955	-0.9	0.264	-0.674
A0A1D5YL67	Glyceraldehyde-3-phosphate dehydrogenase	0.084	0.27	0.12	-0.262	0.269	0.481	1.36	-1.122	0.888	-0.687	0.411	-0.978
A0A1D5YLT8	Aconitate hydratase	0.026	-0.079	0.578	1.134	1.445	1.711	0.973	-1.005	0.74	-0.82	0.275	-0.567
A0A1D5YM24	Cysteine synthase	0.118	-0.331	0.367	0.841	0.262	-0.625	0.251	0.433	0.196	0.348	1.142	-1.306
A0A1D5YMA0	Obg-like ATPase 1	0.282	0.732	0.418	-0.915	0.176	-0.381	0.087	-0.189	0.76	1.098	0.347	0.798
A0A1D5YN89	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	0.094	-0.152	0.189	-0.612	0.239	0.688	0.068	-0.19	0.609	-0.518	0.008	0.016
A0A1D5YP29	Glutathione S-transferase 6, chloroplastic	0.039	0.15	0.146	-0.25	0.21	-0.271	0.768	-0.957	0.378	0.975	0.147	0.477
A0A1D5YTJ8	Lipoxygenase	1.56	1.529	1.625	-1.734	0.707	0.828	2.584	1.955	0.025	0.064	0.068	-0.181
A0A1D5YTX8	Cysteine synthase	0.047	0.172	0.208	0.385	0.059	0.175	0.646	-0.88	0.018	-0.031	0.59	0.953
A0A1D5YVM4	Nucleoside diphosphate kinase	0.515	1.336	0.703	-0.981	0.404	0.539	0.927	-0.355	0.338	0.54	0.726	-1.437
A0A1D5YX23	Glucose-1-phosphate adenylyltransferase	0.051	-0.168	0.029	0.065	0.815	1.503	1.183	-0.737	0.253	0.646	0.468	0.652
A0A1D5Z4N3	Ribulose bisphosphate carboxylase small chain;chloroplastic	0.043	-0.116	0.024	0.073	0.599	0.707	1.243	-1.581	1.602	-1.448	0.951	-1.491
A0A1D5Z8U5	Malate dehydrogenase	0.151	0.521	0.117	-0.335	0.095	-0.151	1.362	-0.525	1.532	1.391	0.003	-0.011
A0A1D5ZA19	Calcium-transporting ATPase	0.254	0.762	1.554	-1.399	0.071	-0.172	0.627	0.554	0.293	0.737	0.39	-0.861
A0A1D5ZA24	Clathrin heavy chain	0.16	-0.346	0.382	-0.881	0.305	-0.763	0.771	-1.293	0.042	-0.102	0.278	-0.512
A0A1D5ZCZ8	Heat shock protein 90	0.655	-1.416	0.571	0.81	0.048	-0.135	0.718	-1.049	1.19	-1.571	0.501	-0.9

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D5ZID1	Peroxidase	0.181	-0.189	0.28	0.546	0.343	0.734	0.003	0.008	0.181	-0.445	0.378	-0.745
A0A1D5ZJM0	Cytochrome b-c1 complex subunit 7	0.776	-0.933	0.648	1.059	0.176	0.362	0.927	-0.797	0.921	0.506	0.105	-0.274
A0A1D5ZKE7	30S ribosomal protein 2, chloroplastic-like	0.335	-0.577	0.425	-0.883	0.792	1.693	0.422	-0.945	0.147	-0.327	0.247	0.224
A0A1D5ZNS0	probable plastid-lipid-associated protein 2, chloroplastic (PAP2)	0.642	1.215	0.432	-1.002	1.171	-1.659	0.026	0.057	1.433	1.029	0.478	0.8
A0A1D5ZNV7	30S ribosomal protein S9, chloroplastic	0.999	1.171	1.792	-1.876	2.103	1.466	0.262	-0.683	0.621	-0.112	0.428	-0.981
A0A1D5ZQF2	Citrate synthase 3, peroxisomal-like	0.419	-0.379	0.648	1.058	0.307	0.616	0.32	-0.895	0.023	0.067	0.924	-1.249
A0A1D5ZRR7	Vacuolar protein sorting-associated protein 35	0.308	-0.609	0.393	-0.937	0.494	1.041	0.278	-0.681	0.322	0.521	0.535	-0.604
A0A1D5ZTS6	Probable 6-phosphogluconolactonase 4, chloroplastic	0.33	-0.697	0.495	1.024	0.032	-0.049	0.008	0.025	0.069	-0.11	0.474	-1.162
A0A1D5ZV70	peptidylprolyl cistrans isomerase FKBP16-4, chloroplastic-like	1.252	-1.426	0.477	0.996	0.192	0.311	0.561	-1.135	0.412	0.733	0.081	-0.263
A0A1D5ZXH9	tetraspanin-8;hypothetical protein;	0.055	0.2	0.876	1.303	1.041	-1.298	0.124	0.26	0.245	0.566	0.88	1.304
A0A1D5ZYH0	heat shock protein 90-5, chloroplastic	0.402	0.988	1.429	-1.45	0.337	0.346	1.321	0.94	0.187	0.494	0.315	-0.845
A0A1D6A2S3	enolase-like isoform X2	0.281	0.812	0.02	-0.054	0.664	1.271	0.012	0.019	0.591	-1	0.283	-0.502
A0A1D6AB89	Carboxypeptidase	0.834	-1.292	0.273	-0.508	0.437	1.095	1.237	-1.318	0.433	0.908	0.691	-0.739
A0A1D6AGT9	Phosphoglycerate kinase	0.06	0.191	1.048	1.675	0.318	0.684	0.106	0.213	0.69	0.897	0.711	-1.185
A0A1D6AIF0	Citrate synthase	0.599	0.926	0.332	0.69	0.264	-0.8	0.322	-0.781	0.235	-0.563	0.374	0.281
A0A1D6AJX2	Lipoxygenase	0.136	0.304	0.674	-1.182	0.164	0.305	0.256	0.577	0.993	-1.322	0.011	-0.036

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D6AKZ2	ATP-dependent Clp protease proteolytic subunit, LOC109754380	0.4	0.935	0.219	0.463	0.194	0.47	0.907	1.088	0.277	0.307	0.094	0.25
A0A1D6AQV8	Aconitate hydratase	0.391	-0.897	0.176	0.513	1.662	-1.381	0.895	1.363	0.355	0.316	1.172	-1.651
A0A1D6AWP4	Ferredoxin--NADP reductase, chloroplastic; chloroplastic	0.134	0.515	0.07	-0.191	0.382	-0.641	0.29	0.431	0.266	0.6	0.099	-0.249
A0A1D6AXF6	Histone H2A	0.118	-0.323	0.212	0.338	0.151	0.286	1.633	-1.736	0.052	-0.18	0.334	0.611
A0A1D6AYW9	Lipoxygenase;Lipoxygenase	0.19	-0.511	0.483	-0.883	0.393	0.522	0.175	0.357	0.518	0.798	0.228	-0.665
A0A1D6B0E1	Histone H2A;	0.316	-0.81	0.501	0.714	0.355	-0.393	1.604	-1.631	0.111	-0.342	0.737	-1.388
A0A1D6B1Q1	Fumarylacetoacetase	0.85	-2.052	0.056	-0.096	0.932	-1.484	1.108	0.645	0.79	-1.418	0.514	-0.801
A0A1D6B2L5	ferredoxin--nitrite reductase, chloroplastic	1.305	2.04	0.063	0.177	0.461	-0.773	0.158	0.198	0.979	1.129	0.587	-1.122
A0A1D6B2M1	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	0.688	-1.378	0.133	-0.382	0.131	0.423	0.096	-0.244	0.065	0.134	0.171	0.139
A0A1D6B2W8	quinone oxidoreductase PIG3	0.327	0.54	0.229	0.621	0.252	0.639	0.803	0.822	0.847	-0.992	0.067	-0.161
A0A1D6B4C8	protein TIC 55, chloroplastic	0.006	0.02	0.488	1.003	0.414	1.027	0.528	-0.681	1.106	1.146	0.042	-0.054
A0A1D6B4Q8	calcium sensing receptor, chloroplastic	1.06	1.686	0.823	1.078	0.551	0.953	0.554	0.961	0.003	0.011	0.511	1.017
A0A1D6B4W1	GrpE protein homolog;uncharacterized protein LOC109736192	0.045	-0.138	0.125	0.401	0.107	0.26	0.447	0.482	0.125	-0.2	0.015	0.046
A0A1D6B7L4	serine protease SPPA, chloroplastic	0.194	0.574	0.237	-0.62	1.314	1.831	0.22	0.284	0.047	-0.098	0.39	0.555
A0A1D6B9K3	uncharacterized methyltransferase At1g78140, chloroplastic-like	0.225	-0.554	0.214	-0.479	0.064	0.225	0.277	0.667	0.379	-0.832	0.35	-0.389
A0A1D6BAG6	hsp70-Hsp90 organizing protein	0.212	0.543	0.189	0.622	0.452	0.297	0.214	-0.382	0.861	-0.541	0.004	-0.015
A0A1D6BAX3	mitochondrial phosphate carrier protein 3, mitochondrial-like	0.564	0.532	0.027	0.067	0.367	0.767	1.305	-1.98	0.875	-1.438	0.602	-1.04
A0A1D6BC24	peroxiredoxin-2E-2, chloroplastic-like	0.188	-0.564	0.428	0.575	1.699	1.14	0.644	-1.07	1.468	-1.856	0.398	-0.693
A0A1D6BE04	glycerate dehydrogenase;	0.091	0.299	0.286	0.795	0.239	0.32	1.65	-0.918	1.379	-1.067	0.303	-0.858

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D6BEB0	Inositol-1-monophosphatase	0.755	1.4	0.177	-0.461	0.419	1.009	0.923	0.637	0.181	-0.366	0.035	0.073
A0A1D6BF30	Ferredoxin--NADP reductase, chloroplastic; leaf isozyme, chloroplastic-like	0.019	0.082	0.343	-0.621	0.317	0.776	0.713	0.836	0.092	0.275	0.06	0.086
A0A1D6BMJ6	Fructose-bisphosphate aldolase	0.349	-1.094	0.041	-0.122	0.661	1.504	0.19	-0.329	0.254	-0.491	0.081	0.088
A0A1D6BR45	40S ribosomal protein S24;	0.278	-0.589	1.395	-1.723	0.053	0.091	0.187	-0.509	0.086	0.195	0.406	-1.014
A0A1D6BR81	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	0.409	1.016	0.064	-0.209	0.185	-0.437	0.241	0.395	0.409	-0.445	0.044	0.126
A0A1D6BU25	Profilin	0.493	-1.36	0.578	0.942	1.515	-1.285	0.101	0.191	1.164	-1.284	0.528	1.115
A0A1D6BU69	Peroxidase	0.144	0.579	0.422	0.691	0.152	-0.392	0.353	0.429	0.026	-0.082	0.225	0.497
A0A1D6BXL7	Ferredoxin--NADP reductase, chloroplastic	0.547	1.232	0.35	-0.505	0.653	1.279	0.404	0.421	0.139	-0.434	0.253	-0.47
A0A1D6CEQ4	Probable alanine--tRNA ligase, chloroplastic;	1.207	1.361	0.048	0.115	0.067	-0.219	0.129	0.359	1.435	1.438	0.495	0.673
A0A1D6CL61	phosphoenolpyruvate carboxylase 1-like	0.583	1.215	0.123	-0.235	0.205	0.585	0.307	0.372	0.554	1.188	0.086	-0.176
A0A1D6CM34	photosystem II stability/assembly factor HCF136, chloroplastic	0.287	0.783	1.059	-1.256	0.112	0.322	0.355	-0.432	0.099	-0.286	0.215	0.452
A0A1D6CPZ4	Peroxidase	0.021	-0.096	0.086	0.202	0.851	1.411	0.478	0.581	0.374	0.772	0.069	0.153
A0A1D6CRA1	Peptidylprolyl isomerase; FKBP13, chloroplastic-like	1.126	-1.72	0.095	0.232	0.088	0.243	1.078	-1.334	0.58	-1.066	0.464	-0.832
A0A1D6CT03	endoplasmin homolog isoform X1	0.092	-0.241	0.085	-0.207	0.331	-0.486	0.574	-0.711	1.882	-1.871	0.685	-1.028
A0A1D6CUN4	Reticuline oxidase-like protein;berberine bridge enzyme-like 27	0.226	-0.86	0.411	1.107	0.441	-0.965	0.138	-0.183	0.064	0.059	0.276	0.551
A0A1D6CUU7	heme oxygenase 1, chloroplastic-like	0.026	-0.072	0.2	0.527	1.095	-0.802	0.02	0.06	0.311	-0.598	0.243	-0.573
A0A1D6CV32	Mg-protoporphyrin IX chelatase;	0.041	-0.153	1.559	-1.608	0.096	-0.187	0.038	0.085	0.394	-0.77	0.258	0.68
A0A1D6CVC4	Ferredoxin; chloroplastic	0.527	-1.286	0.408	1.088	0.283	0.494	1.804	-1.319	2.796	-0.477	0.076	0.172

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D6CVV0	Annexin;	0.291	0.841	0.069	-0.171	0.033	0.117	0.601	0.944	0.056	-0.158	0.382	-0.516
A0A1D6CW84	glutathione S-transferase DHAR3, chloroplastic	0.049	0.185	0.513	1.197	0.732	-0.89	0.242	-0.478	0.365	0.506	0.157	0.444
A0A1D6CZE8	Glutathione peroxidase	0.203	0.497	0.661	-0.584	1.136	1.512	0.312	0.67	1.098	1.199	1.637	1.628
A0A1D6D033	sulfite oxidase	1.357	-2.197	0.359	0.902	0.037	0.085	0.531	-0.378	0.203	0.365	0.481	0.993
A0A1D6D192	Proteasome subunit;proteasome subunit beta type-6-like;beta1 proteasome-7D;beta1 proteasome-7D	0.138	-0.402	0.568	0.954	0.064	-0.177	0.313	-0.603	0.838	-1.357	0.508	-0.946
A0A1D6D218	Carboxypeptidase	0.331	-0.668	0.324	0.733	0.187	0.494	1.139	-1.55	0.924	-1.24	0.742	0.93
A0A1D6D2S9	Chlorophyll a-b binding protein, chloroplastic;	0.244	0.656	0.608	-0.853	0.117	-0.275	0.58	-0.714	0.424	-0.337	0.008	-0.012
A0A1D6D3F0	trigger factor-like protein TIG, Chloroplastic	0.831	1.762	0.425	0.718	0.26	0.516	0.098	0.077	0.227	0.575	0.355	-0.827
A0A1D6D3F8	Chlorophyll a-b binding protein, chloroplastic; chlorophyll a-b binding protein 1B-21, chloroplastic;hypothetical protein	0.151	0.388	0.026	-0.073	0.157	0.397	1.589	-1.571	1.554	-1.675	1.078	-1.403
A0A1D6D5D4	Plastocyanin; chloroplastic	0.819	-0.999	1.324	1.112	0.138	0.243	0.439	-0.403	0.008	0.006	0.587	0.616
A0A1D6D5U3	Aldehyde dehydrogenase family 2 member B4, mitochondrial (ALDH)	0.613	0.526	0.968	-0.753	0.254	-0.371	0.606	-1.077	0.119	-0.388	0.395	-0.941
A0A1D6D5U6	Peptidylprolyl isomerase;Peptidylprolyl isomerase	1.014	1.307	0.009	-0.022	0.109	0.252	0.111	-0.307	0.092	-0.24	0.052	-0.101
A0A1D6D7A2	glucan endo-1,3-beta-glucosidase GII-like	0.058	-0.199	0.029	-0.07	1.458	1.469	0.449	0.201	0.421	-0.893	2.749	-1.507
A0A1D6D7R5	Peroxidase	0.08	-0.205	4.238	1.827	0.146	-0.304	0.017	-0.05	0.193	0.486	0.287	-0.711
A0A1D6D888	Carbonic anhydrase	0.208	0.555	0.232	0.661	1.692	1.476	1.093	-1.195	0.283	-0.446	1.083	-1.106
A0A1D6D8H8	Histone H2B	0.338	0.932	0.031	0.082	0.774	1.239	0.808	1.12	0.767	-0.856	1.117	-1.253
A0A1D6D8L9	Pyruvate, phosphate dikinase 2	0.175	0.602	0.135	0.214	0.517	-0.755	0.391	0.165	0.166	-0.436	0.342	0.863
A0A1D6D9D4	Aminoacylase	0.081	0.219	0.477	1.117	0.146	0.417	0.115	-0.297	0.143	-0.264	0.386	-0.61

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D6D9N7	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex;	0.33	1.053	0.936	-1.374	0.285	-0.881	0.052	0.095	0.208	0.46	0.068	0.042
A0A1D6DED4	Dihydrolipoyl dehydrogenase	0.262	-0.484	0.039	-0.126	0.199	0.573	0.518	-0.822	2.402	-1.493	0.109	-0.217
A0A1D6DEQ5	Peroxidase	0.614	1.394	0.06	0.206	0.29	-0.688	0.287	-0.351	0.564	0.69	0.354	0.699
A0A1D6DEU3	probable ribose-5-phosphate isomerase 3, chloroplastic isoform X2;	0.046	-0.153	0.558	-1.09	0.127	0.355	0.942	1.021	0.375	-0.723	0.164	-0.371
A0A1D6DEW4	Chlorophyll a-b binding protein, chloroplastic	0.22	-0.42	0.133	0.473	1.434	1.25	1.454	-1.506	0.076	-0.037	0.255	-0.626
A0A1D6DFK9	Beta-amylase	0.468	1.055	0.53	-0.74	0.021	-0.065	1.782	1.271	0.155	-0.39	0.358	-0.676
A0A1D6DGJ9	CBS domain-containing protein CBSX2, chloroplastic-like	0.472	0.859	0.586	1.024	0.188	-0.363	1.837	1.357	3.092	-1.904	0.107	0.264
A0A1D6DGS9	monodehydroascorbate reductase 5, mitochondrial isoform X1	0.123	0.415	0.678	0.658	0.575	0.905	0.199	0.41	0.034	0.108	0.69	-1.191
A0A1D6DIM9	Obg-like ATPase 1	0.008	0.037	0.622	1.135	0.533	0.96	0.023	0.035	0.296	0.468	1.138	-1.506
A0A1D6DJH0	Peroxidase	0.545	-1.059	0.873	1.51	0.195	-0.484	0.369	-0.471	0.952	-0.923	0.225	-0.496
A0A1D6DLG2	probable adenylate kinase 5, chloroplastic isoform X1	0.925	1.88	2.176	-2.188	0.38	-1.054	0.161	-0.083	1.908	-0.588	0.303	-0.31
A0A1D6DLJ4	glycine-rich protein 2-like;	0.01	0.028	0.156	-0.405	0.501	0.828	0.315	0.645	2.052	1.693	0.658	1.245
A0A1D6DME9	ATP synthase subunit gamma, chloroplastic	0.18	0.509	0.002	-0.005	0.065	0.214	1.045	-1	0.393	-0.924	0.291	0.48
A0A1D6RDG1	PRA1 family protein	0.164	-0.443	0.248	-0.709	0.523	0.988	0.101	-0.284	0.135	-0.171	0.262	0.59
A0A1D6RDZ8	Phosphoglycerate kinase	0.47	1.043	0.2	0.346	0.479	0.817	0.82	-1.055	0.924	-1.462	1.469	-1.752
A0A1D6RFJ3	Histone H2A	0.085	0.076	0.454	-1.036	0.314	-0.124	0.221	-0.575	0.421	0.669	0.417	1.118
A0A1D6RJM6	Peroxidase;	0.373	-0.631	0.205	-0.412	0.111	0.241	0.584	1.107	0.216	-0.433	1.17	1.636
A0A1D6RMY7	Thioredoxin reductase	0.51	-0.993	0.446	0.868	0.02	0.068	0.756	-1.197	0.543	-1.014	0.144	0.285

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
A0A1D6RP42	Malate synthase;	0.42	-1.345	0.179	-0.51	0.542	0.695	0.446	0.475	1.08	1.129	0.045	-0.154
A0A1D6RPG3	NADPH-protochlorophyllide oxidoreductase	0.189	0.459	0.296	0.475	0.038	-0.122	0.223	0.46	0.151	-0.42	0.34	0.598
A0A1D6RUA1	Dipeptide epimerase	0.072	0.196	0.249	0.52	0.869	-1.129	0.493	1.039	0.5	0.9	0.315	0.757
A0A1D6RUC3	phosphoenolpyruvate carboxylase 1-like	0.029	-0.092	0.67	-0.447	0.093	0.306	0.122	0.33	0.003	-0.01	0.061	-0.116
A0A1D6RXF7	40S ribosomal protein S3a;40S ribosomal protein S3a;	0.079	-0.26	0.55	-0.994	0.072	-0.148	0.341	0.294	0.808	-0.764	0.241	0.669
A0A1D6S3V8	Cysteine synthase;	0.225	-0.594	0.215	0.398	0.305	-0.29	1.923	-1.337	0.281	0.591	0.06	-0.202
A0A1D6S572	Alpha-mannosidase	0.297	-0.741	3.004	2.032	0.482	1.076	0.036	0.08	0.005	0.012	0.201	-0.372
A0A1D6S5I1	long chain acyl-CoA synthetase 8	1.128	1.186	0.192	-0.631	0.191	-0.475	0.253	0.533	0.697	-0.364	0.084	0.177
A0A1D6S5W2	Spermidine synthase	0.233	-0.737	0.614	0.974	1.265	-1.451	0.624	-0.858	0.542	-0.992	0.46	-0.947
A0A1D6S8Q4	Lipoxygenase;	0.375	0.967	1.433	-0.76	1.444	0.878	1.255	0.823	0.188	-0.556	0.222	-0.262
A0A1D6S9T8	Elongation factor Tu	0.042	0.154	0.153	0.5	0.959	1.066	1.225	-0.832	0.01	-0.015	0.097	-0.273
A0A1D6SB81	protein CURVATURE THYLAKOID 1D, chloroplastic-like	1.999	1.758	0.046	0.126	0.05	0.125	0.614	-0.949	0.075	-0.207	0.137	-0.277
A0A1D6SEV8	Catalase, catalase isozyme 1;	0.637	0.85	1.24	-1.918	0.122	0.284	0.634	-1.279	0.217	-0.333	0.73	-1.199
A0A1D6SFF6	Type I inorganic proton-pumping pyrophosphatase	0.532	-0.944	1.481	1.63	0.082	0.239	0.601	-1.179	0.697	-1.068	0.318	-0.613
W4ZM65	Nucleoside diphosphate kinase	0.291	0.881	0.081	-0.124	1.28	-0.98	0.355	-0.606	0.415	1.084	0.609	-1.15
W4ZQ59	60S acidic ribosomal protein P2B	0.065	0.191	0.062	-0.126	0.086	-0.157	0.692	1.169	0.395	0.963	0.24	-0.687
W4ZRQ6	Ribonuclease 1	0.909	1.499	0.049	0.127	2.011	1.384	0.134	0.337	0.293	-0.578	0.188	0.502
W5A6U2	Chlorophyll a-b binding protein, chloroplastic	0.706	-1.395	0.165	0.373	1.789	2.078	0.888	-1.269	1.586	-0.598	0.03	-0.05
W5A8H1	Histone H2A	0.351	-1.283	0.973	1.399	0.341	-0.707	2.376	-0.921	0.33	0.687	0.097	0.263
W5ACP9	26S proteasome non-ATPase regulatory subunit 14 homolog	0.863	1.066	0.322	-0.727	0.156	0.534	0.856	1.493	0.275	0.566	0.098	0.094
W5AFN1	ERBB-3 BINDING PROTEIN 1	0.313	-0.425	1.68	-1.652	0.513	0.966	0.126	0.115	0.238	-0.534	1.833	1.654
W5AGK9	Nucleoside diphosphate kinase	0.074	0.312	0.492	-0.608	0.6	-0.771	0.3	0.437	0.456	-0.958	2.281	-1.257
W5AL94	Peroxidase A2-like	0.288	0.722	0.277	-0.712	0.41	0.967	0.331	0.514	0.221	-0.43	0.165	-0.317

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
W5AM16	6-phosphofructo-2-kinase / fructose-2, 6-bisphosphatase-like isoform X2	0.116	0.331	0.66	-1.132	0.055	0.153	0.774	1.049	0.069	-0.172	0.347	0.703
W5AMG0	Peroxidase 2-like	0.89	1.341	0.784	1.102	0.588	0.99	0.653	1.1	0.153	-0.416	0.356	0.648
W5AMN3	Beta-hexosaminidase 1	0.024	0.079	0.541	1.222	0	0.001	1.191	-1.259	0.741	0.82	0.036	-0.115
W5AN44	ras-related protein RABA2a-like	0.248	0.852	0.322	0.943	0.161	-0.454	1.021	-0.808	0.784	0.513	0.397	-0.619
W5B0Q9	Xylose isomerase	0.061	0.167	0.122	-0.241	0.046	0.137	0.198	-0.464	2.53	2.025	0.019	-0.052
W5B474	Chlorophyll a-b binding protein, chloroplastic	0.222	0.564	1.475	1.505	0.109	-0.32	1.57	-1.198	0.631	1.007	0.315	-0.589
W5B6B7	Zeaxanthin epoxidase, chloroplastic	0.11	0.43	0.78	1.413	0.424	1.005	0.956	0.915	0.056	0.112	0.551	-0.788
W5B8T9	Dihydroorotate dehydrogenase (quinone), mitochondrial	0.587	0.889	0.486	1.256	0.407	0.474	0.335	0.766	0.058	-0.066	0.092	0.302
W5BBF4	Lipoxygenase	0.275	-0.412	0.52	0.419	0.419	1.01	0.474	-1.135	0.097	-0.21	0.007	0.015
W5BBT6	Chlorophyll a-b binding protein, chloroplastic;	0.045	0.125	0.002	0.004	0.027	0.04	1.934	-1.721	1.084	-1.16	1.053	-1.714
W5BEB6	Photosystem I reaction center subunit psaK, chloroplastic.	0.464	-0.992	0.111	0.23	0.325	0.759	2.251	-1.86	0.965	-0.907	0.204	-0.28
W5BFB4	Histone H2B	0.772	1.658	0.027	0.076	1.184	1.795	1.59	1.263	0.48	0.922	0.46	0.674
W5BPU1	40S ribosomal protein SA	0.243	-0.518	0.026	0.054	1.086	-1.667	0.209	0.493	0.06	-0.173	0.245	0.462
W5BQF4	Lactoylglutathione lyase	0.127	-0.321	0.059	-0.075	0.598	0.795	0.081	-0.243	0.539	-1.301	1.186	-1.801
W5BYT8	glycerol kinase;	0.162	0.385	0.088	-0.243	0.849	-0.523	0.921	-1.392	0.323	-0.675	0.173	-0.441
W5C3Z7	germin-like protein 8-14	0.276	0.55	1.496	1.135	0.055	-0.098	0.407	0.762	0.55	-1.166	0.526	-1.203
W5C5X0	Peroxidase 2-like	0.132	0.18	0.568	0.961	0.508	-1.069	0.104	0.303	0.051	0.081	0.026	0.056
W5CN13	Ribosomal protein L11	0.587	-1.179	0.025	-0.063	0.214	0.43	0.038	-0.09	0.379	0.837	0.263	-0.51
W5DL22	Plastocyanin	0.073	-0.289	0.223	0.608	0.503	1.021	0.617	-0.693	0.063	0.074	0.138	0.318
W5DQV9	Adenylyl cyclase-associated protein	0.233	-0.691	0.044	-0.14	0.084	0.232	1.406	1.495	0.654	0.6	0.284	0.614
W5DUG1	Beta-adaptin-like protein	0.821	1.053	0.43	0.653	0.342	0.835	0.149	0.394	0.302	-0.566	0.855	0.818
W5DXC6	Glutathione reductase	0.043	-0.106	0.646	-1.099	0.145	-0.2	0.375	0.897	0.202	0.36	0.551	1.313
W5E2J7	Eukaryotic translation initiation factor 3 subunit I	0.752	1.064	0.014	-0.042	0.164	0.315	0.391	0.914	0.205	0.401	0.986	-1.618
W5E9J6	Proteasome subunit beta	0.369	1.038	0.051	-0.161	0.092	-0.229	0.152	-0.328	0.676	-0.954	0.608	-1.093
W5EEK9	Adenylosuccinate synthetase, chloroplastic	0.062	0.211	0.25	0.346	0.043	-0.136	1.044	1.36	0.987	-1.659	0.553	-0.874
W5EGF1	26S proteasome non-ATPase regulatory subunit 4 homolog isoform X1	0.925	-1.02	0.06	-0.12	0.281	0.787	1.617	-1.854	0.504	-1.147	0.35	0.418
W5EHT8	photosystem II repair protein PSB27-H1, chloroplastic	0.071	0.205	0.294	0.65	0.502	1.086	1.461	-1.492	0.317	0.589	0.006	0.014
W5EI90	ricin B-like lectin R40C1;	0.426	0.743	0.106	0.268	0.456	-1.01	1.155	1.721	0.1	0.258	0.427	-0.609
W5EJC8	50S ribosomal protein L6, chloroplastic	0.319	0.597	2.645	-1.711	0.299	0.666	0.084	-0.233	0.189	0.366	1.567	-1.639

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
W5EJD1	Mitochondrial outer membrane protein porin of 34 kDa	0.595	-1.279	0.012	-0.042	0.086	0.253	1.863	-1.547	0.164	-0.223	0.384	0.58
W5EKJ6	Fumarate hydratase 2, chloroplastic	0.088	0.275	0.209	0.536	1.03	-1.439	1.566	1.47	0.12	0.273	0.377	-0.719
W5EKS7	50S ribosomal protein L11, chloroplastic	0.075	0.318	0.379	0.861	0.431	-0.744	0.031	0.055	0.47	0.818	0.419	-0.943
W5EM06	probable LL-diaminopimelate aminotransferase, chloroplastic;	0.271	-0.721	0.224	-0.499	0.143	0.455	0.275	0.488	0.684	-1.207	0.162	-0.256
W5EM36	Ribulose-phosphate 3-epimerase	0.129	-0.333	0.098	-0.269	0.134	0.275	0.725	-1.2	0.836	-1.133	0.3	0.742
W5EMA7	Proteasome subunit alpha type-6	0.313	0.953	0.446	0.723	0.371	-0.937	0.957	1.06	0.534	1.129	0.145	0.265
W5EMN3	glutathione S-transferase F11-like;hypothetical protein	0.096	-0.26	0.371	0.588	0.202	-0.404	0.921	1.285	0.172	-0.436	0.24	0.633
W5EPM7	4-hydroxy-3-methylbut-2-enyl diphosphate reductase, chloroplastic	0.688	-0.875	0.784	-1.399	0.627	1.081	1.181	-1.566	0.254	0.388	0.109	-0.278
W5EPQ7	photosynthetic NDH subunit of lumenal location 2, chloroplastic-like	0.191	0.534	0.575	1.333	0.325	0.71	0.339	-0.598	0.298	-0.335	0.002	-0.006
W5EQ81	CBS domain-containing protein CBSX3, mitochondrial	0.358	1.244	0.248	0.479	0.329	0.658	1.535	0.612	0.322	-0.761	0.141	0.299
W5EQK1	Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic; isoform X1	0.235	-0.671	0.291	0.712	0.124	0.323	0.987	-1.22	0.529	-0.862	0.29	-0.34
W5ER46	uncharacterized aarF domain-containing protein kinase At4g31390, chloroplastic;	0.072	0.241	0.238	0.296	0.604	-0.983	0.627	0.724	0.011	0.034	0.106	-0.27
W5ERK1	probable zinc metalloprotease EGY3, chloroplastic;	0.323	-0.901	0.066	-0.241	0.348	0.846	0.307	0.246	1.017	0.191	0.019	-0.045
W5ERQ0	alpha-L-arabinofuranosidase 1-like isoform X2	0.746	-1.83	0.045	-0.139	0.093	-0.274	0.156	-0.234	0.308	-0.562	1.141	-1.205
W5ERR3	Oxalate oxidase GF-2.8	0.037	0.098	0.762	1.404	0.153	0.491	0.573	0.556	0.395	0.629	0.441	-0.533
W5ES41	Alpha-1,4-glucan-protein synthase (UDP-forming);UDP-arabinopyranose mutase 1	0.407	-1.031	0.694	1.077	0.462	-0.684	1.832	-1.623	0.654	-1.192	0.691	-1.292
W5ESI8	Adenylyl cyclase-associated protein	0.056	-0.234	1.634	-0.689	0.647	-0.795	0.315	0.447	0.303	0.836	0.9	-1.461
W5FB69	Lysine--tRNA ligase	0.375	-0.853	0.146	0.178	0.241	-0.334	0.195	0.422	0.512	1.123	0.61	-1.357
W5FBQ5	Uridine kinase	0.111	-0.421	0.095	0.228	0.095	0.204	0.59	0.731	0.15	0.084	0.222	-0.607
W5FDZ3	Mitochondrial Rho GTPase	0.624	1.666	0.293	-0.374	0.063	0.214	0.244	0.308	0.744	1.518	0.008	0.015
W5FEQ2	prohibitin-1, mitochondrial-like	0.717	-1.731	0.072	0.169	0.543	1.056	0.363	-0.515	0.546	-0.866	0.679	-0.908
W5FGH0	UTP--glucose-1-phosphate uridylyltransferase	0.291	0.634	0.276	-0.816	0.582	-0.528	0.286	-0.738	0.429	-0.417	0.121	-0.367
W5FI94	Ferritin	0.116	-0.413	0.976	-1.573	0.181	0.444	0	-0.001	0.013	0.031	0.17	0.413
W5FM23	Acyl carrier protein	0.392	0.213	0.566	-0.888	0.154	-0.267	0.005	0.02	0.568	-1.109	0.31	-0.839
W5FQ55	UDP-glucose 6-dehydrogenase 4-like	1.142	-1.341	0.368	-0.796	0.154	-0.291	0.125	-0.341	0.002	-0.006	0.405	-0.386

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
W5FQG6	40S ribosomal protein S26;40S ribosomal protein S26-like	1.757	1.565	0.039	0.137	0.117	-0.333	0.746	-1.135	0.042	-0.075	0.109	0.246
W5FR28	Pantothenate kinase 2	0.534	-1.186	0.61	-1.194	0.25	-0.617	0.662	0.841	0.024	-0.04	1.557	-1.519
W5FSK5	Polyadenylate-binding protein 8-like	1.646	-2.303	0.219	-0.303	0.117	0.347	0.74	0.827	0.1	-0.329	0.003	0.006
W5FTZ9	Chlorophyll a-b binding protein, chloroplastic	0.586	-1.339	0.166	0.373	0.332	0.688	0.295	-0.591	0.991	1.167	0.389	0.824
W5FVP1	PGR5-like protein 1A, chloroplastic	0.158	0.291	0.013	0.032	0.628	0.98	2.216	-2.159	0.08	-0.152	0.482	-0.938
W5FVU1	Eukaryotic translation initiation factor 3 subunit C-like	0.548	1.049	0.81	-1.047	1.012	1.56	0.322	0.43	0.188	-0.493	0.597	0.92
W5FXL5	Dirigent protein 22-like	0.102	0.265	0.266	0.282	0.349	0.679	0.243	-0.36	0.244	-0.747	0.35	-0.76
W5FY62	Glucose-6-phosphate isomerase	0.789	-1.175	0.078	-0.212	0.018	-0.062	0.741	-1.343	1.251	-1.337	0.865	0.761
W5FYM6	ACT domain-containing protein ACR12;	0.311	0.779	0.421	-0.807	0.015	-0.037	0.998	-1.104	0.133	0.332	0.098	-0.294
W5G103	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	0.28	0.727	0.687	0.951	0.334	-0.684	0.647	-0.834	0.191	0.481	0.383	-0.618
W5G1K5	Elongation factor Ts, mitochondrial	0.521	1.182	1.242	-1.561	0.377	-0.638	0.482	-0.67	0.552	-0.893	0.921	-1.461
W5G1U8	haloacid dehalogenase-like hydrolase domain-containing protein At3g48420 isoform X1;	0.075	0.18	0.448	0.888	0.244	-0.494	0.788	1.361	0.024	0.064	0.039	-0.11
W5G2J5	Thioredoxin M-type, chloroplastic	0.148	0.368	0.023	0.063	0.697	-1.089	1.742	-1.552	0.67	-1.082	0.562	-1.054
W5G3N9	polyol transporter 5-like;	0.239	0.606	0.288	-0.809	0.057	-0.098	0.208	0.468	0.611	-0.658	0.258	0.624
W5G4L3	Pyruvate dehydrogenase E1 component subunit beta, chloroplastic-like	0.122	-0.321	0.91	-0.588	0.5	0.885	0.011	-0.034	0.28	-0.729	0.28	-0.664
W5G5A6	Aspartate aminotransferase	0.189	0.549	0.086	-0.212	0.003	0.007	1.012	-0.774	0.413	-0.727	0.475	-1.055
W5G5H3	Glyceraldehyde-3-phosphate dehydrogenase	0.169	-0.526	0.471	1.222	1.134	1.075	1.633	-1.032	0.064	-0.07	0.037	0.122
W5GBB3	Eukaryotic translation initiation factor 3 subunit G	0.433	-1.025	0.172	0.511	0.975	-1.555	0.403	-0.442	0.28	-0.255	0.366	-0.193
W5GCF7	26S proteasome non-ATPase regulatory subunit 2 homolog A-like	0.141	0.447	0.249	-0.552	0.182	0.377	1.248	0.861	0.191	0.474	0.006	0.021
W5GDZ8	4-hydroxyphenylpyruvate dioxygenase	0.454	-1.142	0.426	-0.974	0.119	0.329	1.02	0.78	0.147	-0.27	0.18	-0.278
W5GH00	50S ribosomal protein L21, chloroplastic	0.078	-0.191	0.054	-0.115	0.133	0.328	0.43	0.934	0.943	-1.567	0.439	0.885
W5GHT5	Isocitrate dehydrogenase [NAD] subunit, mitochondrial;, mitochondrial;Isocitrate dehydrogenase (NAD) catalytic subunit 5, mitochondrial;	0.605	-0.977	0.078	-0.105	0.088	-0.246	0.39	-0.917	0.482	-0.92	0.762	-1.038
W5GJ80	Phosphoribulokinase	0.061	0.179	0.082	0.261	0.603	0.717	1.487	-1.39	2.158	-1.362	0.265	-0.715
W5GUI9	Phenylalanine ammonia-lyase;predicted protein	0.055	0.238	1.312	-0.747	0.179	0.377	0.056	0.086	0.628	-1.439	0.312	0.701

	cultivars	Cv2						Cv4					
	stages	Grain filling		flowering		vegetative		Grain filling		flowering		vegetative	
List_Ids	Protein names	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference	-Log (P-value)	Difference
W5GVP7	60S ribosomal protein L12;60S ribosomal protein L1260S ribosomal protein L12	0.498	1.174	0.397	0.977	0.964	1.689	0.09	-0.157	0.462	-0.616	0.469	-0.594
W5GYF2	ADP,ATP carrier protein, mitochondrial;	0.161	-0.612	0.177	-0.456	0.659	-1.113	0.555	0.278	0.37	-0.585	0.105	-0.273
W5GYS4	protein HHL1, chloroplastic;	0.474	-0.653	0.105	-0.133	0.075	-0.211	0.303	-0.817	1.198	1.912	0.043	0.104
W5H0F7	HMG1/2-like protein;	0.479	1.191	0.828	-0.881	0.606	0.735	1.074	1.107	0.228	0.601	0.448	-1.084
W5H0U1	TOM1-like protein 2	0.083	0.233	0.478	-0.858	0.211	0.295	0.986	-1.305	0.051	-0.15	0.576	1.333
W5H174	Glutathione reductase	0.649	-0.861	0.707	1.28	0.019	0.054	0.017	-0.019	0.111	0.101	0.104	-0.248
W5H230	Calnexin-like protein	0.143	0.337	0.658	1.033	0.54	1.072	1.227	1.432	0.554	1.091	1.521	1.265
W5H3N4	Proteasome subunit alpha type -1	0.792	-1.526	1.051	1.309	0.063	-0.169	0.305	0.475	0.058	-0.154	0.151	-0.384
W5H4V8	Aminopeptidase M1-A	2.706	-1.791	0.018	0.061	0.049	-0.084	0.422	-0.85	0.992	-1.089	0.885	1.512
W5HXX0	Elongation factor 1-gamma 2	0.167	0.622	0.099	-0.238	0.503	-0.852	0.067	0.09	0.285	0.686	0.416	-0.745
W5HYN5	malate dehydrogenase, chloroplastic-like	1.207	1.358	0.17	-0.21	0.217	-0.651	0.846	1.041	0.133	0.401	0.082	-0.14
W5HZR7	ATP-citrate synthase beta chain protein 1-like	1.041	-2.092	0.305	0.607	0.403	0.881	0.096	0.057	1.199	-1.638	0.016	0.041
W5I2Y4	polyamine oxidase-like	0.469	0.909	0.575	-1.231	0.132	0.27	0.149	-0.301	0.545	-0.773	0.259	-0.619
W5I329	40S ribosomal protein S18-like	0.183	0.465	0.437	-0.846	0.08	-0.247	1.666	0.939	0.491	-0.827	0.391	0.654
W5I3E6	Aspartate aminotransferase	0.574	-1.275	0.181	-0.6	0.117	0.192	0.205	0.311	0.171	-0.151	0.011	-0.033
W5I3H6	haloacid dehalogenase-like hydrolase domain-containing protein At3g48420 isoform X2;	0.873	1.484	0.43	1.097	0.417	0.666	0.09	0.193	0.531	-0.6	1.129	-1.597
W5I3K2	V-type proton ATPase catalytic subunit A	0.127	-0.308	1.124	-1.132	0.222	-0.489	0.496	0.984	0.077	0.19	0.371	-0.766
W5I3W3	uncharacterized protein At2g34460, chloroplastic	0.32	-0.889	0.372	-0.784	0.083	0.063	0.057	0.118	0.228	-0.494	0.092	0.309
W5I4P6	60S ribosomal protein L13; like60S ribosomal protein L13-1;	0.008	-0.025	1.746	1.223	0.027	0.083	0.236	0.475	0.433	1.049	1.031	1.265
W5I4Y2	Glucose-1-phosphate adenylyltransferase, chloroplastic/amyloplastic isoform X1	0.572	-1.558	0.24	0.725	0.54	0.99	1	-0.827	0.102	-0.084	0.254	0.546
W5I5Y4	Dirigent protein; LOC109765094;hypothetical protein	0.217	0.804	0.35	-0.981	0.475	-0.43	1.029	0.839	0.102	-0.138	0.025	0.07
W5I774	sucrose synthase 1	0.373	0.983	0.287	-0.85	0.078	0.174	0.413	-0.528	0.095	-0.124	1.068	-1.584
W5I877	ruBisCO large subunit-binding protein subunit beta, chloroplastic	0.298	0.829	0.04	0.069	0.019	0.062	0.76	0.739	0.069	-0.19	0.128	-0.265
W5I9L6	Mitochondrial pyruvate carrier 4-like	0.22	0.465	0.492	-1.042	1.103	1.82	0.631	1.175	0.263	0.516	0.62	-0.57

Table S3. 2 Numbers of common DEPs regulated in wheat cultivars (*Triticum aestivum* L.; 193 Najran (Cv2); 377 Rafha (Cv4)) at three growth stages: vegetative, flowering and grain filling, subjected to 15% PEG6000. (n:6; P >0.05)

stages	Regulation	Cv2 cultivar	Cv4 cultivar
Grain filling	up	162	153
	down	147	156
Flowering	up	160	129
	down	149	180
Vegetative	up	184	124
	down	125	185
t-test (stages)		5.34	
p value (0.05)		0.003	

One-Sample test									
								95% Confidence Interval of the Difference	
	N	Mean	Std. Deviation	Std. Error Mean	t	Sig. (2-tailed)	Mean Difference	Lower	Upper
Cv2	6	154.5	19.56272	7.98645	19.339	0	154.45	133.9202	174.9798
Cv4	6	154.5	25.16148	10.27213	15.036	0	154.45	128.0446	180.8554
stages	6	2	0.89443	0.36515	5.34	0.003	1.95	1.0114	2.8886
regulation	6	1.5	0.54772	0.22361	6.485	0.001	1.45	0.8752	2.0248

Appendix D Supporting information

Table S4. 1 Primers used for quantitative real-time PCR analysis.

Gene name	Gene Bank/ NCBI Reference Sequence:	Primer sequence (5′–3′)	Amplicon length (bp)	Amplicon Tm (C◦)	Gene and protein name	biological process
TraesCS2A01G196400	MH376698.1	primer_F CCAACATCCTTGCTGTCCTT primer_R TCGCAAGCATCATGTAGGAG	177	60	Protein submitted name: Phenylalanine ammonia-lyase Gene PAL5	phenylpropanoid metabolic process, cinnamic acid biosynthetic process.
TraesCS6A01G350500	KT633580.1	Primer_F ACCACCGTTGCAGAATCAAT Primer_R ATACAGTGGCTCCTCCAACA	106	60	Protein Submitted name: Salt-induced YSK2 dehydrin 3 Gene DHN3	Response to water-stess.
TraesCS4D01G068100	BT009272.2	primer_F GTTCACCTGCCAACTTCCAT primer_R CAATCAAGCCAGTGCTTTCA	186	60	Protein Serine hydroxymethyltransferase Gene CAMPLR22A2D_LOCUS4912	Circadian rhythm., glycine biosynthetic process Photorespiration. cold.response
TraesCS7B01G050500	XM_020308354.1	Primer_F AGCATGGCTTTCCTGAACAT Primer_R TTGCGGTACACACGGTAGAA	122	59.7	Protein Bidirectional sugar transporter SWEET Gene TRAES_3BF077700030CFD_c1	carbohydrate transport.
wheat Ubiquitin (reference gene)	AY862401.1	Primer_F TACCCTGTGTCGCCTTTGTT Primer_R ACTGTTTGCACCAAACCACA	103	60.5	Ubiquitin UBQ	Regulation of protein turnover

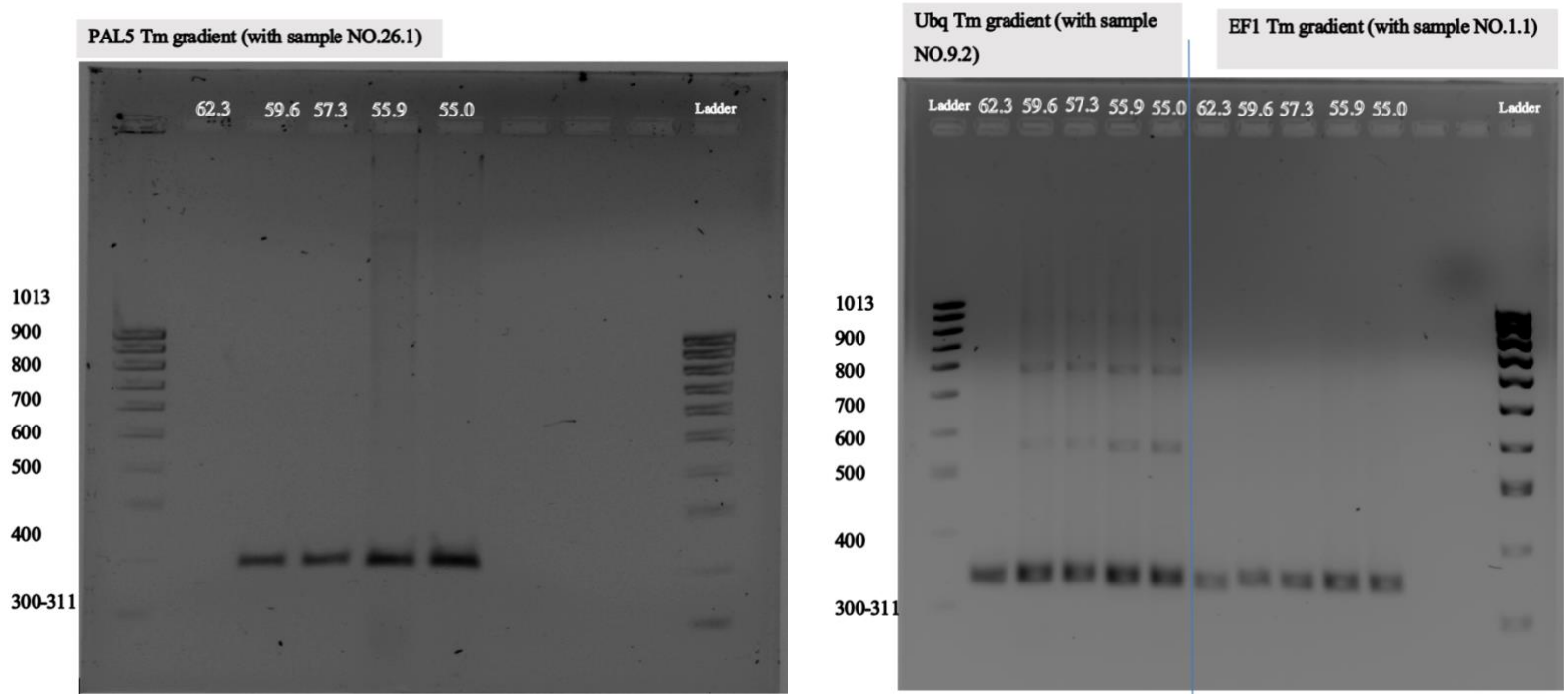
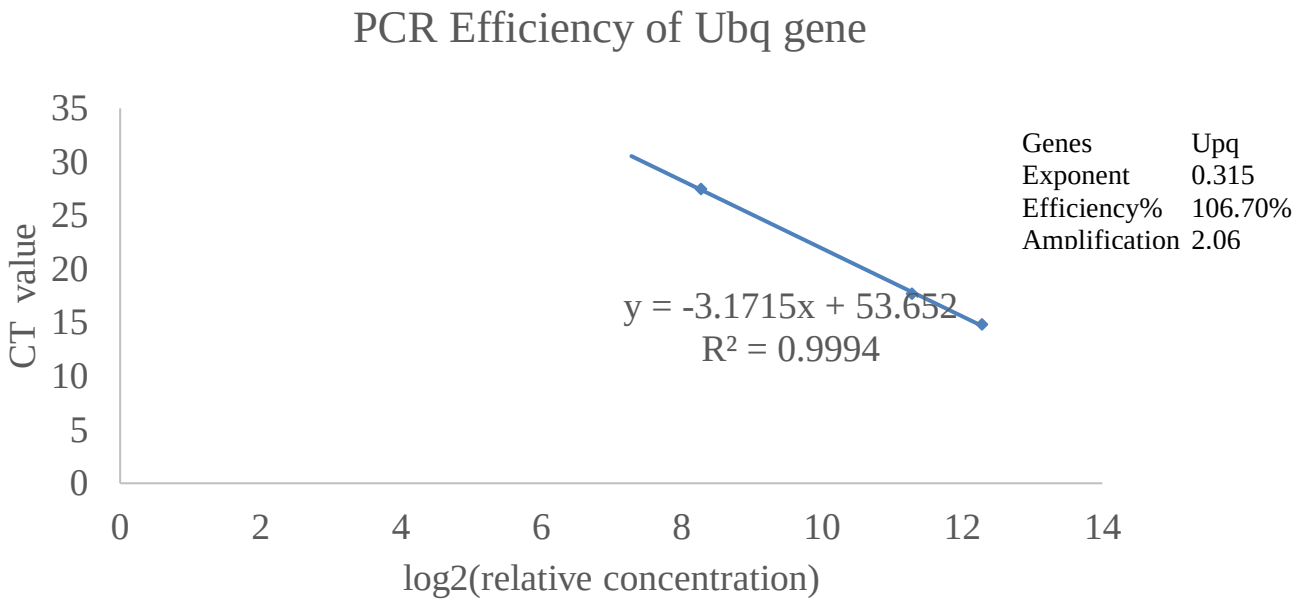


Figure S4. 1 Primer optimization using temperature gradient experiment from 55 to 62.3 C. a. gradient experiment for PAL5 gene. b. gradient experiment for housekeeping genes (Ubq and EF1).

a.



b.

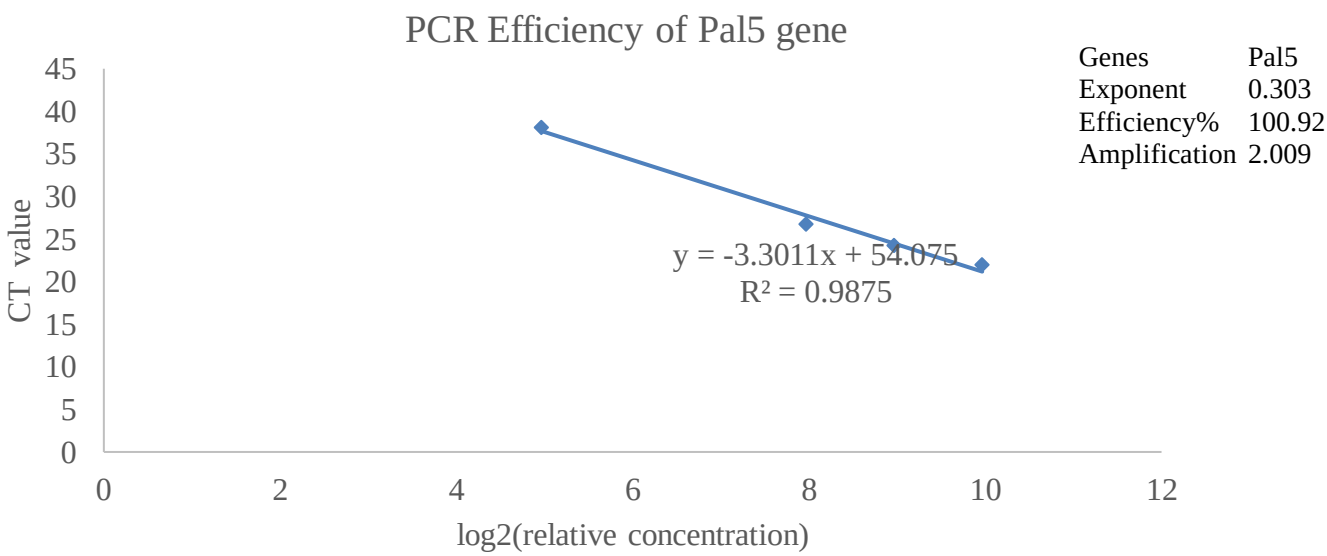
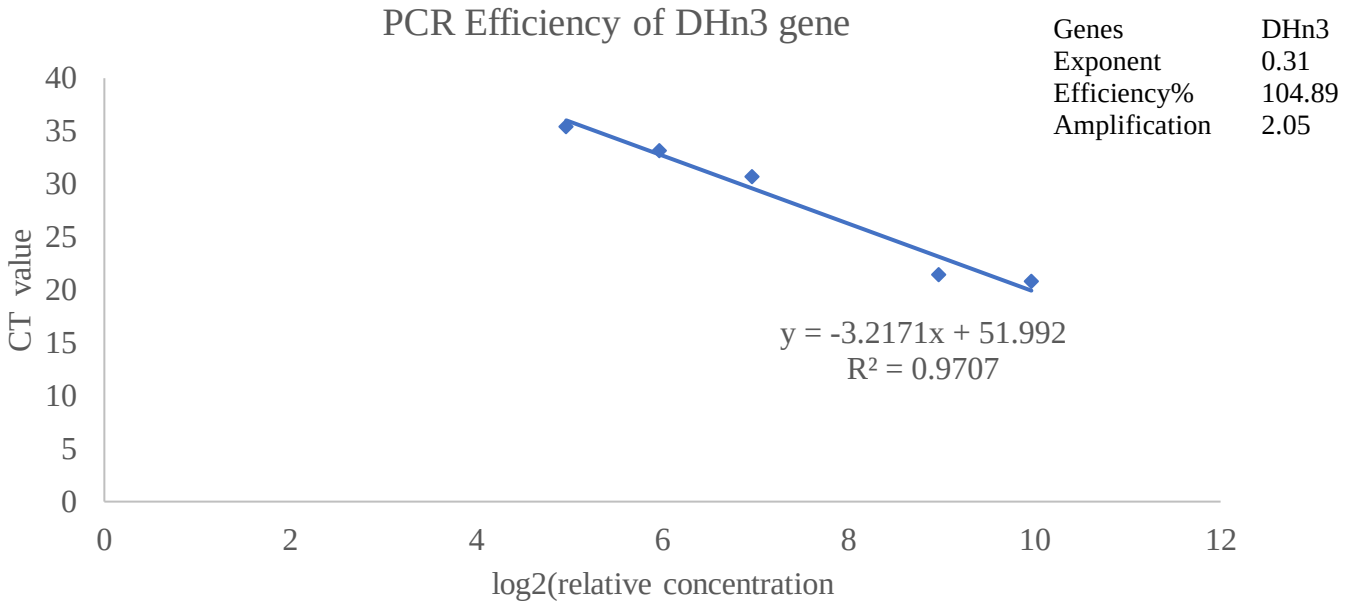


Figure S4. 2 PCR Efficiency of one gene under study (PaL) and a housekeeping gene (Ubq). a. Ubq. b. Pal5. PCR efficiency for each gene was assessed using log2 of relative concentration dilution series of cDNA mixture of all samples. Linear regression was performed in excel file to obtain the slope and R 2. The exponent was calculated from the slope using the formula E = 2^ (1/-slope)-1 and the efficiency was calculated from the slope using =((10^(-1/-slope))-1)*100. https://www.chem.agilent.com/store/biocalculators/calcSlopeEfficiency.jsp?_requestid=212228

a.



b.

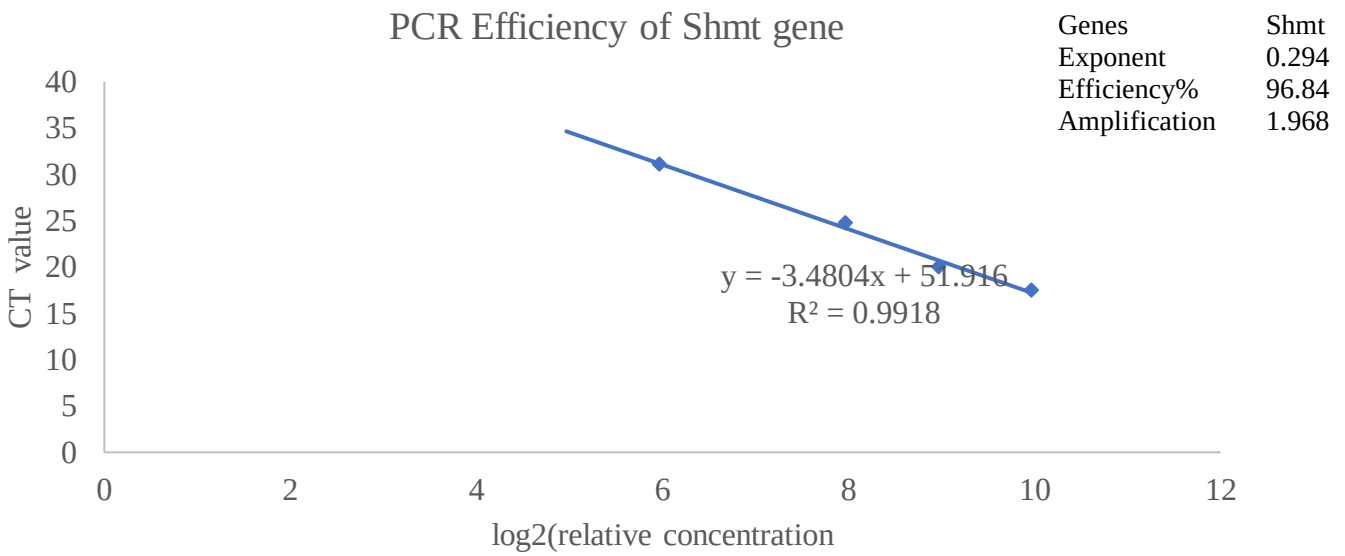


Figure S4. 3 PCR Efficiency of two genes under study. a. DHn3. b.Shmt. PCR efficiency for each gene was assessed using log2 of relative concentration dilution series of cDNA mixture of all samples. Linear regression was performed in excel file to obtain the slope and R 2. The exponent was calculated from the slope using the formula E = 2^ (1/-slope)-1 and the efficiency was calculated from the slope using =((10^(-1/-slope))-1)*100. https://www.chem.agilent.com/store/biocalculators/calcSlopeEfficiency.jsp?_requestid=212228

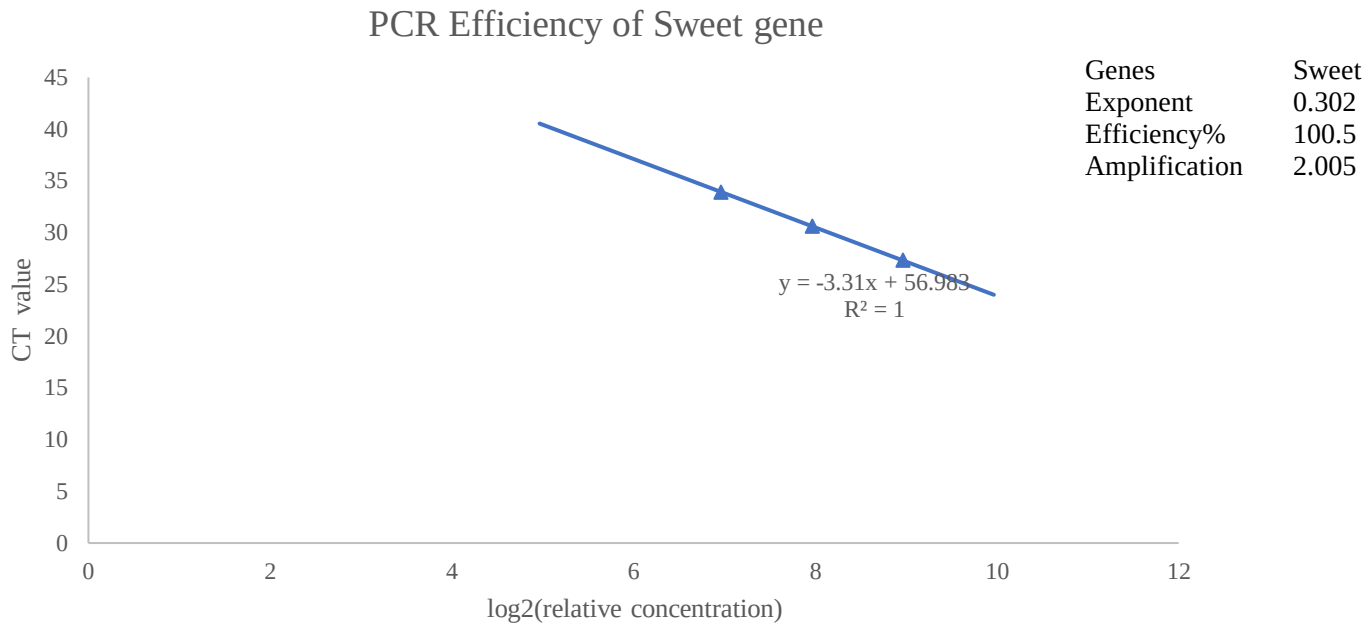


Figure S4. 4 PCR Efficiency of two genes under study (Sweet). PCR efficiency for each gene was assessed using log2 of relative concentration dilution series of cDNA mixture of all samples. Linear regression was performed in excel file to obtain the slope and R 2. The exponent was calculated from the slope using the formula E = 2^ (1/-slope)-1 and the efficiency was calculated from the slope using =((10^(-1/-slope))-1)*100. https://www.chem.agilent.com/store/biocalculators/calcSlopeEfficiency.jsp?_requestid=212228

Table S.4 1 The analysis of the main effects and interaction effects of the four genes within the three methods (RTqPCR, RNAseq and Proteomic) (one way-ANOVA) and the analysis of the main effects and interaction effects of the four genes. Statistical significance was set up to p <0.05 for both analyses.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DHn3	RTqPCR	6	2.9667	4.71952	1.92673	-1.9862	7.9195	-2.21	8.78
	RNAseq	6	3.6767	5.74762	2.34646	-2.3551	9.7084	-4.06	9.90
	Proteomic	6	-.1082	.92407	.37725	-1.0779	.8616	-1.81	.94
	Total	18	2.1784	4.40175	1.03750	-.0105	4.3673	-4.06	9.90
Sweet	RTqPCR	6	17.9333	10.10146	4.12390	7.3325	28.5342	3.47	28.08
	RNAseq	6	2.9533	4.13636	1.68866	-1.3875	7.2942	-1.21	9.67
	Proteomic	6	-1.5483	3.79263	1.54833	-5.5285	2.4318	-9.29	.00
	Total	18	6.4461	10.61649	2.50233	1.1667	11.7256	-9.29	28.08
PAL5	RTqPCR	6	1.6550	3.69200	1.50725	-2.2195	5.5295	-.65	9.10
	RNAseq	6	-.6233	3.72149	1.51929	-4.5288	3.2821	-5.30	4.08
	Proteomic	6	-1.0900	2.37876	.97113	-3.5864	1.4064	-5.67	.70
	Total	18	-.0194	3.35701	.79125	-1.6888	1.6500	-5.67	9.10
Shmt	RTqPCR	6	1.7950	2.56004	1.04513	-.8916	4.4816	-.95	6.24
	RNAseq	6	4.9308	4.71053	1.92307	-.0126	9.8742	.07	11.18
	Proteomic	6	.1808	.84899	.34660	-.7101	1.0718	-1.00	1.23
	Total	18	2.3022	3.57542	.84273	.5242	4.0802	-1.00	11.18

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
DHn3	11.870	2	15	.001
Sweet	4.277	2	15	.034
PAL5	.617	2	15	.553
Shmt	12.009	2	15	.001

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DHn3	Between Groups	48.567	2	24.284	1.297	.302
	Within Groups	280.814	15	18.721		
	Total	329.382	17			
Sweet	Between Groups	1248.401	2	624.201	14.024	.000
	Within Groups	667.665	15	44.511		
	Total	1916.067	17			
PAL5	Between Groups	25.887	2	12.944	1.172	.337
	Within Groups	165.694	15	11.046		
	Total	191.582	17			
Shmt	Between Groups	70.003	2	35.001	3.564	.054
	Within Groups	147.318	15	9.821		
	Total	217.321	17			

Table S4. 2 Gene expression ratio and Log2 Foldchange of Dehydrins gene (DHn3) by qRT-PCR in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. Mean of three replicates ± Std. Deviation. n=3.

Cultivars	Stages	Sample	Mean	Std. Deviation	Log2Foldchange
Cv1	Vegetative	Control	2.65	2.25	1.41
		Treatment	100.99	164.44	6.66
	Flowering	Control	8.06	9.54	3.01
		Treatment	0.3833	0.62	-1.38
	Grain Filling	Control	15.2267	24.74	3.93
		Treatment	25.9533	39.15	4.70
Cv2	Vegetative	Control	35.49	58.14	5.15
		Treatment	439.11	656.88	8.78
	Flowering	Control	209.53	362.20	7.71
		Treatment	383.45	292.90	8.58
	Grain Filling	Control	0.01	0.01	-7.22
		Treatment	0.22	0.36	-2.21
Cv3	Vegetative	Control	2.42	3.29	1.27
		Treatment	27.40	42.70	4.78
	Flowering	Control	7.90	11.80	2.98
		Treatment	22.71	33.38	4.51
	Grain Filling	Control	0.00	0.01	-8.24
		Treatment	0.24	0.22	-2.06
Cv4	Vegetative	Control	41.2267	71.02	5.37
		Treatment	2.68	4.31	1.42
	Flowering	Control	103.35	177.54	6.69
		Treatment	0.47	0.805	-1.09
	Grain Filling	Control	15.3367	24.91	3.94
		Treatment	4.9767	5.43	2.32
Cv5	Vegetative	Control	17.7467	28.20	4.15
		Treatment	6.2267	10.77	2.64
	Flowering	Control	18.3867	31.8	4.20
		Treatment	0.0867	0.15	-3.53
	Grain Filling	Control	0.2633	0.4	-1.93
		Treatment	0.5733	0.9	-0.80
Cv6	Vegetative	Control	0.04	0.06	-4.64
		Treatment	0.06	0.1	-4.06
	Flowering	Control	0.16	0.16	-2.64
		Treatment	4026.44	1991.3	11.98
	Grain Filling	Control	0	0	0.00
		Treatment	4.72	7.67	2.24
The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 10.626, P value > 0.000					

Table S4. 3 expression ratio and Log2 Fold change of Bidirectional sugar transporter gene (Sweet) by qRT-PCR in wheat cultivars (Triticum aestivum L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. Mean of three replicates ± Std. Deviation. n=3

Cultivars	Stages	Sample	Mean	Std. Deviation	Log2Foldchange
Cv1	Vegetative	Control	2.17E+09	3.75E+09	31.01
		Treatment	4.83E+09	8.37E+09	32.17
	Flowering	Control	3.62E+09	6.28E+09	31.75
		Treatment	1.82E+10	3.14E+10	34.08
	Grain Filling	Control	1.00E+10	1.74E+10	33.22
		Treatment	2.16E+08	3.75E+08	27.69
Cv2	Vegetative	Control	4.51E+06	7.81E+06	22.11
		Treatment	8.94E+05	1.55E+06	19.77
	Flowering	Control	4.77E+08	4.13E+08	28.83
		Treatment	2.74E+08	4.74E+08	28.03
	Grain Filling	Control	1.44E+07	1.25E+07	23.78
		Treatment	2.84E+08	4.91E+08	28.08
Cv3	Vegetative	Control	1.48E+01	2.53E+01	3.89
		Treatment	9.70E-01	1.44E+00	-0.04
	Flowering	Control	1.03E+02	1.78E+02	6.68
		Treatment	2.55E+02	4.38E+02	7.99
	Grain Filling	Control	1.13E+02	1.95E+02	6.82
		Treatment	2.17E+09	3.76E+09	31.01
Cv4	Vegetative	Control	8.91E+01	1.53E+02	6.48
		Treatment	3.57E+02	6.15E+02	8.48
	Flowering	Control	1.82E+01	2.34E+01	4.18
		Treatment	1.11E+01	9.94E+00	3.47
	Grain Filling	Control	4.46E+05	7.73E+05	18.77
		Treatment	8.93E+05	7.73E+05	19.77
Cv5	Vegetative	Control	4.10E-01	6.49E-01	-1.29
		Treatment	2.23E+01	2.95E+01	4.48
	Flowering	Control	1.00E+02	1.71E+02	6.65
		Treatment	2.36E+01	4.08E+01	4.56
	Grain Filling	Control	2.81E+01	2.56E+01	4.81
		Treatment	1.64E+02	2.80E+02	7.35
Cv6	Vegetative	Control	2.02E+01	3.36E+01	4.34
		Treatment	6.09E+01	1.05E+02	5.93
	Flowering	Control	3.92E-01	5.85E-01	-1.35
		Treatment	3.23E+02	5.55E+02	8.33
	Grain Filling	Control	1.71E+08	2.96E+08	27.35
		Treatment	1.25E+08	2.16E+08	26.90
The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 0.983, P value > 0.466					

Table S4. 4 Gene expression ratio and Log2 Fold change of Phenylalanine ammonia-lyase gene (Pal5) measured by qRT-PCR in wheat cultivars (*Triticum aestivum* L).; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama (Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. Mean of three replicates ± Std. Deviation. n=3.

Cultivars	Stages	Sample	Mean	Std. Deviation	Log2Foldchange
Cv1	Vegetative	Control	1.29	1.156	0.369
		Treatment	0.61	0.249	-0.722
	Flowering	Control	1.01	0.173	0.011
		Treatment	1.79	0.000	0.841
	Grain Filling	Control	1.55	1.677	0.633
		Treatment	0.34	0.207	-1.577
Cv2	Vegetative	Control	1.54	1.649	0.619
		Treatment	1.37	1.034	0.455
	Flowering	Control	1.36	1.294	0.439
		Treatment	0.77	0.513	-0.378
	Grain Filling	Control	1.06	0.517	0.090
		Treatment	0.64	0.544	-0.651
Cv3	Vegetative	Control	1.05	0.448	0.069
		Treatment	0.04	0.021	-4.538
	Flowering	Control	1.00	0.098	0.003
		Treatment	0.68	0.145	-0.565
	Grain Filling	Control	1.09	0.603	0.120
		Treatment	0.42	0.194	-1.265
Cv4	Vegetative	Control	1.00	0.129	0.006
		Treatment	1.52	1.010	0.605
	Flowering	Control	0.72	0.470	-0.471
		Treatment	1.74	0.328	0.799
	Grain Filling	Control	1.22	0.282	0.286
		Treatment	27.11	1.820	4.761
Cv5	Vegetative	Control	0.83	0.260	-0.264
		Treatment	0.37	0.008	-1.442
	Flowering	Control	0.88	0.177	-0.180
		Treatment	122.74	45.314	6.939
	Grain Filling	Control	0.96	0.061	-0.062
		Treatment	0.98	0.033	-0.026
Cv6	Vegetative	Control	0.81	0.299	-0.302
		Treatment	13.72	0.516	3.778
	Flowering	Control	0.99	0.020	-0.021
		Treatment	0.02	0.000	-5.827
	Grain Filling	Control	0.83	0.261	-0.265
		Treatment	0.08	0.003	-3.568
The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 1.107, P value > 0.000					

Table S4. 5 Gene expression ratio and Log2 Fold change of Serine hydroxy methyl transferase (Shmt) genes measured by qRT-PCR in wheat cultivars (*Triticum aestivum* L.; 181 Jizan (Cv1) ; 193Najran (Cv2); 357 Sama(Cv3); 377 Rafha (Cv4); 562 Ma'ayah (Cv5) and 981 Najd (Cv6)) grown in different regions of Saudi Arabia at three growth stages, vegetative, flowering and filling stages subjected to 15% PEG6000. Mean of three replicates ± Std. Deviation. **n=3**

Cultivars	**Stages**	**Sample**	**Mean**	**Std. Deviation**	**Log2Foldchange**
Cv1	Vegetative	Control	2.2714	2.041	1.18
		Treatment	1.1629	1.3045	0.21
	Flowering	Control	3.6323	4.087	1.86
		Treatment	9.244	11.31	3.21
	Grain Filling	Control	6.8094	7.5	2.76
		Treatment	37.7417	47.9	5.24
Cv2	Vegetative	Control	2.9851	4.06	1.58
		Treatment	0.6367	0.81	0.65
	Flowering	Control	2.6539	3.65	1.41
		Treatment	6.5244	9.19	2.71
	Grain Filling	Control	3.4591	4.82	1.79
		Treatment	75.7022	106.95	6.24
Cv3	Vegetative	Control	1.4194	1.45	0.51
		Treatment	0.042	0.026	-4.57
	Flowering	Control	0.9798	0.74	-0.029
		Treatment	1.6987	1.08	0.76
	Grain Filling	Control	0.9471	0.65	-0.078
		Treatment	0.1284	0.09	-2.96
Cv4	Vegetative	Control	0.9948	0.7	-0.008
		Treatment	0.5173	0.72	-0.951
	Flowering	Control	1.1601	0.83	0.21
		Treatment	4.3364	3.42	2.12
	Grain Filling	Control	1.3033	1.18	0.38
		Treatment	0.0016	0.00219	-9.29
Cv5	Vegetative	Control	1.1848	0.89	0.245
		Treatment	1.2848	1.28	0.362
	Flowering	Control	1.1934	0.92	0.26
		Treatment	216.5889	161.31	7.76
	Grain Filling	Control	1.1373	0.76	0.186
		Treatment	0.0038	0.00254	-8.04
Cv6	Vegetative	Control	1.3533	1.29	0.44
		Treatment	32.1415	11.37	5.006
	Flowering	Control	1.1373	0.77	0.186
		Treatment	1.2573	0.89	0.33
	Grain Filling	Control	1.2231	0.99	0.29
		Treatment	0.111178	0.028	-3.169
The mean difference between (cultivars*stages*sample) is significant by F-test at the .05 level, F= 1 , P value > 0.000					

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