COMPARATIVE METABOLITE PROFILING OF DROUGHT STRESS RESPONSIVE BIOCHEMICAL PATHWAYS IN ROOT AND LEAVES OF TRITICEAE SPECIES

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by
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ABSTRACT

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Keywords: Metabolomics, Organic acids, Biochemical pathway, Plant Genomics

An untargeted metabolite profiling was applied to modern wheat and wild relatives exposed to drought stress using Gas Chromatography-Mass Spectrometry technique. A total of 84 analytes were resolved in the wheat metabolome for which multivariate analyses including supervised (Principal Component Analyses) and unsupervised (Partial Least-Squares-Discriminant Analysis) provided significantly variable dataset under control and drought stress conditions. Around 45 significantly altered metabolites, with possible roles in drought stress, were identified in all species tested through the GC-MS study. The potential drought stress responsive metabolites were further investigated to track genes encoding the enzymes of selected biochemical pathways using FL-cDNA sequences and transcriptome data. It has been hypothesized that if the genes encoding the enzymes that control the biosynthesis of drought stress-specific metabolites have a significant role in tolerance, contrasting genotypes would have a variance in the metabolite content. A small proportion showed a reduction in the metabolite accumulation in the drought sensitive genotypes, indicating that selected genes are directly or indirectly engaged in metabolome-regulative biochemical pathways under water-limiting conditions. These results demonstrated that those specific genotypes with high drought tolerance skills, especially wild emmer wheat, could be genetic model systems for experiments to validate metabolomics–genomics networks.

ÖZET

KOMPARATİF METABOLİT TARANMASI YÖNTEMİYLE TRİTİCEAE TÜRLERİNİN KÖK İLE YAPRAKLARINDA KURAKLIK STRESİNDE BİYOKİMYASAL YOLAKLARIN KARAKTERİZASYONU

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Gaz Kromatografi-Kütle Spektrometri (GC-MS) yöntemiyle hedeflenmeyen metabolit taranması çağdaş buğday çeşitleriyle yabani akraba türlerine uygulanmıştır. Toplam olarak 84 buğday örneklerin metabolomune karakterize edilmiştir. Çok değişkenli analize olan Temel Bileşen Analizi (PCA) ve Kısmi En Az Kare Ayırtaç Analizi (PLS-DA) kullanarak verilerinde kontrol ile kuraklık stres koşulların arasında istatistik olarak anlamlı değişiklikleri tespit edilmiştir. Tüm türlerine bakarken, GC-MS çalışmasında 45 istatistik olarak anlamlı fark gösteren metabolit belirlenmiştir; kuraklık stresinde rol oynadığını düşünülmektedir. Kuraklık stresine tepki gösteren metabolitlerini üreten biyokimyasal yolaklarında yer bulunan enzim kodlayan genleri, FL-cDNA ve transkriptom verilerinden araştırılmıştır. Varsayım bulunmakta ki, eğer kuraklık stresine özel metabolitlerin biyosentezi yapan enzimlerin genleri dayanıklılığında rol oynarsa, farklı genotiplerde metabolit içeriklerinde değişiklik bulunurdu. Kuraklığa hassas genotiplerde, bazı metabolitin birikmesini azaldığını görülmüştür. Belirtilen genler, kısıtlı su koşullarında doğrudan veya dolaylı olarak metabolomu ayarlayan biyokimyasal yolaklarında yer aldığını gösterilmektedir. Sonuç olarak, kuraklık stresine iyi tolerans gösteren genotipler, özellikle yabani gernik buğdayı, ileride metabolomik-genomik ağlarını araştırmak için faydalı genetik model sistemleri olacağını önerilmektedir.

With all my heart,

To my dear parents, brothers, sisters, wife and son (Rayyan Khan)

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ABBREVIATIONS

AT	Aspartate transaminase
CL	Control leaves
CR	Control roots
DTW	days to drought stress
GC-MS	Gas Chromatography-mass spectrometry
PCA	principal component analysis
PCs	principal components
PLS-DA	partial least-squares-discriminant analysis
QTL	Quantitative Trait Locus
RL	root length
SA	surface area
T6PP	trehalose-6-phosphate phosphatase
T6PS	trehalose-6-phosphate synthase
TICs	total ion chromatograms
UDP-glucose	Uridine diphosphate glucose
VIP	variable importance in the projection
WGSS	Wheat Genome Survey Sequences
DSL	drought stress treated leaves
DSR	drought stress treated roots
ICARDA	International Centre for Agricultural Research in the Dry Areas
HSPs	heat shock proteins
WHC	Water holding capacity

LEAs	late embryogenesis abundant proteins
RSS	Root system size
WUE	water use efficiency
DREBs	Dehydration-Responsive Element-Binding proteins
ROS	reactive oxygen species
DSI	drought susceptibility index
MAS	marker-assisted selection
HCA	Hierarchical cluster analysis
KEGG	Kyoto Encyclopaedia of Genes and Genomes

CHAPTER NO.1

1. Introduction

Human beings get 94% of the food from plants worldwide; two-third of which is contributed by cereals. Among all cereals, wheat (*Triticum* ssp.) is one of the major and staple crops, providing 20% of all calories consumed by 75% population of the world. Additionally, it also makessignificant contribution to animal feed worldwide. It is predicted that the consumption of wheat will overcome its production in future due to fast growing global population. With the world's population estimated to reach 9.6 billion by 2050, wheat production will have a crucial bearing on food security and the global economy in the coming decades.

Approximately half a century ago, population growth threatened to overtake food production, and at that point, it was discovered that semi-dwarf mutants of wheat produced much more grain than their taller relatives. A series of research, development and technology transfer initiatives so-called Green Revolution has led to steady annual increases in grain production, in which selective breeding for yield and other important traits played a major role (Kantar et al., 2011a).

Wheat, an outstanding member of Triticeae, attracts more attention than many other crops, particularly in the face of increasing population and the global climate change challenging the food security of future generations (Ergen and Budak, 2009; Lucas et al., 2011b). Drought, affecting more than 70% of arable lands around the world, is the most critical condition for plants among all other environmental stresses that bring the good-yielding crops to a lower production.

The drought stress-related yield loss has gained considerable attention in recent years as agricultural activities have been extended to less fertile or infertile fields to meet the growing food demand. As a result, the enhancement of drought tolerance in plants, especially in the cereal crops, has become the key challenge for today’s wheat agronomists and plant geneticists.

Drought or water shortage is considered as the main factor responsible for the decrease in wheat production. However, this growth may no longer be adequate to meet future demand (Tester and Langridge, 2010). The World is threatened by global

warming resulting in increased incidence of environmental stresses, making stabilizing yields as much of a challenge as increasing them. Climate change has detrimental consequences particularly for crops which hold great economic value (Habash et al., 2009). Drought, arguably the most significant single abiotic stress factor is currently increasing worldwide, effecting progressively more arable land and impacting agricultural production.

Wheat and its related species are of great importance, constituting the primary sources of food and feed consumption. However, domestication of wheat species, followed by years of cultivation, genetics and breeding practices has considerably narrowed gene pools of today's elite cultivars. These practices introduce an artificial selection pressure for yield, ultimately eradicating genetic diversity, resulting in the loss of valuable alleles for drought stress tolerance. Ironically, the semi-dwarfism trait that drastically improved grain yields 50 years ago makes wheat more vulnerable to drought in many cases. Therefore, it is crucial to take initiatives for the next Green Revolution to develop wheat yielding high even under water-limited environments.

Drought tolerance translates not only into the survival skills under water deficit conditions but also the maintenance of high productivity (Budak et al., 2013b; Lucas et al., 2011b). Over the past few decades, there has been a significant effort for the elucidation of the drought stress mechanisms in plants. Although several genes involved in the plant drought stress responses have been identified (Budak et al., 2013a, 2013b), the drought stress response is still a complex phenomenon with several key factors that have yet to be investigated. Comprehensive understanding of the stress adaptation mechanisms in plants and associate them with the genome at the structural and functional level is required to overcome the reduced grain yield.

Various omics fields including biochemistry, physiology, molecular biology, genetics, and metabolomics have been used to clarify the drought tolerance mechanisms in wheat and reveal metabolic pathways that can be manipulated to surmount the adverse effects of water-limited conditions. Plant metabolomics (Fiehn et al., 2000), for instance, has been extensively exercised for investigations of physiological and metabolomic functions of genes, QTL studies, and development of genetic breeding programs (Jacobs et al., 2007).

Metabolomics refers to the quantitative and comprehensive study of metabolites in an organism (Dettmer & Hammock, 2004). It can be described as a snapshot of all

small metabolites constituting an organism's phenotype in its tissues or fluids and is the latest of the "omics"- sciences, preceded by genomics, transcriptomics and proteomics. In contrast to the other "omics" metabolomics provides broader information of the phenotype, with endogenous metabolites reflecting both genetic setup and response, as well as exogenous metabolites from exposure to environmental factors. The untargeted metabolomics approach is primarily a tool generating new hypotheses and prediction models (Kell, 2004).

The main hypothesis when using the untargeted approach is that there is asystematic variation of metabolites, e.g. between treatment group and control or between baseline and later time points or between treatments. The aim when choosing analytical methods for metabolomics is to find a method that detects and quantities as many metabolites as possible, rather than aiming at the in-depth exploration of an a priori defined set of metabolites.

In this study, a GC-MS-based metabolomics approach was implemented for determination of low-molecular-weight drought stress-responsive metabolites in leaf and root tissue samples of wild and domesticated wheat relatives. The metabolic content of control and drought-stressed leaf and root tissues from different Triticeae species were compared to explore the effects of drought stress on a metabolomic level and to track the genes that are encoding enzymes involved in the biochemical pathways, using the transcriptome and Wheat Genome Survey Sequences (WGSS).

Finally, the mechanisms of plant adaptation to drought stress were observed through morphological examination of the sample roots. The outcomes of this study provide a valuable source for metabolome of modern and wild wheat species, which could eventually contribute to the future genetic and metabolomic studies of the domesticated crops.

CHAPTER NO. 2

2. Review of Literature

2.1. Triticeae, The tribe

The tribe *Triticeae* belongs to the grass family (*Poaceae*) includes nearly 400 perennial and 100 annual taxa. *Triticeae* has played a precious role in human civilization, and it includes species that are indispensable for human welfare. It encompasses forage and lawn grasses as well as several agriculturally important domesticated major crops from the genera *Hordeum* (barley), *Triticum* (wheat) and *Secale* (rye), which are traditionally cultivated in the temperate zone. These species have been used as staple food and beverages in various ways throughout the history of humanity. *Triticeae* species have a complex evolutionary history being subjected to domestication (Middleton et al., 2014).

Triticeae tribe has a basic chromosome number of seven and comprises diploids ($2n=2x=14$), as well as species with varying degrees of polyploidy up to duodecaploids ($2n=12x=84$). Allopolyploidization, a cytogenetic process during hybridization resulting in the presence of complete chromosome sets of both parents in the progeny, has been and still is the major driving force in this tribe's evolution. Hence, this natural process has been utilized to artificially create species through intergeneric or interspecific hybridization, increasing the genetic variability within the tribe.

For instance, Triticale (*Triticosecale)*, a currently commercial crop was synthesized by artificial hybridization to develop a crop with high grain quality and quantity of wheat, and superior stress tolerance of rye. Elucidation of molecular mechanisms underlying differential yield and stress characteristics of *Triticeae* genera, species, subspecies and cultivars and their integration into breeding programmes is crucial for further improvement of their agronomic performance and ameliorate the effects of climate change (Wang et al., 2010; Wang and Lu, 2014; Middleton et al., 2014).

2.2. Wheat as a Staple Food

Wheat is currently the most extensively grown crop in the world covering 30% of the agricultural area (approximately 218 million hectors) used for cereal cultivation. With a global annual production of over 713 million tones, wheat is the third most abundantly produced crop, following maize and rice (based on FAO statistics of 2013;http://faostat.fao.org). Wheat is a fundamental source of protein, vitamins and minerals for human food consumption, providing almost 20% of the human dietary energy supply in calories (http://www.fao.org, 2011).

Wheat cultivation and domestication has been directly associated with the spread of agriculture. Cultivated wheat refers mainly to two types: hexaploid bread wheat (*Triticum aestivum* L.; AABBDD, 2*n*=6*x*=42) accounting for about 95% of world wheat production, and the tetraploid durum wheat (*T.turgidum* ssp. *durum;* AABB, 2*n*=4*x*=28) accounting for the remaining 5%. Domesticated tetraploid durum is one of the oldest cultivated cereal species in the world and its domestication from wild emmer wheat (*T. turgidum* ssp. *dicoccoides*; AABB, 2*n*=4*x*=28) in the Near East Fertile Crescent, dates to approximately 10,000 year ago.

Allohexaploid bread wheat is originated from hybridization between cultivated allotetraploid emmer wheat and diploid goat grass (DD, *Aegilops tauschii*) approximately 8,000 years ago in the Near East Fertile Crescent. The three diploid genome progenitors: *Triticum urartu* (AA)*, Aegilops tauschii* (DD) an unknown BB progenitor (possibly *Sitopsis* section species similar to *Aegilops speltoides*) radiated from a common *Triticeae* ancestor 2.5-4.5 million years ago and, AABB tetraploids arose less than 0.5 million years ago (Feldman, 2001; Brenchley et al., 2012; Kurtoglu et al., 2014).

2.3. Abiotic Stress Factors and Drought

To meet the demands of the ever-growing population, world food production needs to be doubled by the year 2050 (Tilman et al., 2002; Qin et al., 2011). Abiotic stresses, as the primary causes of agricultural loss worldwide, are estimated to result in an average yield loss of more than 50% for most crops (Boyer, 1982; Bray et al., 2000; Akpinar et al., 2013; Qin et al., 2011). Global environmental warming, with the prospect of increasing environmental stresses, threatens the world's food supply, making

stabilizing yields as much of a challenge as increasing them (Nevo and Chen, 2010; Kantar et al., 2011a). Drought in crop production results from a shortage of water in the root zone (Salekdeh et al., 2009; Nevo and Chen, 2010). Constant and sproadic periods of drought is currently the most prominent andwidespread abiotic stress, accounting for a significant portion of the yield loss resulting from abiotic factors and effecting more than 10% of arable land (Akpinar et al., 2013; Kantar et al., 2011a; Bray et al., 2000).

2.4. Drought Stress Tolerance

Drought stress tolerance is the ability of a plant to access soil water and use it efficiently to live, grow and reproduce satisfactorily under conditions of limited water supply or under periodic conditions of water deficit (Fleury et al., 2010; Turner, 1979; Richards et al., 2010; Munns et al., 2010; Kantar et al., 2011a). Tolerance strategies include resistance mechanisms, which enable plants to survive osmotic stress, and avoidance mechanisms, which prevent plants' exposure to dehydration through growth habits like deeper rooting for better access soil water, or shortened growth span through faster development and maturation (Fleury et al., 2010; Kantar et al., 2011a; Nevo and Chen, 2010).

Most plants have developed strategies to cope with drought stress having evolved in habitats with limited water availability (Kantar et al., 2011a). However, modern crop species, have drastically lost their tolerance to environmental stresses, including drought through the process of domestication, followed by centuries of cultivation (Tang et al., 2010; Nevo, 2004; Dubcovsky and Dvorak, 2007; Reynolds and Condon, 2007; Kantar et al., 2011a; Nevo and Chen, 2010).

The capacity of plants to tolerate drought depends largely on the drought adaptation mechanisms within their genomes, and how efficiently these mechanisms are activated when plants are exposed to stress. Few agronomic traits are controlled by single genes or isolated biological pathways. Likewise, genetic control of plant response to drought is a complex trait controlled by an intermingled network of gene interactions regulated at multiple levels and highly effected by environmental factors. Elucidation, the complete molecular basis of drought response and tolerance, is highly challenging, yet crucial.

2.5. Effects and Responses to Drought Stress

The drought has a multitude of detrimental effects on plant cellular function. Drought responses of plants include attenuated growth and suppression of core metabolism. Exposure to drought is followed by a decrease in osmotic potential and cellular dehydration, causing reduced cytosolic and vacuolar volumes. With the suppression of core metabolism, reactive oxygen species (ROS) (e.g. singlet oxygen and hydrogen peroxide) are highly accumulated majorly from chloroplasts and to some extend from mitochondria, causing oxidative stress, resulting in cellular and protein damage (Ergen et al., 2009; Kantar et al., 2011a).

Plant response to drought aims to minimize these harmful effects for the continuation of plant survival, growth and reproduction. This includes stimulation of multiple signal transduction cascades consisting of a network of protein interactions mediated by reversible phophorylation (e.g. mitogen activated protein kinases, sucrose nonfermenting-like kinases, phosphotases) and release of secondary messengers (e.g. phospholipid and calcium signalling) triggering cellular, metabolic and physiological changes. Following dehydration, compatible solutes, sugars, sugar alcohols, amino acids, or other nontoxic molecules (e.g. proline, glycine betaine), are highly accumulated in the cytoplasm and are believed to confer osmotic adjustment without interfering with the metabolism (Bartels and Sunkar, 2005; Valliyodan and Nguyen, 2006; Barnabás et al., 2008).

The level of different chemicals including ascorbate, carotenoids and enzymatic antioxidants (superoxidase dismutase, catalase), which cope with oxidative damage by scavenging ROS, are also drought induced (Shinozaki and Yamaguchi-Shinozaki, 2007). To ameliorate the effects of oxidative damage, late embryogenesis abundant proteins (LEAs) (e.g. dehydrin) and molecular chaperones like heat shock proteins (HSPs) also accumulate during osmotic stress aiding in the functional protection of essential proteins (Wang et al., 2003; Mahajan and Tuteja, 2005). Drought response is a complex process, in which several other cellular mechanisms have been implicated including signalling through molecules like salicyclic acid, or nitric oxide; as well as regulation of transport through aquaporins and ion channels.

Activation of various cellular mechanisms for triggering drought response demands the synthesis of new proteins and degradation of existing ones that are not or less essential in this environment (Bartels and Sunkar, 2005; Barnabás et al., 2008;

Mahajan and Tuteja, 2005). These alterations in expression profiles are regulated elaborately in multiple levels: transcriptional, post-transcriptional, post-translational. Transcriptional regulation of drought-induced gene products is achieved through activation of several transcription factors and trancriptional regulators; and abscisic acid (ABA)-dependent and -independent pathways are two well-established transcriptional regulatory circuits induced by drought.

Plant genes involved in drought response are also known to be regulated at the post-transcriptional level. Similarly, some post-translational modifications (e.g. ubiquitination, small ubiquitin-like modifier-ylation, isoprenylation) with different cellular roles have also been shown to contribute to regulation in response to drought (Kantar et al., 2011a; Ergen et al., 2009).

2.6. Wild and Domesticated Crops

As the availability of water for agriculture is becoming limited, there is growing emphasis on the need to identify and dissect novel drought-response mechanisms to utilize in the genetic improvement of cultivated crops for stress tolerance. Domestication of crops, followed by centuries of cultivation has considerably narrowed the gene pools of today's elite cultivars, drastically reducing their stress tolerance. Common agricultural practices favour breeding under tightly controlled conditions, which introduces an artificial selection pressure for production yield, which eradicates the crop germplasm diversity in the long run and leads to the loss of valuable alleles for stress tolerance.

For the development of high yielding cultivars under stress conditions, investigation of naturally occurring relatives of modern crops hold great potential as these drought- resistant ancestors are valuable sources harbouring advantageous stress adaptation and tolerance pathways. As progenitors of cultivated wheat and barley: *T. dicoccoides* and *H. spontaneum* have recently gained prominenence as genetic resources for novel drought mechanisms (Kantar et al., 2010; Ergen et al., 2009; Akpinar et al., 2013; Nevo and Chen, 2010).

2.6.1. Wild Emmer Wheat

Triticum turgidum ssp. dicoccoides is the tetraploid progenitor of both bread wheat and domesticated tetraploid durum wheat. It is thought to have originated in

north-eastern Israel and the Golan and diversified into the Near East Fertile Crescent, through adaptation to a spectrum of ecological conditions. As revealed by the analysis of allozyme and DNA marker variations, wild emmer wheat populations exhibit a high level of genetic diversity, showing significant correlation with environmental factors. Hence *Triticum turgidum ssp. dicoccoides* gene pool harbours a rich allelic repertoire of agronomically important traits (Nevo and Beiles, 1989; Nevo et al., 1982; Fahima et al., 1999, 2002; Dong et al., 2009; Wang et al., 2008) including drought (Peleg et al., 2005, 2008).

Some of its accessions are even fully fertile under extreme arid environments (Nevo et al., 1984) and compared to durum wheat; several thrive better under water limitation (Ergen and Budak, 2009; Peleg et al., 2005). Two highly promising drought tolerant varieties originating from south-eastern Turkey where the climate is characterized by long drought periods are TR39477 and TR38828 evident by morphological observations and physiological measurements in response to slow dehydration stress (Ergen and Budak, 2009).

Although *Triticum turgidum ssp. dicoccoides* genome sequence is currently unavailable, information regarding transcript, protein and metabolite profiles of Turkish (drought tolerant TR39477; drought sensitive TTD-22) and Isralean (drought tolerant: Y12-3 and drought sensitive: A24-39) varieties is swiftly accumulating, revealing biochemical pathways unique to dehydration tolerant wild emmer wheat (Krugman et al., 2010, 2011; Ergen and Budak, 2009; Ergen et al., 2009; Budak et al., 2013a).

Some of the drought related gene candidates discovered in these studies (integral transmembrane protein inducible by tumor necrosis factor-α; dehydration responsive element binding factor 1, autophagy related protein 8) were even further functionally characterized in relation to their roles in dehydration and drought stress in wheat (Kuzuoglu-Ozturk et al., 2012; Lucas et al., 2011a, 2011b). With its high drought tolerance and compatibility in crossing with durum and bread wheat (Feldman and Sears, 1981), wild emmer wheat is an important reservoir of novel drought-related mechanisms and highly suitable as a donor for improving drought tolerance (Xie and Nevo, 2008; Peng et al., 2011b, 2011a; Nevo and Chen, 2010; Budak et al., 2013b).

2.7. Improvement of Drought-Tolerant Cultivars

In modern era of 21st century, modern wheat better achieved drought stress tolerant characteristics on molecular level due to advancements in molecular and genetic tools to identify and characterize drought responsive characters more quickly. The engineering of drought related components could be achieved by using marker assisted selection (MAS) or transgenetic tools (Budak et al., 2013b; Nevo and Chen, 2010). Components integral to several stress related pathways are the most appealing targets for crop improvement since their introduction can potentially enhance tolerance to multiple environmental threats (Budak et al., 2013b).

A more established method for crop improvement is molecular breeding, which utilizes molecular markers for the screening of specific traits across cultivars. Loci that are targeted in marker-assisted selection (MAS) are most often derived from QTL mapping studies of quantitative traits. MAS is most often performed based on physio-morphological characteristics related to yield under drought stress conditions. Most commonly used molecular markers in such a context include SSR (simple sequence repeat) markers (Budak et al., 2013b). For instance, SSR marker, *gwm*312 is being routinely used in durum breeding programs (James et al., 2006) to transfer and select for the presence of sodium (Na^+) exclusion (Nax) genes, which are involved in sequestration of Na^+ in the vacuole compartment, enhancing osmotic adjustment capability and ameliorating the negative effects of drought (Brini et al., 2005).

Currently, the major challenge to MAS is that most of the potential drought related genes which can be used for selection purposes belong to large gene families (Wei et al., 2008). Hence, identification and successful isolation of a single drought-related lociarecomplicated by the members of the same family with high sequence similarity and in the case of bread wheat its complex, polyploid genome. However, in the very near future, completion of wheat reference genome will pace the identification of specific loci and the development of markers to be used in selection during breeding processes (Witcombe et al., 2008).

Recent increase in sequence availability has already contributed to the discovery of drought-related QTLs and provided several high quality genetic markers for breeding (Bennett et al., 2012c, 2012b, 2012a; Bonneau et al., 2013). Up until now, no drought tolerant wheat or barley genotype has been produced through conventional and molecular approaches, which has found its way to the farmer's field. However, it is not unreasonable to predict in the following decades; such cereals will be transferred to the fields as a common commercial crop owing to recent efforts and advances.

CHAPTER NO.3

3. Materials and Methods

3.1. Plant growth conditions, experimental design and drought stress treatments

Wild and domesticated wheat genotypes from different ploidy levels that our group has used in several previous studies were combined for comparison (Budak, Akpinar, et al. 2013; Budak, Kantar, et al. 2013; Lucas et al. 2011; Kantar et al. 2010; Ergen & Budak 2009). The list of the species used was presented in Table 1. The seeds of all genotypes were pre-germinated (20 plants from each genotype) in Petri dishes after surface sterilization with 70% ethanol for 5 min, washing with water (3X), immersing in 1% NaOCl for 10 min and rinsing with water (10X). The plants were subjected to 80% soil Water holding capacity (WHC) served as control and 30% WHC served as drought stress treatment (Boutraa et al., 2010).

Maintenance of the water treatments was made by daily weighing of the pots replacing the water lost by transpiration and evaporation from the pot and plant surface. Three plastic pots (2 kg) were used for each genotype for each treatment, and each pot contained ten plants. After 16 days of drought stress treatment, three biological replicates from each genotype across each treatment (control vs. drought stress) were sampled, whereas for each replicate with six seedlings, an equal amount of sample from randomly selected five individual plants were pooled.

All leaf and root samples were immediately frozen in liquid nitrogen after harvesting and stored at -80°C until the extraction of metabolites. Another three plants with uncut roots from each genotype across each condition (control vs. drought stress) were used to analyse different morphological parameters of root development.

3.1.1. Measurement of root morphology dynamics

Full roots of three replicates from each genotype across each condition (control vs. drought stress) were collected following a 16 days drought stress treatment (16DTW), thoroughly washed, dried, and used to determine root morphological parameters. The root length (RL), average root diameter, surface area (SA), number and length of lateral roots, number of tips, number of forks and crossings (overlapping parts) were measured with WinRHIZO 4.1 system (Regent Instruments Inc; Quebec, Canada) (Himmelbauer et al., 2004; Wang and Zhang, 2009; Bauhus and Messier, 1999). Lateral root initiations and the diameter of primary roots were measured under optical light microscope illumination (10X-lense) (Chen and Xiong, 2005; Yamaguchi, 2002).

3.1.2. Extraction and derivatization of wheat leaf and root metabolites

Standard mixtures used for the optimization of GC-MS studies were prepared in 1000 µg/ml methanol and stored at -20 °C. Working standard solution was diluted up to 50 µg/ml from the main stock solution. Polar metabolites were extracted with 350 µl of 100% methanol and suspended in 20 µl of internal polar standard (Ribitol; 0.2 mg/ml in water) (Jacobs et al., 2007). The mixture was incubated at 70 °C for 15 min and well-mixed with 1 volume of distilled water. Chloroform (300 µl) was added to the mixture to separate polar and non-polar metabolites, followed by centrifugation at 14000 rpm for 10 min.

The supernatant was taken and washed again with chloroform. Aliquots of the leaf and root polar phases (100 µl and 5 µl) were used for the analysis of high and low abundance metabolites while the non-polar phase was discarded. All aliquots were dried under vacuum, re-dissolved and derivatized at 37 °C for 2 hours in methoxy-amine-hydrochloride (40 µl of 30 mg/ml in pyridine). Trimethylsilylation was performed at 37 °C for 30 min with N-methyl-N-[trimethylsilyl] trifluoroacetamide (70 µl; MSTFA) (Orata, 2012).

3.1.3. Metabolite profiling using GC-MS technology

GC-MS-QP2010 Ultra Gas Chromatograph Mass Spectrometer with an AOC-20i auto-injector GC Ultra and a DSQ quadruple MS (SHIMADZU Corporation, Tokyo, 101-8448, Japan) was used for metabolite profiling. The MS was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). GC was performed on a 30-m MDN-35 capillary column with 0.25 mm inner diameter and 0.25 μm film thickness (Varian Inc, Victoria, Australia).

The injection temperature was set at 230 °C, the MS transfer line at 280 °C, and the ion source at 250 °C. Helium 99.99% purity was used as a carrier gas with 1 ml/min flow rate. The analysis was performed under the following oven temperature program: injection at 70 °C followed by 1 °C/min ramp to 76 °C, and then by 6 °C/min to 330 °C, finishing with 10 min isothermal at 330 °C. The samples obtained were injected into the GC-MS column in the splitless mode, using the hot needle technique. The GC-MS system was then temperature-equilibrated for 1 min at 70 °C before injection of the next sample (Warren et al., 2011). The workflow of the method was presented in Figure 1a.

3.1.4. Metabolomics data analysis and metabolite identification

Data was acquired with Advanced Scanning Speed Protocol (ASSPTM) integrated into GC-MS-QP2010 Ultra at a speed of 20,000 μ/s and 100 Hz. Both chromatograms and the mass spectra of the eluted compounds were identified using the AMDIS program (version 2.72) with the mass spectral reference NIST library comprised of the spectra of 191436 general compounds, and Wiley Registry of Mass Spectral Library accompanied by the corresponding structural information, enabling the discovery of new components as well as the targeted ones. Authentic standards were used to analyse and verify all matching spectra (Witt et al., 2012).

The pseudo peaks, originating from the internal standards or caused by noise, column and derivatization procedure, were removed from the dataset. The peaks with similarity index higher than 70% were considered effective metabolites in the experiments, while those with lower than 70% similarity index were regarded as unknown metabolites and removed from the data. Following the deconvolution of resulting chromatograms, 45 metabolic compounds including amino acids, organic acids, and sugars were identified (Figure 2 and Figure 3).

Each metabolic compound was given a specific trace to be used in the quantification (Alvarez et al., 2008). The resulting peak areas were normalized to the area of a specific trace of the internal standard resulting in relative response ratios, which were further normalized by the fresh weight of each sample (Table 2 and Table 3).

3.1.5. Statistical data analysis

The complete metabolomics data were mean-centered for Principal Component Analysis (PCA) and Partial Least-Squares-Discriminant Analysis (PLS-DA). Hierarchical Cluster Analysis (HCA) was performed usingCluster (version 3.0). Total explained variance (R^2) and predictability (Q^2) values were extracted from the metabolomics data by using unit variance scaling method. A two-sample t-test was applied to find the level of significance between the metabolites and the inter-connection between significantly altered metabolites was analyzed by using R software.

The Cytoscape software was used to reveal metabolite-metabolite interaction and gene-metabolite networks by integrating the data (Lopes et al., 2010; Shannon et al., 2003; Kopka, 2006).

3.1.6. Identification and location of genes in wheat genome

For the validation of data, the full-length cDNA from *Oryza sativa* (The Rice Full-Length cDNA Consortium, 2013) was used to extract orthologous of genes encoding the enzymes of biochemical pathways responsible for the biosynthesis of drought-specific metabolites. Later, TBLASTX search (e-value<$3e^{-106}$) was adopted by using annotated rice orthologous cDNA sequences to identify corresponding wheat FL-cDNAs from the Chinese spring collection (Kawaura et al., 2009) and the transcriptome data (Akpinar et al., 2015; Alptekin and Budak, 2016).

Finally, BLASTN search was performed against WGSS, and the chromosomal location was identified based on a threshold value of 85% sequence identity. The workflow of the study was presented in Figure 1b.

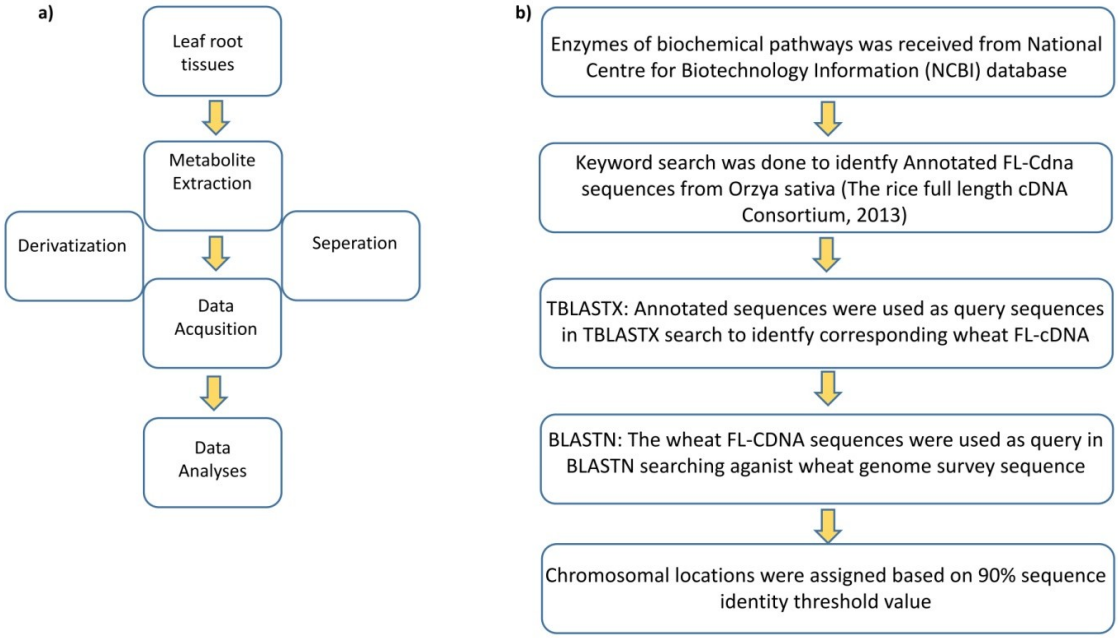

Figure 1 The outlines of methodologies used for (a) metabolite extraction and analysis and (b) BLAST search for target gene identification.

CHAPTER NO. 4

4. Results

4.1. Morphological responses of roots to drought stress

A statistically significant difference ($P<0.05$) was observed in all morphological parameters measured in this study for all genotypes grown under control and drought stress conditions. The average root length and surface area were increased in TR39477, IG132864 and Bolal as a tolerance response to the drought stress while few to no lateral root formation and reduction in the diameters of primary and secondary roots were observed in genotypes mentioned above after 16DTD.

Morphological changes were practically reverse in the sensitive genotypes TTD-22, Tosunbey, Ligustica, and Meyeri. For example, the mean values of RL in wild emmer (TR39477), domesticated einkorn (IG132864) and bread wheat (Bolal) increased after the drought stress induction, whereas the mean values of RL in wild emmer (TTD-22), wild einkorn (Meyeri), einkorn (Ligustica) and bread wheat (Tosunbey) plants decreased (Figure 2a and 2b).

Similar results were obtained related to SA parameter, presented here for two wild emmer genotypes contrasting in response to the drought. The mean value of SA in wild emmer wheat (TR39477) increased, whereas the average value of SA in drought-sensitive wheat (TTD-22) decreased after 16DTD. The diameters of primary and secondary roots were found to be smaller in the drought stress-tolerant plants (mean value, 13.8 μm) upon drought stress than the same genotypes under well-watered conditions (average value, 19.17 μm) (Figure 2c-h).

Other morphological parameters including the number of tips and forks were less common in the drought stress tolerant wheat genotypes as compared to the well-watered plants of the same cultivars (Table 4).

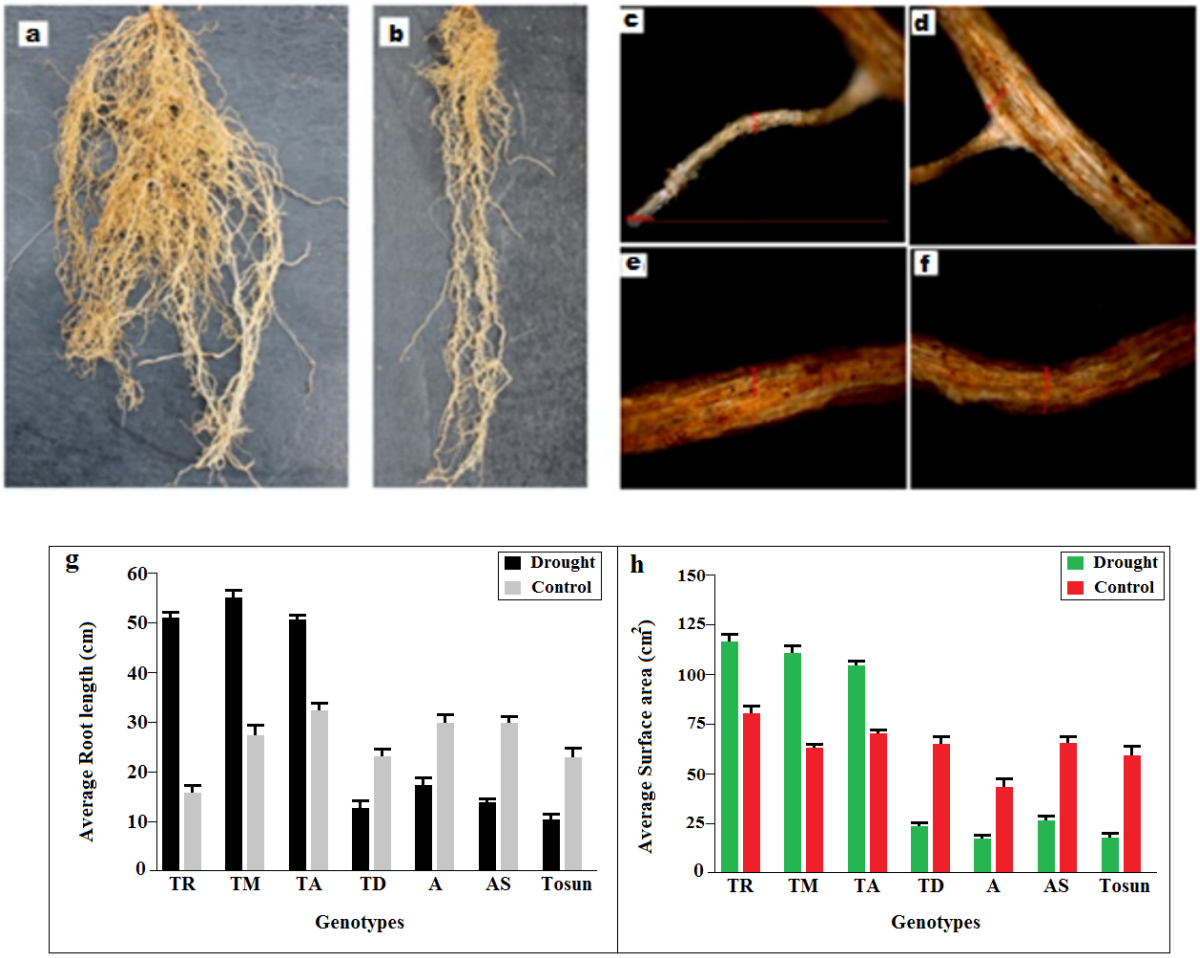

Figure 2 Root Morphology of normal and drought-stressed Triticum aestivum (Bolal) as a representative sample (a) Sample under normal conditions (b) root samples under drought stress conditions (c) Light Microscopy (10X) images of lateral root length and diameter (d) Primary root diameter (19.17 µm) taken from normal roots (e & f) Primary & secondary root diameters (13.8 µm) from drought stress treated sample (g) Average root length measured in centimeters (cm) and (h) average surface area measured for all seven genotypes.

4.1.1. Metabolic profile analysis upon control and drought stress treatments

Different levels of drought stress involving control (80%), mild (50%) and severe drought stress (30%) Water holding capacity, were set to investigate the changes in morphology of the roots and metabolic variations amongst different genotypes.

Interestingly, no obvious morphological differences were observed between control samples and individuals exposed to mild 50% WHC for maximum 16 days, whereas 30% WHC caused severe effects on the morphology and physiology of the drought-sensitive plants.

Therefore, severe drought stress treatment (30% WHC) was chosen to compare the morphological and metabolic responses of the samples from different ploidy with control treatment (80% WHC), wherein metabolomics analysis coupled with the transcriptomics data, previously reported by our group (Akpinar et al., 2015), was used. Metabolites were extracted from the leaf, and root tissue samples in triplicates from all seven Triticeae species for each of the four experimental groups, including drought stress treated leaves (DSL), drought stress treated roots (DSR), control leaves (CL) and control roots (CR) (Figure 3 and Figure 4). All four groups presented distinct chromatographic patterns, and 45 metabolic compounds were differentially accumulated, embracing amino acids, organic acids, sugars, organic compounds and organic antioxidants and compatible solutes as presented in Table 5.

PCA, an unsupervised data analysis method, was performed to reduce the dimensionality of the metabolomics data generated by GC-MS. The explanation and predictability values measured for first two PCs were 71.2% and 42.6%, respectively. PCA analysis is presented discriminations between the 80% WHC and 30% WHC samples, but, an overlap was observed between the DSL and DSR samples (Figure 5a). PCA analysis was also applied separately for each of the remaining three groups including CL vs. DSL, CR vs. DSR and DSL vs. DSR in order to contrast the datasets for better understanding.

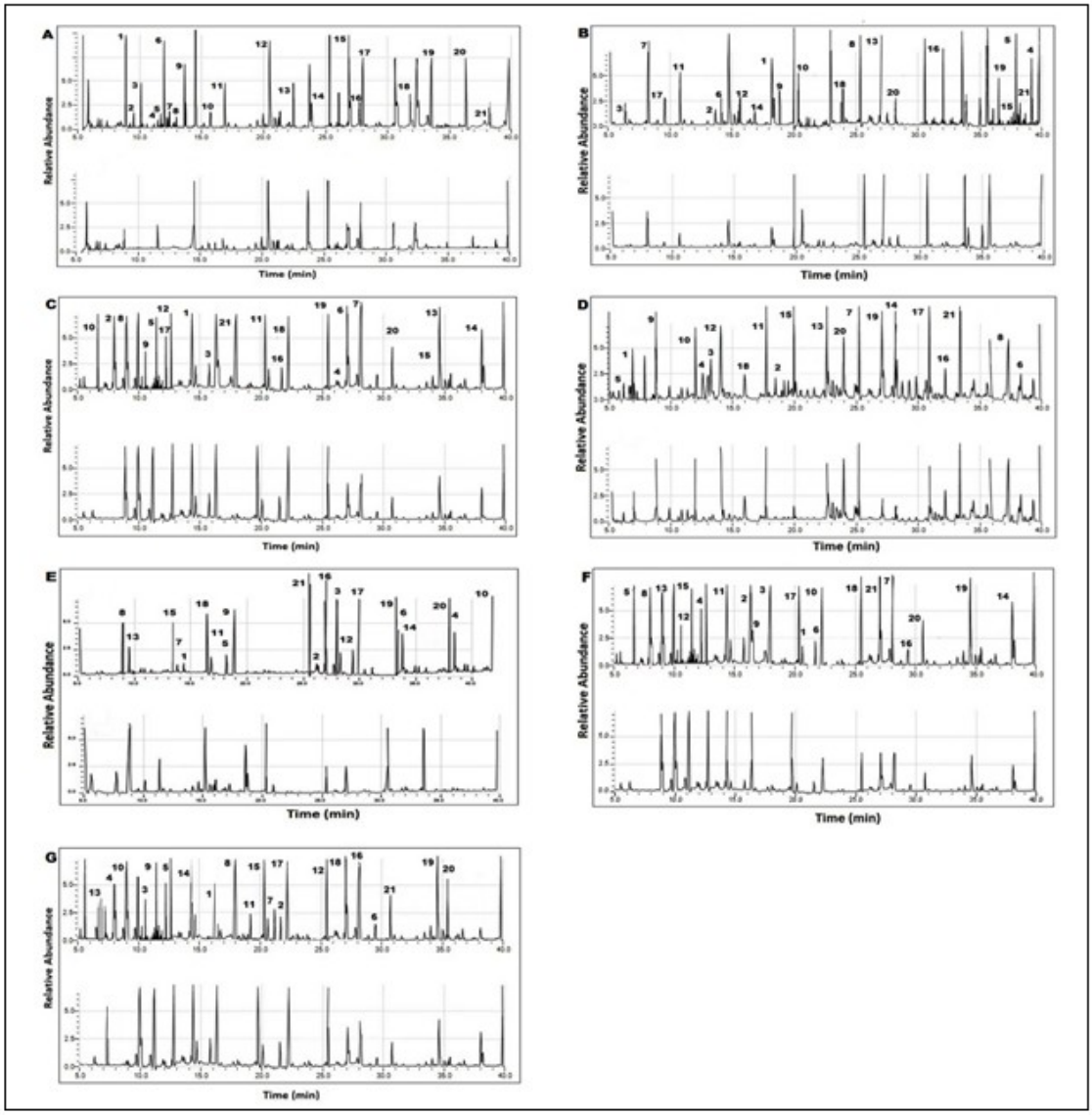
A
B
C
D
E
F
G
Relative Abundance
Time (min)

Figure 3 GC-MS spectra for a typical (representative) sample in control (lower pannel) and water-stress treated (upper pannel) leaves of (A) Aegilops speltoides (B) Triticum dicoccoides (TR39477) (C) Triticum dicoccoides (TTD-22), (D) Triticum aestivum (Bolal). 1. Sucrose, 2. Trehalose, 3. Mannitol 4. Maltose, 5. Proline, 6. Glutamate, 7. Alanine, 8. Lycine, 9. Asparagines, 10. Methionine, 11. Threonine, 12. Phenylalanine, 13. Homocysteine, 14. Serine, 15. Valine 16. Tyrosine, 17. Succinate, 18. Citrate, 19. Aspartate 20. Gluconate 21. Glutathione (E) Triticum aestivum (Tosunbey), (F) *Triticum monococcum*, and (G) *Aegilops tauschii*. Complete chromatographic time was 5.0-40.0 min. 1. Sucrose, 2. Trehalose, 3. Mannitol 4. Maltose, 5. Proline, 6. Glutamate, 7. Alanine, 8. Lycine, 9. Asparagines, 10. Methionine, 11. Threonine, 12. Phenylalanine, 13. Homocysteine, 14. Serine, 15. Valine 16. Tyrosine, 17. Succinate, 18. Citrate, 19. Aspartate 20. Gluconate 21. Glutathione

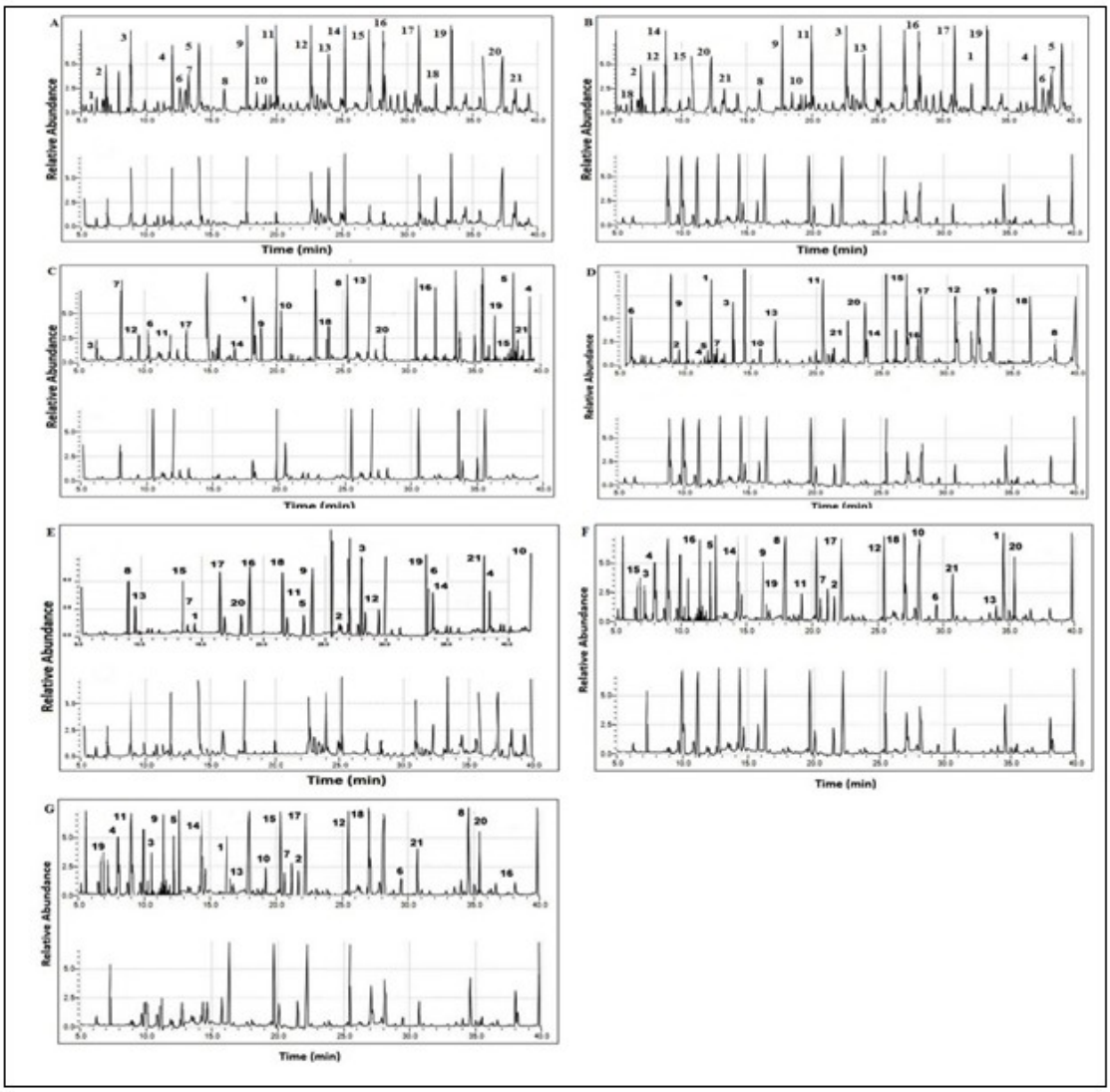

Figure 4 GC-MS spectra for a typical (representative) sample in control (lower panel) and water-stress treated (upper panel) roots of (A) Aegilops speltoides (B) Triticum dicoccoides (TR39477) (C) Triticum dicoccoides (TTD-22), (D) Triticum aestivum (Bolal). 1. Sucrose, 2. Trehalose, 3. Mannitol 4. Maltose, 5. Proline, 6. Glutamate, 7. Alanine, 8. Lysine, 9. Asparagine, 10. Methionine, 11. Threonine, 12. Phenylalanine, 13. Homocysteine, 14. Serine, 15. Valine 16. Tyrosine, 17. Succinate, 18. Citrate, 19. Aspartate 20. Gluconate 21. Glutathione (E) *Triticum aestivum* (Tosunbey), (F) *Triticum monococcum*, and (G) Aegilops tauschii. 1. Sucrose, 2. Trehalose, 3. Mannitol 4. Maltose, 5. Proline, 6. Glutamate, 7. Alanine, 8. Lysine, 9. Asparagine, 10. Methionine, 11. Threonine, 12. Phenylalanine, 13. Homocysteine, 14. Serine, 15. Valine 16. Tyrosine, 17. Succinate, 18. Citrate, 19. Aspartate 20. Gluconate 21. Glutathione

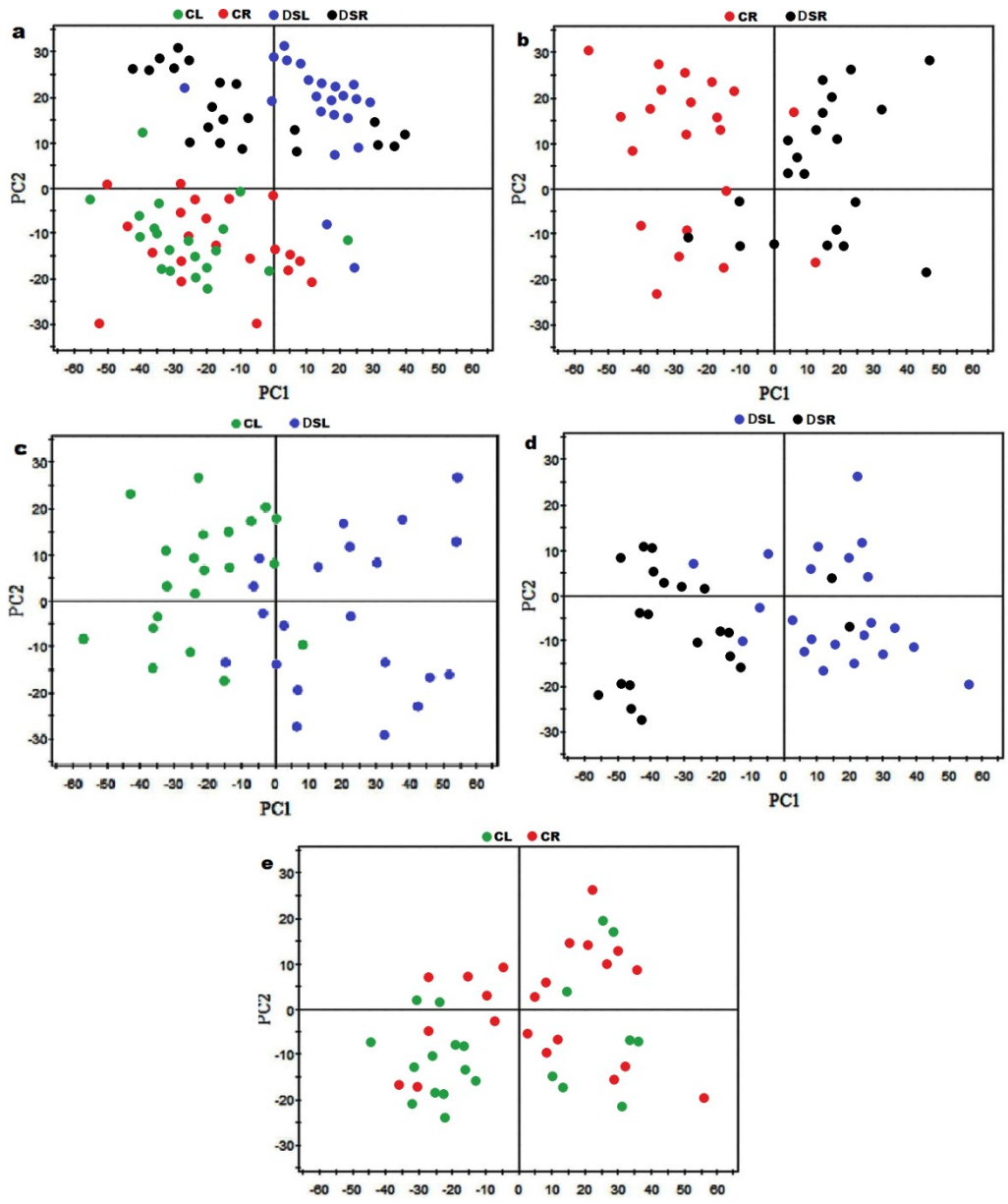

Figure 5 Principal component analysis (PCA) score plots of metabolite profiles in wheat leaves and roots under control and drought stress conditions. (a) PCA score plot for control leaves (CL; green), drought stress leaves (DSL; blue), control roots (CR; red) and drought stress roots (DSR; black) samples, (b) PCA score plot for CR and DSR samples, (c) PCA score plot for CL and DSL samples, (d) PCA score plot for DSL and DSR samples and (e) PCA score plot for CL and CR samples.

The R^2X and Q^2 values presented in Table 6 demonstrates the differences between the groups. As given in Figure 5b-e, a discriminative boundary between every two groups aforementioned was not achievable. Therefore, a supervised multivariate method called PLS-DA was applied to classify the observations in the groups by giving the largest predicted indicator variable (Figure 6a). The prediction results were satisfactory when only two principal components were obtained using the data from the control and drought stress-treated samples, whereas both drought stress-treated groups were clearly separated from the control groups along the first principal component, PC1 (Figure 6b and 6c).

In addition to the overlapping, DSL, and DSR samples were separated in the PLS-DA score plot with two PCs (Figure 6d and 6e). The comparison among similar treatments such as drought stress treated groups (DSL-DSR) and control groups (CL-CR) presented values 0.482 and 0.461 for R^2Y whereas 0.375 and 0.058 for Q^2, respectively (Table 6), indicating a minor metabolic change between the same treatments as compared to the respective controls.

HCA, on the other side, was performed to reveal the accumulation patterns of the metabolites. Figure 7 shows the accumulation patterns of 45 significantly altered metabolites after the exposure of plants to 30% WHC. On the basis of metabolite accumulation pattern, HCA presented two main clusters from all samples exposed to the drought stress. The smaller cluster consisted of two genotypes *Triticum turgidum* ssp. *dicoccoides* genotype TR39477 (TR) and *Triticum monococcum* ssp. *monococcum* genotype IG132864 (TM); *Triticum aestivum* ssp. *aestivum* genotype Bolal (TA) placed next to them whereas the remaining four genotypes *Aegilops tauschii var. Meyeri* (A), *Aegilops speltoides var. Ligustica* (AS), *Triticum turgidum* ssp.*dicoccoids* genotype TTD-22 (TD) and *Triticum aestivum* ssp. *aestivum* genotype Tosunbey (Tosun) together formed a bigger cluster as a result of their similar metabolite accumulation patterns.

4.1.2. Identification of the drought stress-responsive metabolites in wheat roots and leaves

The altered metabolites with significant ($P<0.05$) and highly significant ($P<0.01$) fold changes were obtained from the X-loading plots of the PC1 in PLS-DA. Variable importance in the projection (VIP) values were calculated for each altered metabolite

and a cut-off point made for all metabolites obtained from the GC-MS analysis. The metabolites are having VIP values greater than one was considered as the most relevant ones for the drought stress.

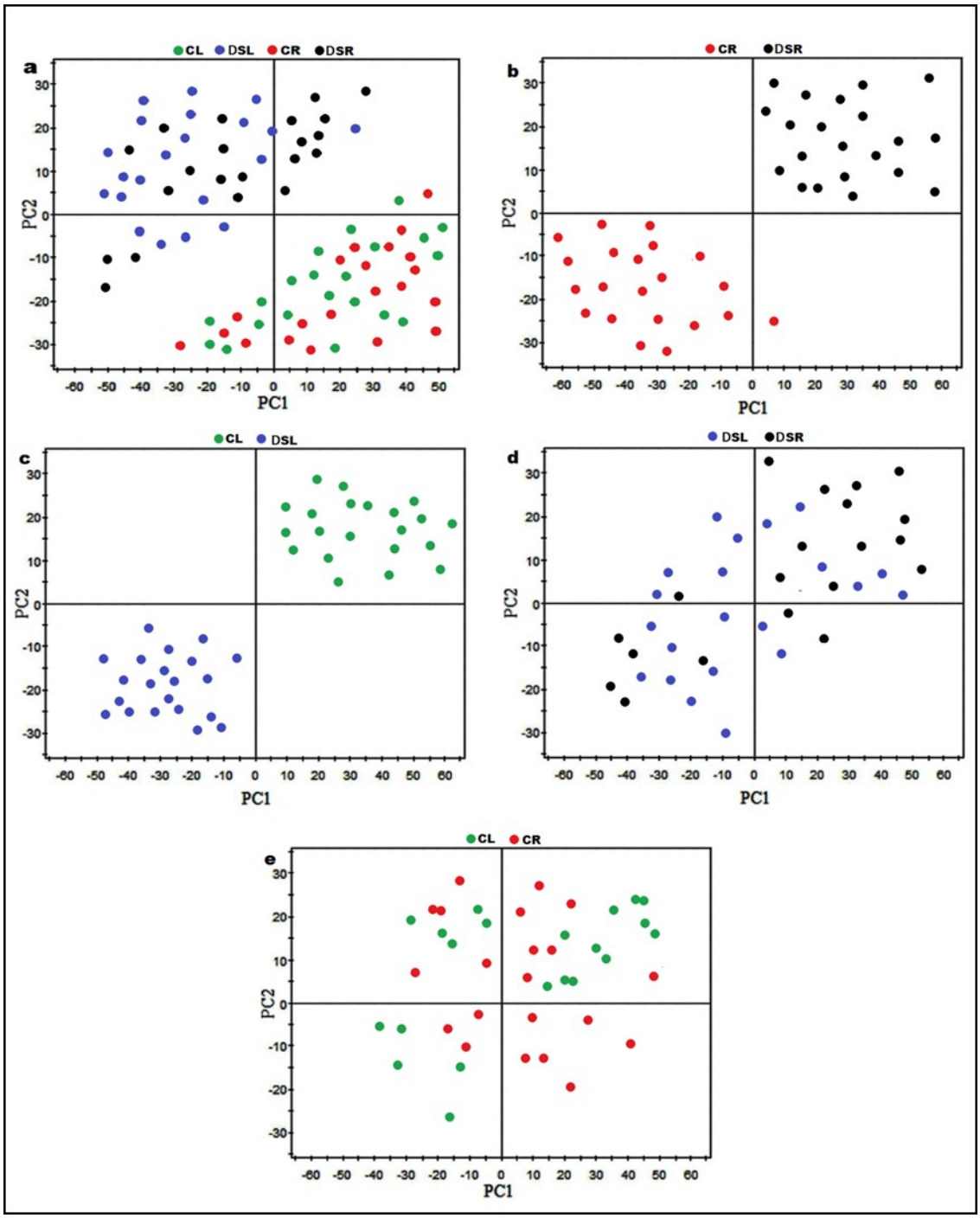

Figure 6 Partial least squares-discriminate analysis (PLS-DA) score plots of metabolic profiles in wheat leaves and roots under control and drought stress conditions. (a) PLS-

DA score plot for control leaves (CL; green), drought stress leaves (DSL; blue), control roots (CR; red) and drought stress roots (DSR; black) samples, (b) PLS-DA score plot for CR and DSR samples, (c) PLS-DA score plot for CL and DSL samples, (d) PLS-DA score plot for DSL and DSR samples and (e) PLS-DA score plot for CL and CR samples.

Figure 7 Hierarchical cluster analysis (HCA) revealed the differentially accumulated metabolites in seven Triticeae species after exposure to drought stress (30% Water holding capacity). The colour scale is red, high accumulation; black, moderate/normal accumulation; green, low accumulation.

On the basis of the cut-off point VIP>1, 45 drought stress responsive metabolites were identified in the leaves and root tissue samples, as presented in Table 7 and Table 8, respectively. As shown in Figure 8, the amount of 21 metabolites out of 45 including sugars or its derivatives (sucrose, trehalose, mannitol and maltose), amino acids (proline, glutamate, alanine, glycine, asparagines, methionine, threonine, phenylalanine, homocysteine, serine, valine and tyrosine), organic acids (succinate, citrate, aspartate and gluconate) and low molecular weight compounds (glutathione) increased in both leaf and root samples of TR39477, IG132864 andBolal under drought stress, contrasting to TTD-22, Tosunbey, Ligustica and Meyeri samples under drought stress.

The coordinated decrease in the accumulation levels of ɣ-Aminobutyric acid (GABA), pyruvate, α-ketoglutarate, was found both in the leaf and root tissue samples of all seven genotypes. The accumulation levels of 10 metabolic compounds including glucose, inositol, galactose, fructose, mannose, glyceric acid, quinic acid, malonic acid, oxalic acid, phthalic acid presented a decrease in the roots of TR39477, IG132864 and Bolal whereas these metabolites (mainly sugars) were present in normal levels in the leaf samples.

The remaining four genotypes (TTD-22, Tosunbey, Ligustica, and Meyeri) presented a lower standard of accumulation for glucose, inositol, galactose, fructose, mannose, glyceric acid, quinic acid, malonic acid, oxalic acid, the phthalic acid in the leaf and root samples.

On the other hand, accumulation level of the other 11 metabolic compounds (pimelic acid, shikimic acid, malic acid, adipic acid, oleic acid, ascorbic acid, fumaric acid, mandelic acid, lysine, leucine, and cysteine) decreased in the leaf samples of

TR39477, IG132864 and Bolal compared to the root and control samples whereas 5 metabolites (shikimic acid, adipic acid, lysine, cysteine, fumaric acid), 4 metabolites (fumaric acid, mandelic acid, lysine and leucine), 2 metabolites (shikimic acid and malic acid) and 3 metabolites (oleic acid, ascorbic acid and fumaric acid) were accumulated in moderate to high levels in the leaf and root tissue samples of TTD-22, Tosunbey, Ligustica and Meyeri, respectively (Figure 8 and 9).

The metabolites such as pimelic acid, malic acid, oleic acid, ascorbic acid, mandelic acid and leucine in TTD-22; pimelic acid, shikimic acid, malic acid, adipic acid, oleic acid, ascorbic acid, and cysteine in Tosunbey; pimelic acid, adipic acid, oleic acid, ascorbic acid, fumaric acid, mandelic acid, lysine, leucine and cysteine in Ligustica; and pimelic acid, shikimic acid, malic acid, adipic acid, mandelic acid, lysine, leucine and cysteine in Meyeri, were found in decreased amounts upon drought stress treatment as compared to the control treatment.

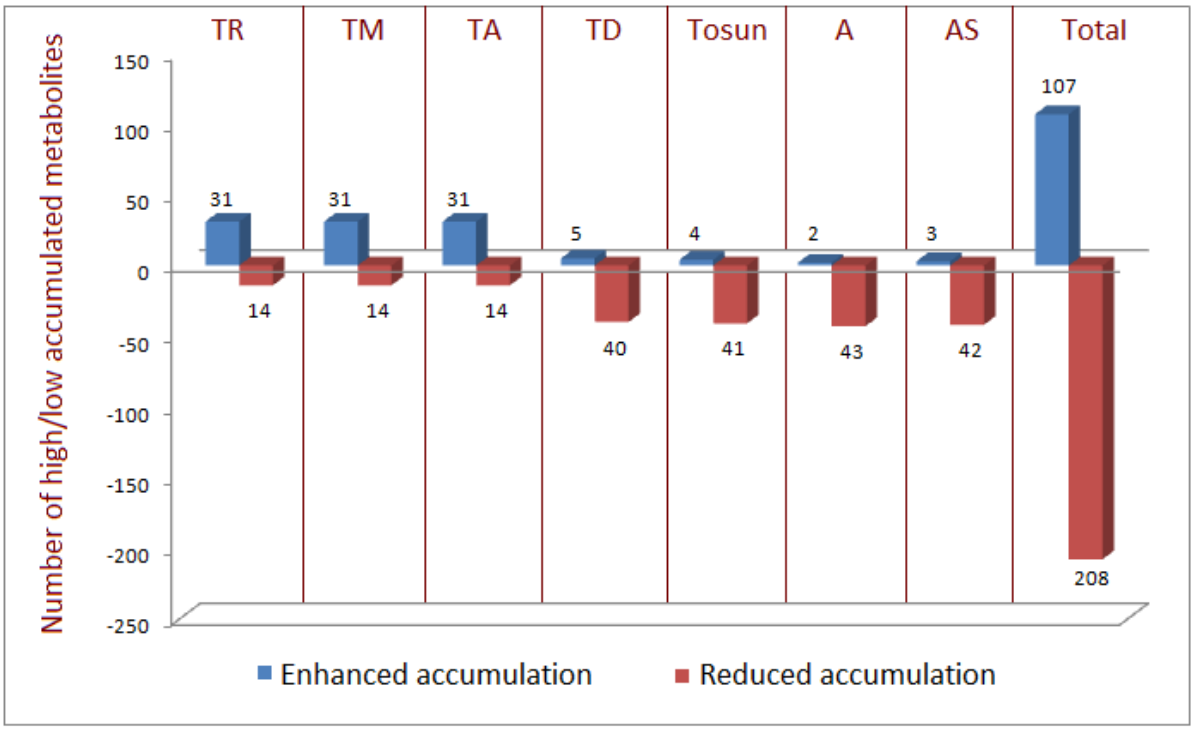

Figure 8 The number of high and low accumulated metabolites in seven Triticeae species under drought stress treatment (30% Water holding capacity). Each group consists of a blue (high accumulated metabolites) and red column (low accumulated metabolites). The numeral over the column is the number of high/low accumulated metabolites in each genotype.

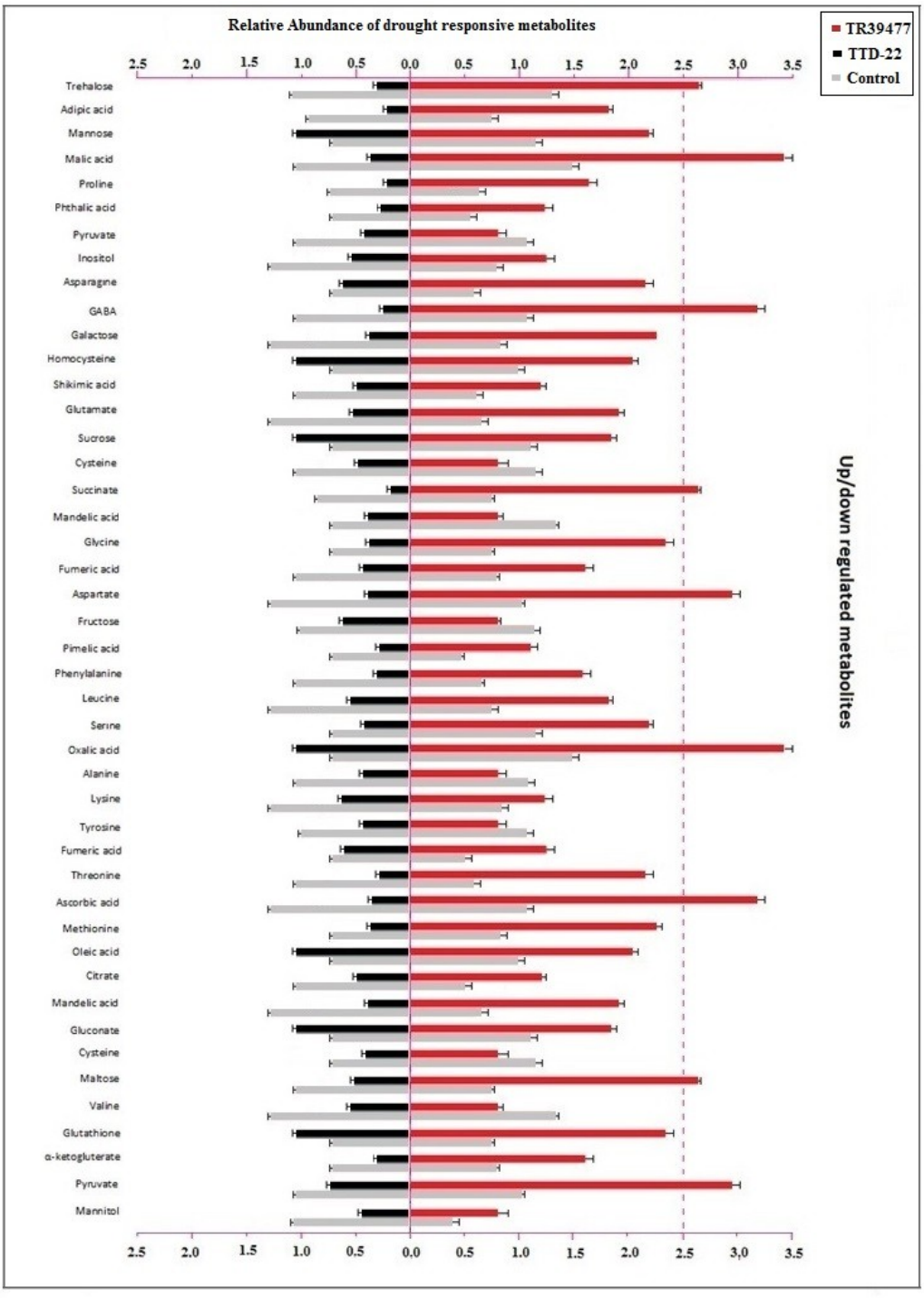

Figure 9 The relative abundances of metabolites increased and decreased in their accumulation in Triticeae species. TR39477 (Red) is taken as a representative sample for drought tolerant, while TTD-22 (black) represents drought sensitive genotypes among seven *Triticeae* species. Their Controls are shown in Gray colour.

4.1.3. Pathway mapping and the metabolite-to-metabolite network visualization

All the metabolites affected by the drought stresses were mapped to the biological pathways involved in the KEGG database, which was assigned to 12 pathways in either treatment (Table 9). The results showed that three pathways were enriched with the affected metabolites, as a consequence of the water stress.

Furthermore, a metabolite-to-metabolite interaction network was constructed using all the altered metabolites as inputs that comprised metabolites for the drought stress exposure in wheat and its wild relatives. The biochemical pathways presenting the metabolites accumulated at high levels in the leaf and root samples were shown in Figure 10 and 11, respectively.

4.1.4. Putative genes controlling the accumulation of succinate, aspartate, and trehalose

Three metabolites including succinate, aspartate and trehalose were selected for further genome analysis due to their dramatically increased levels in TR39477, IG132864, and Bolal upon drought stress treatment as well as their major role in energy producing biochemical pathway (TCA cycle). The increased metabolite levels were possibly related to the drought stress treatment, as shown in Figure 12.

Succinate is controlled by a relatively simple biochemical pathway involving three enzymatic steps where α-ketoglutarate is a substrate for conversion to succinyl-CoA by an enzyme α-ketoglutarate dehydrogenase which, in turn, is used to synthesize succinate through succinyl-CoA synthetase activity (Wang et al., 2015). Furthermore, succinate is converted to form fumarate molecules (Figure 13). Potential wheat genes encoding the two enzymes were searched in the WGSS and root transcriptome data.

Full-length wheat cDNA sequences with E-values <$3e^{-106}$ were identified, including three with homology to α-ketoglutarate dehydrogenase and four with significant homology to succinyl-CoA.

Analysis of the draft wheat genome sequence using wheat FL-cDNA as query sequence identified three copies of α-ketoglutarate dehydrogenase and four copies of succinyl-CoA related genes on the long arm of homologous chromosomes 1, 3 and 5 (Table 10). Of the wheat genotypes (TTD-22) that might suppress the succinyl CoA-related sequences under drought stress, showed the expected decrease in succinate level. Expression of the genes in RNA-sequence data was also inspected where succinyl CoA-related genes exhibited a significant drop in the expression level in TTD-22 (Akpinar et al., 2015).

On the other hand, most genes in this pathway exhibited more gradual, yet significant, increased expression levels in TR39477 (Akpinar et al., 2015).

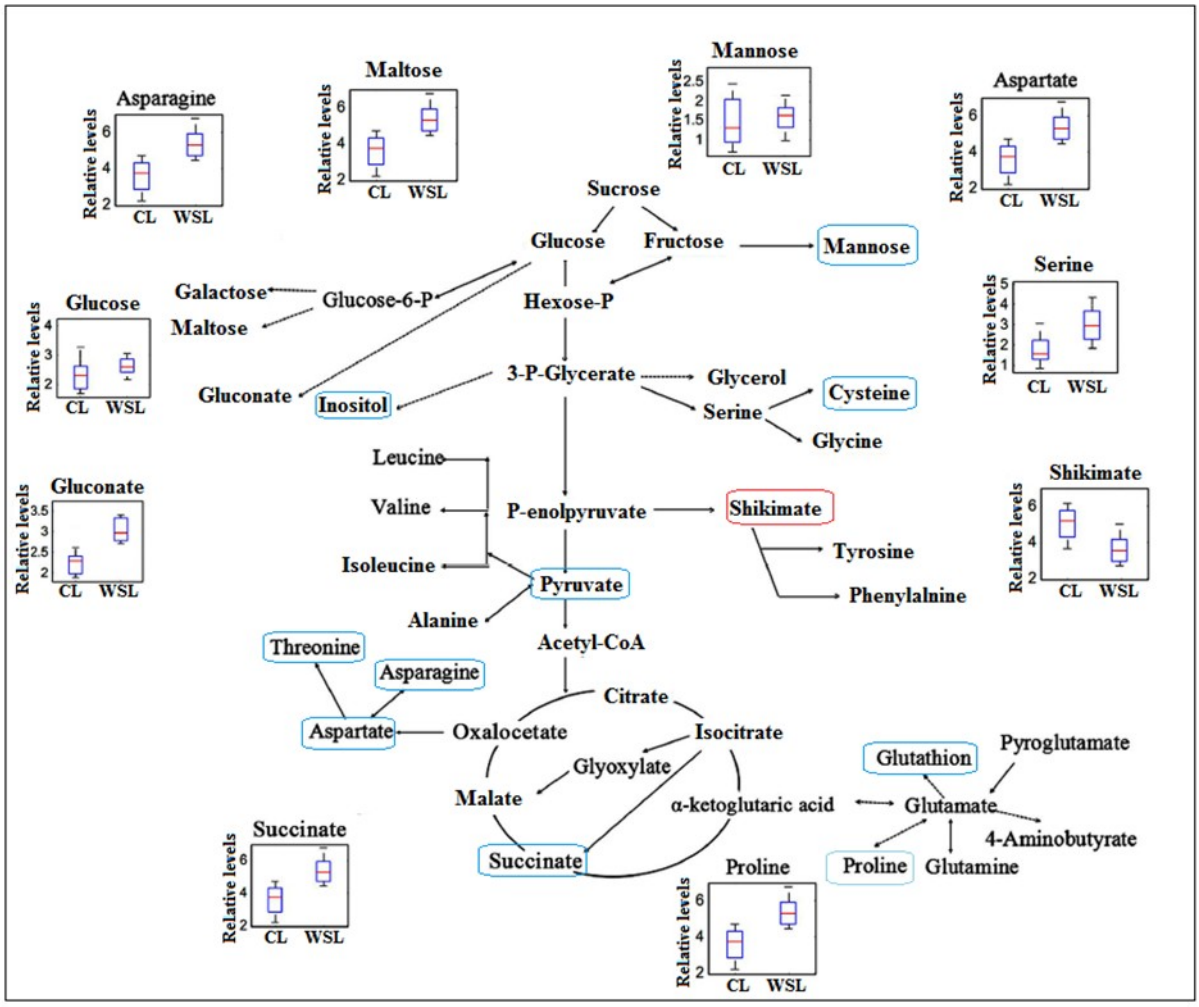

Figure 10 Metabolites involved in the primary pathways in leaves of Triticeae and its wild relatives under drought stress. The significant (P<0.05) and highly significant (P<0.01) up-regulated metabolites were indicated in red and blue circles, respectively.

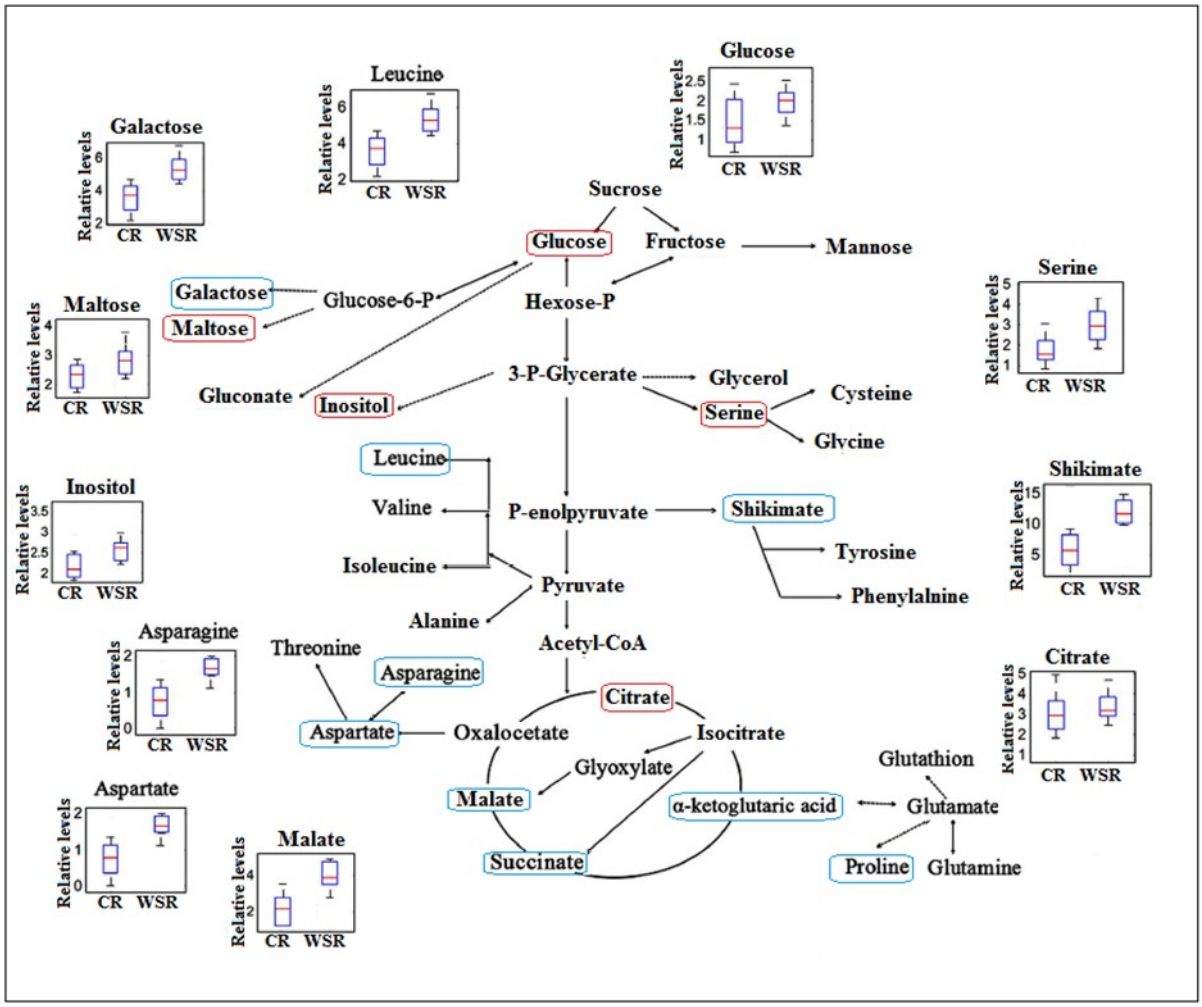

Figure 11 Root metabolites involved in the primary pathways in Triticeae under drought stress. The significantly (P<0.05) increased metabolites are represented by red circles and the metabolites with highly significant (P<0.01) up-regulation are encircled in blue boxes.

The other two most important drought stress specific metabolites selected were aspartate and trehalose. During drought stress, aspartate transaminase enzyme was found to be responsible for the biosynthesis of aspartate from glutamate. Our previous

studies indicates that aspartate transaminase belongs to a multi-gene family of which different homologous chromosomes (1, 3 and 5) contain almost six copies of these genes instead of each copy present on 3AS and 3DS (Budak et al., 2013a; Lucas et al., 2011b; Akpinar et al., 2015).

A very simple biochemical pathway having three enzymatic steps control accumulation of trehalose and uridine diphosphate glucose (UDP-glucose) and glucose-6-phosphate acts as substrates for the conversion to trehalose through trehalose-6-phosphate phosphatase (T6PP) activity and further, trehalose, an enzyme converts trehalose molecules into two glucose molecules (Figure 14). An additional enzyme involved in the biosynthesis of trehalose (not shown in the pathway) is trehalose-6-phosphate synthase (T6PS).

The putative wheat genes encoding all enzymes involved in both biochemical pathwayswere identified in the WGSS. For comparative purposes, the identification of wheat cDNAs encoding aspartate transaminase, T6PP, T6PS and trehalase were performed. Analysis of the draft wheat genome sequence revealed different copy numbers of an above-mentioned enzyme related genes on the long and short arms of different chromosomes of TR39477 and other drought stress tolerant genotypes.

Of the TTD-22 and Tosunbey that lacked the drought, stress-related sequences showed the expected decrease in metabolite levels (Budak et al., 2013a; Lucas et al., 2011b; Akpinar et al., 2015).

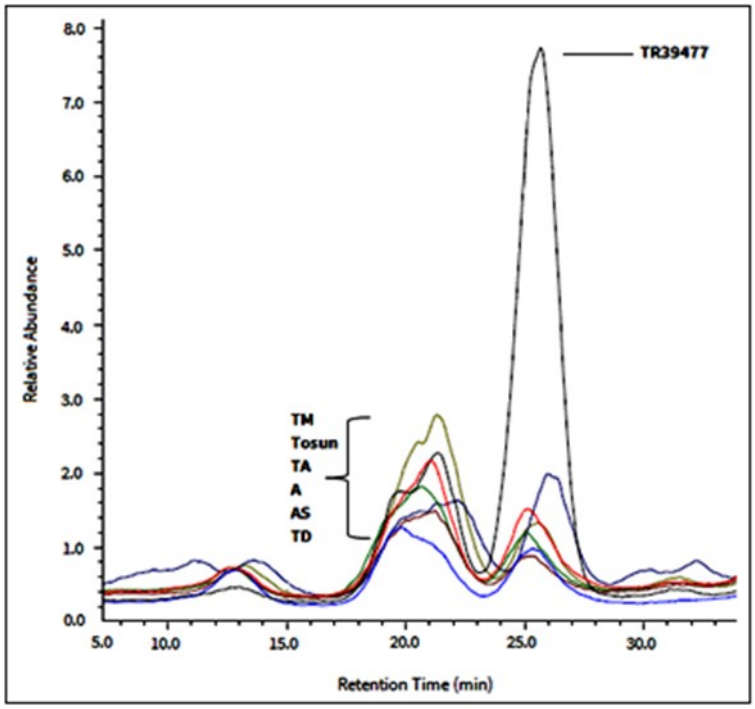

Figure 12 Total ion Chromatograms (TICs) for the comparison of Triticeae on the basis of succinate accumulation after the drought stress treatment (30% Water holding capacity).

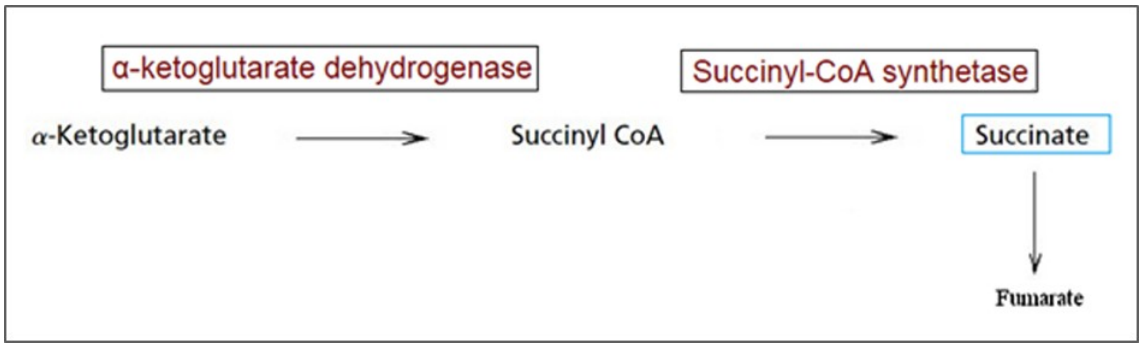

Figure 13 Schematic diagram of the biochemical pathway for succinate (succinic acid) accumulation. Blue box highlights succinate detected in the untargeted metabolite analysis. Enzyme names are indicated in brown colour.

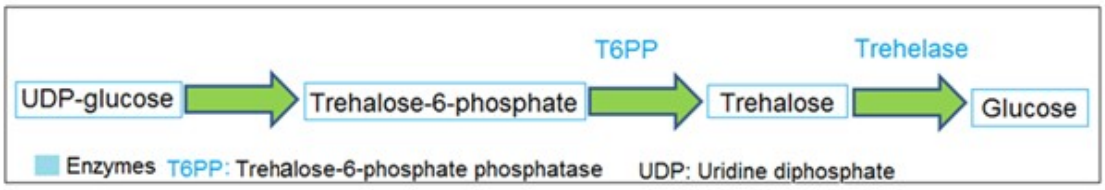

Figure 14 Flow chart showing the biochemical pathway for trehalose synthesis.

CHAPTER NO.5

5.1. Discussion

The development of drought stress-tolerant crops seems to be the only promising solution to increase wheat crop yield under water-limited conditions, especially to fulfil the food requirement for increasing animal and human population (Akpinar et al., 2012; Budak et al., 2015a, 2015b, 2013b). We have investigated a spectrum of morphological and metabolic responses from different Triticeae species grown under drought stress condition and standard condition with a regular supply of water. Increased metabolic levels of the selected metabolites were correlated with potential biochemical pathways, enzymes or gene locations for a better evaluation of the experimental results.

Roots are the first site to come into contact with water deficiency and therefore should be the place to trigger a response to the drought stress, yet there is no genetic explanation for the adaptive response of root under drought stress. Few studies documented the root response to the drought stress in different plant species .For instance, *Arabidopsis thaliana* root hairs became short and swollen in response to the water deficiency (Xiong et al., 2006; Schnall and Quatrano, 1992), whereas the presence of very short and hairless root development under drought stress was also reported in soil-grown *A. thaliana* (Vartanian et al., 1994). Other studies indicated the drought stress response in roots of citrus plants (Zaher-Ara et al., 2016), *Zea maize*(Jiang et al., 2012), and sunflower (Rauf and Sadaqat, 2007).

In this study, TR39477, IG132864, and Bolal represented significant tolerance to the drought by elongating the root length deep into the soil in search of water while keeping their surface area large and average diameter short to absorb and store water. Among all genotypes, TR39477 (wild emmer) (Akpinar et al., 2015; Budak et al., 2013a) had presented strong tolerance-associated morphological dynamics. Several studies with different plants have also shown the inhibition of lateral roots after drought stress for the purpose to go deeper to take water instead of spreading horizontally in the

soil (Xiong et al., 2006). The ability of plants to access water from depths through vertical root growth has been found beneficial for crop productivity under water deficiency (Comas et al., 2013).

Observation of the significantly altered metabolites accumulated upon drought stress was monitored with a non-targeted metabolite profiling analysis in Triticeae species using GC-MS technique. The most significant changes were observed in metabolites in the form of amino acid, organic acid, and sugars, of which approximately half increased statistically in TR39477, IG132864, and Bolal samples. TR39477, IG132864, and Bolal were found to be more tolerant against severe drought stress (30% WHC) by accumulating proline, trehalose, glycine and some other amino acids, considered as drought stress-specific markers and osmoprotectants.

The increased accumulation of these metabolites was reported in other studies conducted on different plant species in which these metabolites were found responsible for drought stress tolerance and had an osmoprotective function (Sanchez et al., 2012; Norouzi et al., 2008; Charlton et al., 2008; Rampino et al., 2006; Boyer et al., 2008; Nanjo et al., 1999; Redillas et al., 2012; Guimarães et al., 2008; Witt et al., 2012). Proline accumulation functions as an electron sink mechanism can reduce the amount of singlet oxygen present, which causes lipid peroxidation of thylakoid membranes, providing evidence that it is a significant contributor to cellular redox balance (Alia et al., 1997; Szabados and Savouré, 2010; Sharma and Dietz, 2006).

The branch chain amino acids such leucine, valine, alanine also increased significantly in TR39477, IG132864 and Bolal samples unlike to other genotypes under drought stress exposure. The increased accumulation of these branch chain amino acids was also reported in previous studies on Arabidopsis (Urano et al., 2009; Rizhsky et al., 2004; Taylor et al., 2004; Malatrasi et al., 2006). Less and Galili (2008) reported that catabolic enzymes of amino acids increase rapidly in response to drought stress and have an important role in amino acid metabolism under drought stress conditions (Less and Galili, 2008).

On the other side, sugar and its derivatives such as galactose, mannose, fructose, mannitol and other non-reducing sugars and oligosaccharides provide a hydration shell around proteins under drought stress (Hoekstra et al., 2001). The increase in the amount of these sugars may provide an initial defensive state against further water loss.

Succinic acid or succinate is the basic, intermediate component of ATP pathway, the citric acid cycle (Krebs cycle), which plays a vital role in energy production and involve in the regulation of mitochondrial tricarboxylic acid cycle (TCA cycle) (Cavalcanti et al., 2014). The overproduction of NADH under drought stress inhibits all dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and citrate synthase) except the succinate dehydrogenase in TCA cycle which converts succinyl-CoA to succinate (Tretter and Adam-Vizi, 2005). By over synthesis of succinate, mitochondria get more ATPs and store them for unfavourable conditions (Oestreicher et al., 1973).

The elevated level of succinate found in wild emmer genotype, TR39477 which is characterized by its high tolerance against drought stress (Kantar et al., 2011b; Ergen and Budak, 2009; Akpinar et al., 2015; Budak et al., 2013a), might be related to the efficient use of TCA cycle to produce more energy (ATPs) under water-limited conditions.

Succinic acid (succinate), trehalose and aspartic acid (aspartate) were selected for further genome analysis because of their potential involvement in biochemical pathways linked to drought stress specific response (Jain, 2013; Golldack et al., 2014). Our main focus for genomic analysis were drought stress tolerant genotypes, most specifically TR39477 due to the higher elevation of succinate level.

The results of genome analyses demonstrated that droughts stress tolerant wheat genotypes might be worthy of endorsing gene-to-metabolite networks. Therefore the alteration in metabolic levels in sensitive and drought stress tolerant genotypes under control and drought stress conditions can be attributed to gene suppression or overexpression from the related chromosome arms. Of the wheat genotypes that lacked the succinyl CoA-related sequences, TTD-22 and Tosunbey were among the wheat genotypes which showed a decrease in the level of metabolites, suggest that succinyl-CoA synthetase on 1AS, 5AL, 6BL, and 3DL might be a rate-limiting step in succinate accumulation.

However, the near to absent succinate level did not show a similar effect in *Triticum aestivum ssp. aestivum* (Tosunbey), *Aegilops speltoides ssp. speltoids* (Ligustica) and *Aegilops tauschii ssp. tauschii* (Meyeri), indicating that succinyl-CoA synthetase genes might be playing a different role rather succinate biosynthesis. A 3-

fold increase in the accumulation of succinate in wild emmer wheat (TR39477) indicated that some unknown genes from primary biochemical pathways were regulating the accumulation of succinate in wheat.

The metabolism of trehalose accumulation was controlled by post-translational modification pathways and regulatory networks (Ramakrishna and Ravishankar, 2011). Therefore, it is suggested that pathway-specific genes might be located on 1AL, 1BL 1DL, 3BL, 3DL, 5AS, 6AL, 6BL and 6DL which were involved in the up-regulation of trehalose in TR39477. As discussed previously in the literature, the proteomic (Budak et al., 2013a) and transcriptomic (Akpinar et al., 2015) analyses of these cultivars have identified candidate genes for the genetic manipulation of wheat cultivars in order to enhance drought stress tolerance, and the metabolite data further validate these results.

5.2. Conclusion

Drought stress affects the structure of plant cells and tissues. Hence a comprehensive omics approaches (genomics, transcriptomics, proteomics, and metabolomics) will enhance our understanding of the underlying mechanisms of water deficiency in Triticeae, which will in turn help breeders to identify the responsive genes, proteins, metabolites for drought stress tolerance. This study indicated that drought stress treated leaves and roots of wheat and its wild genotypes have distinct mechanisms of metabolite accumulation and regulation, which is valuable for the better understanding of overall abiotic stress tolerance mechanisms.

Triticeae species with high crop yields under the drought stress are expected to be developed in the future through the genetic transformation of novel genes identified in large-scale studies including metabolomics research.

APPENDIX A

Chemicals and Enzymes

6X DNA loading dye	Thermo Scientific	R0611
Absolute ethanol	Riedel de Haen	32221
Agarose	PRONA	8016
Ampicillin	Sigma	A9393
Boric acid	Sigma	B6768
Calcium chloride ($CaCl_2$)	Sigma	B6768
Calcium nitrate ($Ca(NO_3)_2$)	Sigma-Aldrich	237124
Chloroform	Merck	102.445
EDTA iron (III) sodium salt	Sigma-Aldrich	E6760
Ethidium bromide	Applichem	A1151
Ethylenediaminetatraaceticacid (EDTA)	Calbiochem	324503
Hoagland's No.2 basal salt mixture	Sigma-Aldrich	H2395
Isopropanol	Merck	1.09634
Magnesium chloride ($MgCl_2$)	Fluka	63063
Nuclease free water	Qiagen	129114
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich	P0662
Potassium sulfate (K_2SO_4)	Sigma-Aldrich	P0772
Sodium acetate (CH3COONa)	Sigma-Aldrich	S2889
Sodium hypochlorite (NaClO)	Sigma-Aldrich	425044
Taq DNA polymerase (recombinant)	Thermo Scientific	EP0401
Zinc sulfate ($ZnSO_4$)	Sigma-Aldrich	96495

APPENDIX B

Equipments

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Nüve 0T 032, TÜRKİYE
Balance:	Sartorius, BP221S, GERMANY
	Schimadzu, Libror EB-3 200 HU, JAPAN
Camera	Olympus C-7070, JAPAN
Centrifuge:	Microfuge 18 Centrifuge Beckman Coulter, USA
	Kendro Lab. Prod., Heraeus Multifuge 3S-R, GERMANY
	Kendro Lab. Prod., Sorvall RC5C Plus, USA
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Deepfreeze:	-20 ºC Bosch, TURKEY
	-80 ºC Thermo electron corporation, USA
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Labnet Gel XL Ultra V-2, USA
	Biogen Inc., USA
	Biorad Inc., USA
Fiter papers:	Whatman General Purpose Filtration Paper WHASE1141,
	Sigma, MO, USA
Gel Documentation:	Biorad Universal Hood II F1-F2 Fuses Type T2A, USA
	Biorad, UV-Transilluminator 2000, USA
Glassine crossing bags:	Focus Packaging & Design Ltd, North Lincolnshire, UK
Growth chamber:	Digitech DG12, Ankara, TURKEY
Heating block:	HDV Life Sciences, AUSTRIA
	Thermostat Bio TDB-100, LATVIA
Hydroponic tanks:	GroWell, UK

Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Innova 4330, USA
	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Laminar Flow:	Holten LaminAir Model 1.8 82034000, DENMARK
	Heraeus, Modell HS 12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ITALY
Microarray:	Custom-made by LC Sciences, Houston, TX, USA
Microarray analysis:	GenePix 4000B Microarray Scanner, Axon Instruments, USA
	Array-Pro™ Analyzer, Media Cybernetics, Silver Spring, MD, USA
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microscope	Olympus SZ61, JAPAN
	Olympus LG-PS2, JAPAN
Microwave digestion:	CEM-MARS Xpress system, USA
Microwave Oven:	Bosh, TÜRKİYE
Nitrogen tanks:	Linde Industrial Gases, TURKEY
Oven:	Memmert D06062 Modell 600, GERMANY
pH Meter:	WTW, pH540, GLP MultiCal, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
Real-Time PCR:	iCycler iQ Multi Color Real Time PCR Detection System, Bio-Rad, USA
Refrigerator:	+4 ºC Bosh, TÜRKİYE
Sequencer:	Roche 454 GS FLX Sequencer, Basel, SWITZERLAND
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., InnovaTM 4330, USA
	New Brunswick Scientific Excells E24, USA
Spectrophotometer:	Amersham Biosciences Ultraspec 2100 pro, USA
	Nanodrop, ND-1000, USA
Sterilizer:	Steri 350, Simon Keller Ltd., SWITZERLAND
Thermocyler:	Eppendorf, Mastercycler Gradient, GERMANY

	Biorad Gradient Cycler DNA Engine, USA
Tissue Lyser:	Qiagen Retsch, USA
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Vortex Mixer:	VELP Scientifica 2X3, ITALY

Table 1 List of Triticeae species used in our study.

Species	Common Name	Genome (s)	Genotype	Abbreviations	
				Drought	Control
Aegilops speltoides ssp. speltoides	Wild einkorn	BB	Ligustica	AS	ASC
Aegilops tauschii ssp. tauschii	Wild einkorn	DD	Meyeri	A	AC
Triticum turgidum ssp. dicoccoides	Wild emmer	AABB	TR39477	TR	TRC
Triticum turgidum ssp. dicoccoides	Wild emmer	AABB	TTD-22	TD	TDC
T. monococcum ssp. monococcum	Einkorn Domesticated	AA	IG132864	TM	TMC
Triticum aestivum ssp. aestivum	Bread wheat	AABBDD	Bolal	TA	TAC
Triticum aestivum ssp. aestivum	Bread wheat	AABBDD	Tosunbey	Tosun	TosunC

Table 2 Identified water-stress responsive metabolites in leaf samples of wheat using the GC-MS

m/z	RT (min)	SI (%)	Metabolite	DSL-Average	CL-average	SDE.DSL	SDE.CL	VIP	Fold change	T-test
132.0205	5.499	96	Sucrose	251431.2845	390449.1170	10 35 47.26 56	51986.2213	1.752	2.5124	0.0069
101.7112	6.234	89	Trehalose	25964.1344	90523.4619	70 46 6.3 13 8	22455.3902	1.359	3.5821	0.0018
116.0193	6.776	99	Glucose	5591221.797	10130466.42	50 45 29	5045293.597	0.5704	0.9179	0.0011

						3.597				
219.9998	8.599	93	Maltose	409006.3770	541189.2128	4925 2.417 4	4925 2.4174	1.5704	1.9059	0.0011
255.9982	9.572	95	Proline	66222.9467	113220.9117	3940 3.234 2	17961.5473	1.321	3.9542	0.0026
66.0865	10.433	92	Glutamate	104787.8757	165384.8123	6064 9.205 5	35147.9899	1.1819	1.1574	0.0374
132.9994	11.688	99	Malonic acid	2622465.079	3239226.214	2271 92.27 93	227192.2793	0.2131	2.3454	0.0256
73.0719	13.533	94	Glycine	15308773.50	21576528.21	5291 598.252	1821437.269	1.4334	1.3621	0.0008
219.0470	14.397	95	Asparagine	272193.0932	428909.0846	1418 86.45 77	79207.2451	1.2545	1.3501	0.0027
358.9890	15.033	96	Methionine	181826.9050	309026.2336	1022 79.51 46	74211.3542	1.2879	2.1841	0.0176
393.99	15.642	98	Oleic acid	116696.791	247305.41	1173	75224.51	0.54	-1.14	0.0195

73				0	45	73.1155	14	09	14	
372.9999	16.085	99	Ascorbic acid	892584.5688	1727014.140	383612.8697	398008.6959	0.5939	-1.0944	0.0015
433.9814	17.066	98	Homocysteine	23664.3172	36203.5948	10026.7400	7955.5609	1.2974	1.3052	0.0199
465.0958	18.061	99	Serine	99669.7410	17378.6878	153353.7229	9805.3277	1.3457	0.9621	0.0069
307.1537	18.756	97	Lysine	222271193.82	146100888.56	662105 0.428	1041945.072	0.7221	-2.1965	0.0001
87.0059	19.967	91	Leucine	536858.6475	356788.9754	90358.2018	60873.7700	0.4523	-2.6475	0.0161
319.1864	20.393	99	Succinate	43354641.49	36692643.80	4508559.663	100392.6735	1.2116	1.2954	0.0338
424.0809	21.032	89	Phthalic acid	5382.4716	1205.0130	821.7256	821.7256	0.2974	1.3052	0.0199
319.11	21.413	88	Aspartate (Aspartic	2304711.31	3300932.3	2817	2817 55.3	1.54	2.1965	0.0001

01			acid)	9	50	55.353	53	8		
337.1527	22.926	98	Mannose	59060.7969	40803.9515	1131 4.0262	1426 1.8330	0.4334	1.3621	0.0108
79.0102	23.467	99	Glyceric acid	625910.6571	477717.6815	8589 4.0185	116963.662	0.2545	1.3501	0.0227
132.0205	24.940	96	Threonine	251431.2845	390449.1170	1035 47.2656	51986.2213	1.2131	2.3454	0.0056
101.7112	26.330	95	Phenylalanine	25964.1344	90523.4619	7046 6.3138	22455.3902	1.821	2.1414	0.0012
116.0193	27.053	98	α-ketoglutaric acid	5591221.797	101304 66.42	5045 293.597	5045293.59	0.263	-0.835	0.0453
219.9998	28.165	95	Inositol	409006.3770	541189.2128	4925 2.4174	4925 2.4174	0.321	0.9542	0.0126
255.9982	28.929	96	Galactose	66222.9467	113220.9117	3940 3.2342	17961.5473	0.1819	1.1574	0.0374
66.	29.9	98	Fructose	10478	1653	60	3514	0.	1.31	0.0

0865	97			7.8757	84.8123	649.2055	7.9899	3418	14	077
132.9994	31.367	99	Alanine	2622465.079	3239226.214	2271 92.2793	2271 92.279	1.3418	1.3114	0.0077
73.0719	32.270	98	Tyrosine	15308773.50	21576528.21	5291598.252	1821437.26	1.5939	1.0944	0.0015
219.0470	32.867	99	Quinic acid	272193.0932	428909.0846	1418 86.4577	7920 7.2451	0.2879	2.1841	0.0176
358.9890	33.300	97	Citrate (Citric acid)	181826.9050	309026.2336	1022 79.5146	7421 1.3542	1.6205	1.6365	0.0003
393.9973	33.430	98	Oxalic acid	116696.7910	247305.4145	1173 73.1155	7522 4.5114	0.5243	2.1414	0.0012
372.9999	33.626	95	Gluconate	892584.5688	1727014.140	3836 12.8697	3980 08.695	1.4523	2.6475	0.0161
433.9814	34.689	89	Pimelic acid	23664.3172	36203.5948	1002 6.740	7955 .5609	0.3457	-0.9621	0.0069

						0				
465.0958	35.012	99	Mannitol	99669.7410	17378.6878	1533.57229	9805.3277	1.263	1.835	0.0453
307.1537	35.233	93	Fumeric acid	22227193.82	14610088.56	662105 0.428	1041945.07	0.2116	-1.2954	0.0338
87.0059	35.427	95	Mandelic acid	536858.6475	356788.9754	90358.2018	60873.7700	0.6205	-1.6365	0.0003
319.1864	36.069	92	Valine	43354641.49	36692643.80	4508559.663	100392.6735	1.5409	1.1414	0.0095
424.0809	36.299	99	Cysteine	5382.4716	1205.0130	821.7256	821.7256	0.3901	-1.3912	0.0144
319.1101	36.435	94	Shikimic acid	2304711.319	3300932.350	281755.3536	281755.3536	0.7221	-2.1965	0.0001
337.1527	38.628	97	Glutathione	59060.7969	40803.9515	113140.2622	14261.8330	1.3901	1.3912	0.0044
79.0102	38.867	92	ɣ-Aminobutyric acid (GABA)	625910.6571	477717.6815	858940.018	116963.6621	0.2767	-0.5124	0.0069

						5				
319.1101	39.064	94	Pyruvate	181826.9050	309026.2336	1022 79.5146	74211.3542	0.1003	-0.7251	0.0377
337.1527	39.471	81	Malic acid	116696.7910	247305.4145	1173 73.1155	75224.5114	0.4523	-2.6475	0.0161
79.0102	39.793	83	Adipic acid	892584.5688	1727014.140	3836 12.8697	398008.6959	0.3901	-1.3912	0.0144

Table 3 Identified water-stress responsive metabolites in root samples from the GC-MS.

Species	**Treatment**	**ARA**	**ARW**	**ARH**	**RL**	**PA**	**SA**	**AvD**	**LPV**	**RV**	**Tips**	**T-test**
Aegilops speltoides ssp. speltoids	Control	54.804	36.363	94.548	32.47	50.243	84.446	10.156	24.557	21.707	32	0.016
	20% PEG	49.669	77.845	72.183	51.52	54.390	109.268	23.823	83.172	40.058	18	0.001
Aegilops tauschii ssp. tauschii	Control	60.208	22.768	11.675	32.17	45.410	71.558	39.461	39.999	10.626	31	0.014
	20% PEG	10.726	27.151	21.179	18.97	42.717	44.338	60.140	14.105	34.592	20	0.010
Triticu	Contr	77.	10.	12.	33.	60.	81.2	17.	42.	14.	1	0.0

m	ol	340	513	394	64	052	76	742	182	588	1	12
turgidum ssp. dicoccoides	20% PEG	10.035	45.464	93.878	54.12	80.979	119.206	13.932	60.362	17.134	10	0.037
Triticum turgidum ssp. dicoccoides	Control	15.222	43.817	14.442	32.99	43.784	61.642	98.228	29.642	13.845	62	0.017
	20% PEG	41.791	82.788	78.486	11.70	23.051	36.368	68.957	19.102	33.781	25	0.010
T. monococcum ssp. monococcum	Control	82.807	97.626	13.706	31.76	47.232	81.539	19.972	98.725	88.597	25	0.022
	20% PEG	88.676	38.592	14.976	16.78	62.258	28.376	10.625	28.842	25.167	30	0.012
Triticum aestivum ssp. aestivum	Control	19.716	21.787	36.322	37.97	94.239	76.419	23.606	46.977	34.526	13	0.025
	20% PEG	70.981	10.325	12.284	61.63	38.028	120.378	48.782	18.048	25.518	27	0.001
Triticum aestivum ssp. aestivum	Control	40.857	40.949	61.472	32.21	51.929	71.461	12.127	31.445	88.597	18	0.011
	20% PEG	52.966	18.525	10.709	14.75	74.338	53.381	19.972	11.044	25.167	22	0.061

Table 4 Morphological parameters of roots of all genotypes measured by WinRHIZO system. ARA (cm^2) Analysed Region Area (cm^2); ARW (cm) Analysed Region Width (cm); ARH (cm) Analysed Region Height (cm); RL (cm) Root Length (cm); PA (cm^2) Projected Area (cm^2); SA (cm^2) Surface Area (cm^2); AvD (mm) Avg Diameter (mm); LPV (cm/m^3) Length per Volume (cm/m^3); RV (cm^3) Root Volume (cm^3).

m/z	RT (min)	SI (%)	Metabolite	DSR-aveage	CR-aveage	SDE.DSR	SDE.CR	VIP	Fold change	T-test
132.0205	5.504	96	Sucrose	1321775.27	390449.12	127883.36	51986.22	1.10	3.51	0.01
101.7112	6.241	95	Trehalose	20693.48	90523.46	25373.72	22455.39	1.37	2.58	0.00
116.0193	8.854	94	Glucose	3968285.96	10130466.42	3710097.47	5045293.60	0.58	-0.92	0.01
219.9998	10.140	95	Maltose	317053.95	541189.21	250371.68	49252.42	1.59	2.92	0.00
255.9982	11.360	96	Proline	43128.38	113220.91	29605.79	17961.55	1.33	3.95	0.02
114.0049	12.201	98	Glutamate	112089.62	243641.28	130356.50	35147.99	1.15	2.16	0.00
132.9994	14.200	99	Malonic acid	1816819.88	3239226.21	982936.81	227192.28	0.37	-2.11	0.03
73.0719	15.187	98	Glycine	11263933.05	21576528.21	6131715.67	1821437.27	1.26	3.36	0.01
158.0967	16.093	99	Asparagine	6756505.08	2175441.83	1018227.63	1608227.02	1.01	4.35	0.00

142.0977	18,610	97	Methionine	2762313.35	11664490.61	2695770.93	8309725.61	1.06	1.18	0.02
393.9973	20.392	96	Oleic acid	12241.74	247305.41	24404.78	117373.12	0.56	1.14	0.02
100.9987	21.000	95	Ascorbic acid	128052.78	445599.61	174469.43	377787.57	0.97	1.09	0.00
372.9999	21,407	96	Homocysteine	313390.60	172701414.14	277807.35	383612.87	1.02	3.31	0.02
152.9097	22.272	94	Serine	42275.50	69213.73	23054.00	20014.46	1.11	3.96	0.01
182.9957	24.518	93	Lysine	74527.96	156391.99	52675.11	76181.02	0.66	2.20	0.00
260.0053	25.451	98	Leucine	43756.75	107924.65	46282.75	67801.62	0.12	2.56	0.02
172.0850	26.136	93	Succinate	393737.40	786351.12	321374.31	342415.86	1.70	3.30	0.03
451.0000	26.903	92	Phthalic acid	132370.38	1181980.18	225439.02	99434.98	0.12	-1.31	0.02
595.0824	27.193	91	Aspartate (Aspartic acid)	7462.27	16839.37	6936.30	9805.33	1.86	2.15	0.00
173.0187	27.698	99	Mannose	1268376.95	2779319.56	579978.06	1041945.07	0.90	-1.36	0.01
227.9750	28.171	89	Glyceric acid	114902.08	183897.79	53732.92	60873.77	0.46	-1.35	0.02
132.0205	28.629	88	Threonine	132175.27	390449.12	127883.36	51986.22	1.98	3.35	0.01
101.7112	29.179	98	Phenylalanine	20693.48	90523.46	25373.72	22455.39	1.40	2.15	0.00
116.0193	30.004	99	α-ketogluteric acid	3968285.96	10130466.42	3710097.47	5045293.60	0.85	0.84	0.05
219.9998	31.423	96	Inositol	317053.95	541189.21	250371.68	49252.42	0.74	-0.95	0.00
255.	31.7	95	Galactose	43128	11322	2960	1796	0.	-1.16	0.

998 2	94			.38	0.91	5.79	1.55	3 2		0 4
114. 004 9	32.1 14	98	Fructose	11208 9.62	24364 1.28	1303 56.50	3514 7.99	0. 6 5	-1.23	0. 0 1
132. 999 4	32.5 63	98	Alanine	18168 19.88	32392 26.21	9829 36.81	2271 92.28	1. 7 5	2.31	0. 0 1
73.0 719	32.7 82	97	Tyrosine	11263 933.0 5	21576 528.2 1	6131 715.6 7	1821 437.2 7	1. 9 5	3.09	0. 0 0
158. 096 7	33.1 96	96	Quinic acid	67565 5.08	21754 41.83	1018 227.6 3	1608 227.0 2	0. 7 2	-2.18	0. 0 2
142. 097 7	33.4 33	88	Citrate (Citric acid)	27623 13.35	11664 490.6 1	2695 770.9 3	8309 725.6 1	1. 9 7	1.64	0. 0 0
393. 997 3	33.6 35	96	Oxalic acid	12241 .74	24730 5.41	2440 4.78	1173 73.12	0. 5 6	-2.14	0. 0 0
100. 998 7	34.0 09	95	Gluconate	12805 2.78	44559 9.61	1744 69.43	3777 87.57	1. 4 5	3.65	0. 0 1
372. 999 9	34.3 06	94	Pimelic acid	31339 0.60	17270 14.14	2778 07.35	3836 12.87	0. 0 1	0.13	0. 0 1
152. 909 7	34.6 99	95	Mannitol	42275 .50	69213 .73	2305 4.00	2001 4.46	1. 8 9	2.84	0. 0 1
182. 995 7	34.9 59	96	Fumeric acid	74527 .96	15639 1.99	5267 5.11	7618 1.02	0. 7 5	1.26	0. 0 3
260. 005 3	35.2 01	98	Mandelic acid	43756 .75	10792 4.65	4628 2.75	6780 1.62	0. 5 6	1.64	0. 0 0
172. 085 0	35.5 67	99	Valine	39373 7.40	78635 1.12	3213 74.31	3424 15.86	1. 8 9	3.14	0. 0 0
451. 000 0	37.8 74	98	Cysteine	13237 0.38	11819 80.18	2254 39.02	9943 34.98	0. 3 4	1.65	0. 0 2
595. 082 4	38.2 33	99	Shikimic acid	7462. 27	16839 .37	6936. 30	9805. 33	0. 2 2	1.20	0. 0 0
173. 018 7	38.3 68	97	Glutathione	12683 76.95	27793 19.56	5799 78.06	1041 945.0 7	1. 8 5	2.39	0. 0 0
227. 975	38.5 42	96	ɣ-Aminobutyric acid (GABA)	11490 2.08	18389 7.79	5373 2.92	6087 3.77	0. 5	0.75	0. 0

0								6		0
595.0824	38.839	95	Pyruvate	7462.27	16839.37	6936.30	9805.33	0.19	0.34	0.03
173.0187	39.067	96	Malic acid	1268376.95	2779319.56	579978.06	1041945.07	0.70	1.89	0.02
227.9750	39.797	89	Adipic acid	114902.08	183897.79	53732.92	60873.77	0.23	2.85	0.01

Table 5 Names of 45 significantly altered and differentially accumulated metabolites detected and identified by GC-MS from leaf and root tissue samples from all seven genotypes.

Adipic acid	Phenylalanine	Glutamate
Asparagine	Pimelic acid	Glyceric acid
Aspartic acid (aspartate)	Quinic acid	Leucine
Ascorbic acid	Shikimic acid	Glucose
Gluconate (Gluconic acid)	Succinic acid (Succinate)	Methionine
Pyruvate	Trehalose	Glycine
Homocysteine	Alanine	Oleic acid
Inositol	Citric acid (Citrate)	Oxalic acid
Lysine	Cysteine	Proline
Malic acid	D-mannose	Glutathione
Malonic acid	α-ketogluteric acid	Serine
Maltose	Fructose	Mannitol
Mandelic acid	Fumaric acid	Threonine
γ-Aminobutyric acid (GABA)	Galactose	Tyrosine
Phthalic acid	Sucrose	Valine

Table 6 Explanation and predictability values used for different analysis such as CL-DSL-CR-DSR were analyzed using the drought stress treated leaves and roots as well as their controls; CL-DSL, analyzed using the control and drought stress treated leaf samples; CR-DSR, analyzed using the control and drought stress treated root samples; DSL-DSR, analyzed using the drought stress treated leaf and root samples; CL-CR, analyzed using control leaf and root samples.

		CL-DSL-CR-DSR	**CL-DSL**	**CR-DSR**	**DSL-DSR**	**CL-CR**
PCA	R^2X	0.712	0.691	0.689	0.662	0.651
	Q^2	0.426	0.382	0.452	0.469	0.472
PLS-DA	R^2X	0.522	0.412	0.685	0.656	0.694
	R^2Y	0.513	0.891	0.971	0.482	0.461
	Q^2	0.361	0.551	0.642	0.375	0.058

Table 7 Leaf metabolites, the fold changesx in the concentrations of each metabolite between CL and DSL groups using the formula log2(Drought treated/Control) and variable importance in the projection (VIP) of the typical/representative sample (TR39477). "*" and "**" indicate the significant ($P<0.05$) and highly significant ($P<0.01$) differences compared to the control, respectively.

Metabolite	VIP	Fold change[X]	T-test
Sucrose	1.752	2.5124	0.0069**
Trehalose	1.359	3.5821	0.0018**
Glucose	0.5704	0.9179	0.0011**
Maltose	1.5704	1.9059	0.0011**
Proline	1.3210	3.9542	0.0026**
Glutamate	1.1819	1.1574	0.0374*
Malonic acid	0.2131	2.3454	0.0256*
Glycine	1.4334	1.3621	0.0008**
Asparagine	1.2545	1.3501	0.0027**
Methionine	1.2879	2.1841	0.0176*
Oleic acid	0.5409	-1.1414	0.0195*
Ascorbic acid	0.5939	-1.0944	0.0015**
Homocysteine	1.2974	1.3052	0.0199*
Serine	1.3457	0.9621	0.0069**
Lysine	0.7221	-2.1965	0.0001**
Leucine	0.4523	-2.6475	0.0161*
Succinate	1.2116	1.2954	0.0338*
Phthalic acid	0.2974	1.3052	0.0199*
Aspartate (Aspartic acid)	1.5480	2.1965	0.0001**
Mannose	0.4334	1.3621	0.0108*
Glyceric acid	0.2545	1.3501	0.0227*
Threonine	1.2131	2.3454	0.0056**
Phenylalanine	1.8210	2.1414	0.0012**
α-ketogluteric acid	0.2630	-0.8350	0.0453*
Inositol	0.3210	0.9542	0.0126*
Galactose	0.1819	1.1574	0.0374*
Fructose	0.3418	1.3114	0.0077**
Alanine	1.3418	1.3114	0.0077**
Tyrosine	1.5939	1.0944	0.0015**
Quinic acid	0.2879	2.1841	0.0176*
Citrate (Citric acid)	1.6205	1.6365	0.0003**
Oxalic acid	0.5243	2.1414	0.0012**
Gluconate	1.4523	2.6475	0.0161*
Pimelic acid	0.3457	-0.9621	0.0069**
Mannitol	1.2630	1.8350	0.0453*
Fumaric acid	0.2116	-1.2954	0.0338*
Mandelic acid	0.6205	-1.6365	0.0003**
Valine	1.5409	1.1414	0.0095**
Cysteine	0.3901	-1.3912	0.0144*
Shikimic acid	0.7221	-2.1965	0.0001**
Glutathione	1.3901	1.3912	0.0044**
ɣ-Aminobutyric acid (GABA)	0.2767	-0.5124	0.0069**
Pyruvate	0.1003	-0.7251	0.0377*
Malic acid	0.4523	-2.6475	0.0161*
Adipic acid	0.3901	-1.3912	0.0144*

Table 8 Root metabolites, the fold changesx in the concentrations of each metabolite between CR and DSR groups using the formula log2 (Drought treated/Control) and variable importance in the projection (VIP) of the typical/representative sample (TR39477). "*" and "**" indicate the significance ($P<0.05$) and highly significance ($P<0.01$) level.

Metabolite	VIP	Fold change[x]	T-test
Sucrose	1.1042	3.5124	0.0091**
Trehalose	1.3656	2.5821	0.0025**
Glucose	0.5840	-0.9179	0.0089**
Maltose	1.5874	2.9179	0.0002**
Proline	1.3298	3.9542	0.0156*
Glutamate	1.1547	2.1574	0.0031**
Malonic acid	0.3652	-2.1148	0.0256*
Glycine	1.2578	3.3621	0.0088**
Asparagine	1.0058	4.3501	0.0037**
Methionine	1.0587	1.1841	0.0176*
Oleic acid	0.5587	1.1414	0.0185*
Ascorbic acid	0.9658	1.0944	0.0015**
Homocysteine	1.0174	3.3052	0.0199*
Serine	1.1145	3.9621	0.0069**
Lysine	0.6580	2.1965	0.0001**
Leucine	0.1178	2.5574	0.0161*
Succinate	1.6985	3.2954	0.0338*
Phthalic acid	0.1238	-1.3052	0.0199*
Aspartate (Aspartic acid)	1.8579	2.1487	0.0001**
Mannose	0.8974	-1.3621	0.0108*
Glyceric acid	0.4587	-1.3501	0.0227*
Threonine	1.9817	3.3454	0.0056**
Phenylalanine	1.3971	2.1458	0.0012**
α-ketogluteric acid	0.8529	0.8350	0.0453*
Inositol	0.7412	-0.9542	0.0026**
Galactose	0.3214	-1.1574	0.0374*
Fructose	0.6541	-1.2289	0.0077**
Alanine	1.7458	2.3114	0.0077**
Tyrosine	1.9531	3.0944	0.0015**
Quinic acid	0.7159	-2.1841	0.0176*
Citrate (Citric acid)	1.9657	1.6365	0.0003**
Oxalic acid	0.5558	-2.1414	0.0012**
Gluconate	1.4503	3.6475	0.0061**
Pimelic acid	0.0085	0.1289	0.0069**
Mannitol	1.8866	2.8350	0.0053**
Fumaric acid	0.7488	1.2598	0.0338*
Mandelic acid	0.5553	1.6365	0.0003**
Valine	1.8871	3.1414	0.0035**
Cysteine	0.3366	1.6524	0.0184*
Shikimic acid	0.2219	1.1965	0.0001**
Glutathione	1.8536	2.3912	0.0004**
ɣ-Aminobutyric acid (GABA)	0.5577	0.7452	0.0019**
Pyruvate	0.1854	0.3365	0.0274*
Malic acid	0.6974	1.8874	0.0178*
Adipic acid	0.2298	2.8547	0.0126*

Table 9 The KEGG pathways (R-software) of the altered metabolites exposure to drought stress in wheat leaves and root samples.

Label	P-value	Adjusted p-value	Background_count	Count	Compounds
Galactose metabolism	0.00	0.00	8	3	Glucose, galactose , maltose
Starch and sucrose metabolism	0.00	0.00	6	2	Sucrose, trehalose
Citrate cycle (TCA cycle)	0.01	0.04	9	7	Aspartate, mannose, serine, succinate, glutamate, proline, asparagine
Pentose phosphate pathway	0.02	0.07	5	3	Galactose, maltose, glucose-6-phosphate
Glycine, serine and threonine metabolism	0.04	0.11	8	2	Serine, threonine
Cysteine and methionine metabolism	0.05	0.13	9	2	Cysteine, methionine
Biosynthesis of phenylpropanoids	0.12	0.28	3	1	Phenylalanine
Biosynthesis of alkaloids derived from shikimate pathway	0.21	0.46	7	3	Shikimate, tyrosine, phenylalanine
Valine, leucine and isoleucine degradation	1.00	1.00	5	1	Leucine
Inositol phosphate metabolism	1.00	1.00	9	1	Inositol
Alanine, aspartate and glutamate metabolism	1.00	1.00	14	2	Aspartate, glutamate
Pyruvate metabolism	1.00	1.00	5	1	Pyruvate

Table 10 The changed genes and metabolites involved in gene-to-metabolite network upon water stress exposure in wheat.

Gene	Annotated	Gene annotation	Compound	Wheat	Wheat

ID	FL-cDNA		name	FL-cDNA	Chromosome locations
15223122	D83378	Aspartate transaminase	Aspartate	AK333183	1AL, 1BL, 1DL
				AY621539	5AL, 5BL, 5DL
				AK334107	5AL, 5BL, 5DL
				BT009245	5AL, 5BL, 5DL
				BT009049	3AS, 5BS, 3DS
15229223	AK331389	α-ketoglutarate dehydrogenase	Succinate (Succinic acid)	AK330986	1AL, 3BL, 5DL
15228368	AK331389	Succinyl CoA synthetase		BT009368	1AS, 5AL, 6BL, 3DL
15227257	AK103775	Trehalose-6-phosphate synthase	Trehalose	FJ167677	1AL, 1BL, 1DL, 5DL
				AK331389	1AL, 1BL, 1DL, 5DL
				FJ167677	1AL, 1BL, 1DL, 5DL
				AK331389	1AL, 1BL, 1DL, 5DL
				FJ167677	1AL, 1BL, 1DL, 5DL
				AK331389	1AL, 1BL, 1DL, 5DL
				FJ167677	1AL, 1BL, 1DL, 5BL
				AK331389	1AL, 1BL, 1DL, 5BL
22330456	AK072132	Trehalose-6-phosphate phosphatase		AK333853	1AL, 1BL, 1DL, 3AL, 3BL, 3DL
				AK334843	1AL, 1BL, 1DL, 5AS, 5BS, 5BL
				FN564426	1AL, 1BL, 1DL, 5AS, 5BS, 5BL
				AK332212	1AL, 1DL, 3AL, 3AL, 3BL, 3DL
				AK331757	1AL, 1BL, 1DL
				BT009244	6AL, 6BL, 6DL

22331857	AK108163	Trehalase		AK331310	1AL, 1BL, 1DL

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