ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PROTEOME ANALYSIS OF *BRACHYPODIUM DISTACHYON* LEAVES UNDER DROUGHT STRESS

M.Sc. THESIS

Özge TATLI

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Assoc. Prof. Dr. Gizem DİNLER DOĞANAY

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KURAKLIK STRESİ ALTINDA *BRACHYPODIUM DISTACHYON* YAPRAKLARININ PROTEOM ANALİZİ

YÜKSEK LİSANS TEZİ

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ARALIK 2015

Özge TATLI, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 521131129, successfully defended the **thesis** entitled "**PROTEOME ANALYSIS OF *BRACHYPODIUM DISTACHYON* LEAVES UNDER DROUGHT STRESS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Assoc. Prof. Dr. Gizem DİNLER DOĞANAY** Istanbul Technical University	……..
Jury Members :	**Assoc. Prof. Dr. Eda TAHİR TURANLI** Istanbul Technical University	.....
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Date of Submission : 27.11.2015
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To my lovely family,

FOREWORD

Above all, I would like to thank my supervisor Assoc. Prof. Dr. Gizem DİNLER DOĞANAY with all my heart for her valuable guidance and patience all along this study.

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December 2015 Özge TATLI

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ABREVIATIONS

2D : Two-dimensional
ALFC2 : Fructose bisphosphate aldolase
APS : Ammonium persulphate
APX : Ascorbate peroxidase
ATP : Adenosine triphosphate
BSA : Bovine serum albumin
CAHC : Carbonic anhydrase
CAT : Catalase
CBB : Coomassie brilliant blue
CHAPS : 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Cy : Cyanine
DEP : Differentially expressed protein
DHAR : Dihydroxy acetone kinase operon regulatory protein
DIGE : Difference gel electrophoresis
DMF : Dimethyl formamide
DTT : Dithiothreitol
DW : Dry weight
EDTA : Ethylenediaminetetraacetic acid
FAO : Food and agricultural organization
FW : Fresh weight
GAP : Glyceraldehyde-3-phosphate
GAPDH : Glyceraldehyde-3-phosphate dehydrogenase
GE : General electric
GO : Gene ontology
GST : Glutathione S-transferase
HCl : Hydrochloric acid
HSF : Heat shock factor
HSP : Heat-shock protein
IEF : Isoelectric focusing
IPG : Immobilized pH gradient
KCL : Potassium chloride
kDa : Kilodalton
KEGG : Kyoto Encyclopedia of Genes and Genomes
Lea : Late embryogenesis abundant protein
MALDI : Matrix-assisted laser desorption/ionization
Mn : Manganase
mRNA : Messenger ribonucleic acid
MS : Mass spectrometry
MW : Molecular weight
NADPH : Nicotinamide adenine dinucleotide phosphate
OsRMC : Rice root meander curling protein
pH : Power of hydrogen
pI : Isoelectric point
PAGE : Polyacrilamid gel electrophoresis

PMF **:** Peptide mass fingerprinting
PMSF **:** Phenylmethylsulfonyl fluoride
PsbO **:** Oxygen evolving enhancer protein 1
PsbP **:** Oxygen evolving enhancer protein 2
RBS2 **:** Rubisco small subunit
RCAB **:** Rubisco activase B
RNA **:** Ribonucleic acid
ROS **:** Reactive oxygen species
Rubisco **:** Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC **:** Relative water content
SDS **:** Sodium dodecyl sulphate
SHMT **:** Serine hydroxymethyltransferase
SOD **:** Superoxide dismutase
SSP **:** Standart spot numbers
TEMED **:** Tetramethylethylenediamine
TKTC **:** Transketolase
TOF **:** Time of flight
TW **:** Turgid weight
UniProt **:** Universal protein resource
USA **:** United States of America
ZmICDH **:** Maize Phosphate-dependent isocitrate dehydrogenase

SYMBOLS

CO_2	**:** Carbon dioxide
CO_3^{-2}	**:** Carbonate
g	**:** Gram
H_2O_2	**:** Peroxide
HCO_3	**:** Bicarbonate
L	**:** Litre
M	**:** Molar
O_2	**:** Oxygen
v	**:** Volume
w	**:** Weight
µ	**:** Micro

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PROTEOME ANALYSIS OF *BRACHYPODIUM DISTACHYON* LEAVES UNDER DROUGHT STRESS

ABSTRACT

Among the environmental stress factors, drought is the one that has the most severe effect on agricultural production. Hence, improving the yield under drought stress is one of the most important challenges in agriculture. Drought or water deficit has a direct impact on the cellular metabolism of plant. This impact significantly reduces the growth index and crop yield.

A number of studies highlighted the existence of a complex network in the cell upon drought stress. Changes in protein expression play the major role in this complex network. The main strategy to improve crop yield is to integrate all the properties that are required to sustain yield under drought stress and accumulate the most powerful genes and proteins in elite genotypes without any harmful effect on the potential crop yield. This approach will provide the improvement of new plant cultivars that are stable and exhibit high yield potential under drought conditions. In plants, there are number of studies on transcriptome level to elucidate the mechanisms underlying stress response. However, alterations in mRNA levels do not always correlate well with protein levels in the cell due to the post-transcriptional and post-translational modifications. Therefore, transcriptome studies are insufficient for understanding the complex network of stress response, causing a lack of knowledge for the enlightenment of the mechanisms related to stress response.

Brachypodium distachyon, model plant for monocot species, exhibits a close relationship to agriculturally and economically important crops. It is also known that *Brachypodium distachyon* displays high variation in the response against drought stress. This variation will potentially lead to identify new stress-responsive genes and thus proteins. Ongoing transcriptomic analyses in *Brachypodium distachyon* are available. However, requirement of proteomic analyses is an emerging subject for this plant species.

In this study, changes in protein repertoire of *Brachypodium distachyon* leaves after exposing individuals to time-dependent drought stress (4, 8 and 12 day application of drought) were described. Based on two-dimensional difference gel electrophoresis (2D-DIGE), constructed with 3 replicates, and combined mass spectrometry (MS) experiments, a proteomic approach was carried out for this purpose. The comparison of the resulting proteomic data highlighted differentially expressed proteins (DEPs) upon drought stress. 37 differentially expressed proteins were detected via statistical evaluation of relative spot densities and among these one, thirteen DEPs were identified by MS and classified according to their functions. The role and relation of DEPs in drought response of *Brachypodium distachyon* were discussed. The biological functions of DEPs included roles in photosynthesis, protein folding, antioxidant mechanism and metabolic processes. It has been highlighted that there is a significant degree of overlapping between metabolic alterations induced by drought stress. The identified proteins in this study could serve as potential biomarkers for the selection of drought-tolerant *Brachypodium distachyon* plants as well as its relatives like wheat, barley and rye. Furthermore, identified proteins will contribute on the studies on development of drought-resistant crop species such as wheat, oat and barley, which are close relatives of *Brachypodium distachyon*.

KURAKLIK STRESİ ALTINDA *BRACHYPODIUM DISTACHYON* YAPRAKLARININ PROTEOM ANALİZİ

ÖZET

Çevresel stres faktörleri arasında kuraklık tarımda en şiddetli etkiye sahip olan faktörler arasındadır ve buna bağlı olarak kuraklık stresi altında verimi arttırmak tarımda en önemli hedeflerden biri haline gelmiştir. Kuraklık ya da su yetersizliği bitkinin hücresel metabolizmasını doğrudan etkiler. Büyüme indisinde ve böylece verimde ciddi bir azalmaya yol açar. Pek çok çalışma, kuraklık stresine cevap sürecinde hücrede karmaşık bir ağın varlığını vurgulamıştır. Protein ekspresyonundaki değişiklikler bu ağda esas rolü üstlenmektedir. Tarımsal verim artışının sağlanmasında ana strateji, kuraklık altında verimin sürdürülebilmesi için gereken özelliklerin bir araya getirilmesi ve en etkili genlerin ve dolayısıyla proteinlerin, verim potansiyeli üzerinde zararlı etkileri olmadan elit genotiplerde biriktirilmesidir. Bu yaklaşım, kurak çevrede stabil kalabilen ve yüksek verim potansiyeli sunabilen yeni bitki varyetelerinin geliştirilmesine olanak tanıyacaktır. Bitkilerde kuraklık stresine cevap mekanizmasının aydınlatılabilmesi için transkriptom düzeyinde çok sayıda araştırma gerçekleştirilmiştir. Ancak mRNA seviyesindeki değişiklikler transkripsiyon ve translasyon sonrası modifikasyonlara bağlı olarak, her zaman protein seviyesiyle korelasyon göstermemektedir. Bu nedenle transkriptom çalışmaları, stres cevabına ilişkin ağın anlaşılmasında yetersiz kalmaktadır. Dolayısıyla kuraklık stresine ilişkin mekanizmaların aydınlatılabilmesi için proteom düzeyinde bilgiye ihtiyaç vardır.

Akdeniz ve Ortadoğu bölgesi yerel bitkisi olan *Brachypodium distachyon*, buğday, arpa, yulaf ve çavdar gibi beslenmede temel öneme sahip tahılların da içinde bulunduğu çim ailesinin (Poaceae) bir üyesidir. *Brachypodium distachyon*, kompakt bir genoma sahip olma (272 Mb), 5 çift kromozom (2n=10), kısa yaşam döngüsü (~12 hafta), kolay gen aktarımı, kendi kendine döllenmesi ve basit büyüme koşulları gibi özellikleri ile model organizma olmak için gereken bütün vasıflara sahiptir.

Model bitki özellikleri ve önemli tahıllar ile yakın akrabalığı, *Brachypodium distachyon*'u tüm serin iklim tahıl türlerinin, özellikle de hububatların geliştirilmesi için dikkat çekici bir model bitki haline getirmektedir. Ayrıca, yabani bir tür olan *Brachypodium distachyon*'un kuraklık stresine cevapta fizyolojik olarak geniş varyasyon gösterdiği bilinmektedir ve bu çeşitlilik stres koşulları altında ifade edilen yeni genlerin, dolayısı ile çeşitli proteinlerin bulunabilmesine olanak sağlayacaktır. *Brachypodium distachyon*'un süregelen transkriptomik analizleri mevcuttur ancak yukarıda belirtildiği gibi transkriptomdaki değişiklikler proteom seviyesindeki sonuçları her zaman birebir yansıtmadığından, proteomun incelenmesine yönelik analizlerin gerekliliği bu bitki için de söz konusudur.

Proteinler sinyalizasyonun ve biyokimyasal yolakların önemli bileşenleridir, bu nedenle protein seviyesindeki çalışmalar bitkilerin büyüme, gelişme ve çevreyle etkileşimlerinin altında yatan moleküler mekanizmaların aydınlatılması için gereklilik arz etmektektedir. Örnek hazırlama, yüksek çözünürlüklü protein ayrıştırma, protein karakterizasyonu için MS yazılımı ve donanımı ve biyoinformatik araçlardaki gelişmelere büyük ölçüde bağlı olarak bitki proteom çalışmalarında son yıllarda büyük bir ilerleme kaydedilmiştir. Bitki proteomu çalışmalarında amaç protein türlerinin kataloglanması ile sınırlı değildir, fonksiyonel biyoloji altında proteinlerin kalitatif ve kantitatif analizlerini de kapsar. Proteomik çalışmalardan elde edilen zengin verisetleri protein kimliği, miktarı, hücredeki lokalizasyonu, proteinin post-translasyonel modifikasyonu, protein-protein etkileşimleri ve kompleks proteomun oluşumunu kapsar. Bitki proteomik çalışmalarının sistem biyolojisiyle bütünleşmesi pek çok moleküler mekanizmanın anlaşılmasına öncülük edecek ve sonunda bitki verimi, gıda ve savunma için önemli olan süreçlerin mühendisliğini sağlayacaktır.

Bu çalışmada, *Brachypodium distachyon* bireylerinin zamana bağlı kuraklık uygulamasına maruz bırakılmasının ardından (4, 8 ve 12 günlük kuraklık uygulaması), protein repertuarında meydana gelen değişiklikler belirlenmiştir. Söz konusu amaç için, iki boyutlu jel elektroforezi (2D-DIGE) ve kütle spektrometrisine (MS) dayalı proteomik yaklaşım gerçekleştirilmiştir. Elde edilen proteom verilerinin karşılaştırılması sonucunda kuraklığa bağlı farklı düzeylerde anlatım gösteren proteinlerin varlığı saptanmıştır. Otuz yedi protein spotu kuraklık koşulu altında anlamlı varyasyon göstermiştir. Kütle spektrometresi ile tanımlanan on üç protein

fonksiyonlarına göre sınıflandırılmış ve *Brachypodium distachyon'*un kuraklığa cevap mekanizmasındaki rol ve ilişkileri tartışılmıştır. Kuraklık stresine cevap olarak çakışan metabolizmaların varlığı belirlenmiştir. Çalışmanın neticesinde tespit edilen proteinler kuraklığa tolerans gösteren bitkilerin belirlenebilmesi için biyomarkör olma potansiyeli teşkil etmektedir. Ayrıca, belirlenen proteinler *Brachypodium distachyon*'un yakın akrabalık gösterdiği tahıllarda (buğday, arpa, yulaf, çavdar vb.) kuraklık stresine direnç geliştirilmesi çalışmalarına katkıda bulunacaktır.

1. INTRODUCTION

Increasing yield has become a major challenge in agriculture, depending on the adverse effects of climate change as well as increasing world population and the need for natural resources. Only 3.5% of land resources in the world can be considered to be free of any stress factors (Velthuizen *et al*., 2007). Abiotic stres factors including drought, salinity, heavy metals, which cause up to 50% decrease in yield of several economically important crops are major obstacles for the improvement of agricultural production (Nouri and Komatsu, 2013). According to the report of the Intergovernmental Panel on Climate Change, in the near future these stress factors will continue to grow significantly with the effect of global warming (URL1). On the other hand, there is another parameter that worsens the scenario. The global population is estimated to reach 9 billion by 2050, which probably will result in a severe competition for the use of cultivable land. Therefore, improved productivity of existing land resources has an urgency to feed this population. According to the FAO's warning, annual cereal production will need to rise to about 3 billion tonnes from 2.1 billion today (FAO, 2009). Moreover, the fact that several cereals are currently being used for biofuel production, limits to increase the inadequate food supply in the world. All this information highlights that "producing more with less" has become a critical need (Abreu *et al*., 2013).

1.1 Stress Factors

Any environmental stimulus may result in the activation of several signaling pathways and interactions in the cell. These pathways are evolved biological processes that lead to appropriate stress response of the cell (Wrzaczek *et al*. 2011). Like all living organisms, plants need optimal environmental conditions for their normal growth and development, which reveals their potential performance. This optimal growth is achieved under optimal quantity or intensity, like in greenhouse. However, optimal conditions rarely occur for plants and their sessile nature makes them be much more exposed to environmental changes, which deviate from the

optimal conditions and prevent plants to maximize their physiologically achievable performance. Therefore, with respect to animals, plants are pressured to evolve more efficient and complex strategies to respond and adapt environmental changes. Inducers that retain the plants from their normal growth and development are defined 'stress' and they cause decreases in plant productivity. As shown in Figure 1.1, these inducers are subdivided into two major groups as abiotic stress factors and biotic stress factors (Schulze *et al*., 2005).

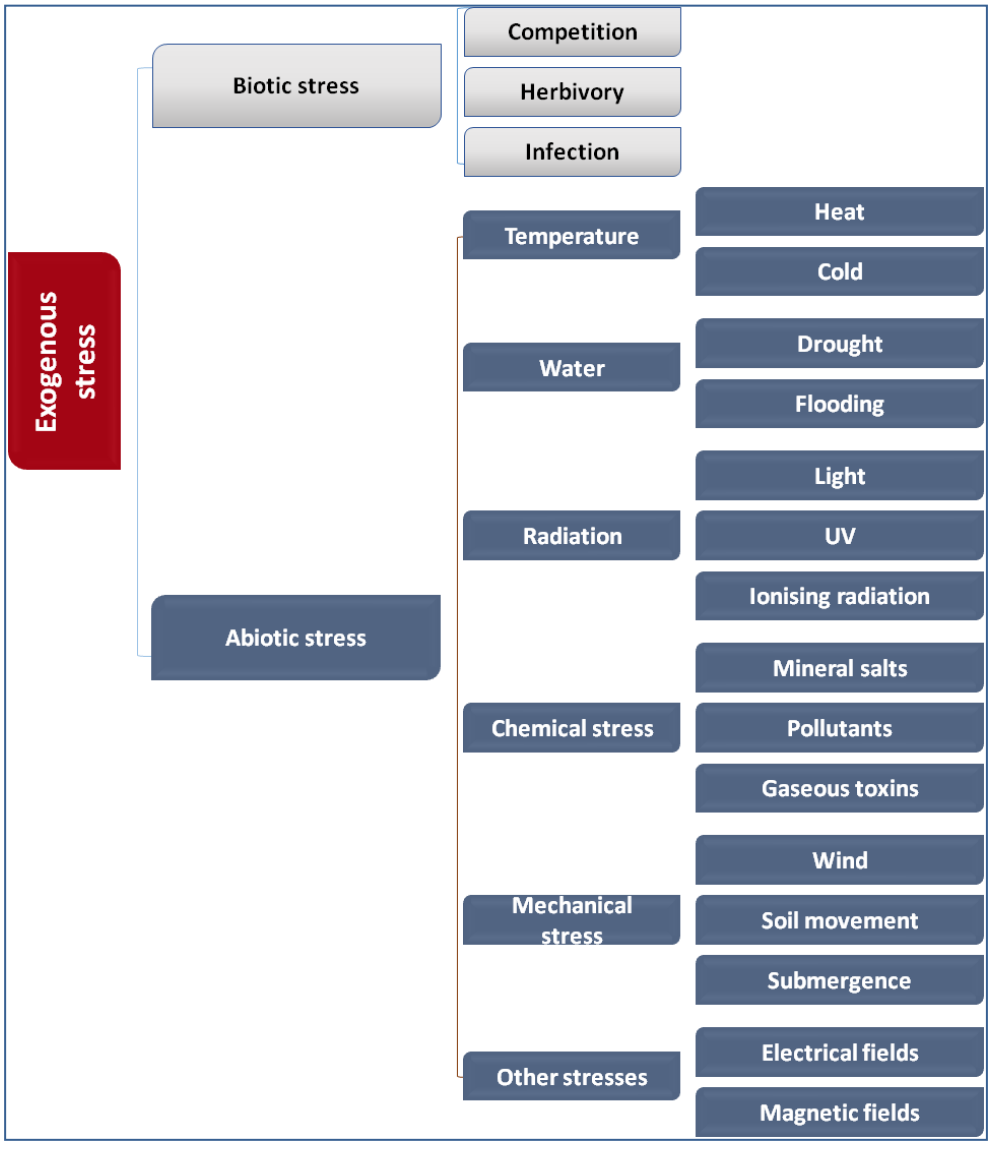


Figure 1.1: Exogenous stress factors

Many scientific studies have been focused on understanding biological responses of plants to the abiotic and biotic stressors and identifying specific genes, proteins or metabolites, which are responsible for tolerance phenotypes.

At molecular, biochemical and physiological levels, a number of mechanisms are activated upon exceeded stress condition. Stress response studies in plants show that the crosstalk among signaling networks increases during stress response (Fraire-Velàzquez *et al*. 2011). Initially, a number of kinase reactions and signaling cascade reactions, which result in the activation of reactive oxygen species production, the accumulation of hormones like abscisic acid, jasmonic acid, salicylic acid are induced. Thus, stress-responsive genes are expressed and overall stress response of plant is created. Following stress control, a new physiological state is established in the plant (Wrzaczek *et al*., 2011).

1.1.1 Biotic stress factors

The term 'biotic stress' refers to biological interactions of plants with other organisms. Infection of the plant or a mechanical damage to a particular part of the plant, caused by bacteria, viruses, fungi, nematodes, parasites, insects and weeds are biotic stressors. These stress factors severely result in losses in plant yield and therefore to economic problems. As an example to highlight that cereals are constantly endangered due to these biotic stress factors, the hemibiotrophic fungus, which causes maize anthracnose characterized by necrotic tissue is responsible for annual losses of up to one billion dollars in the U.S (Frey *et al.*, 2011).

1.1.2 Abiotic stress factors

'Abiotic stress' consists of several stressing factors caused by environmental changes as non-living factors. Some of these factors could be classified as temperature, drought, high salinity, oxidative stress, heavy metals, changes in photoperiod, light intensity and quality, nutrient abundance and starvation, freezing and flooding, air and soil pollution. According to the report of the Intergovernmental Panel on Climate Change, in the near future these stress factors will continue to grow significantly with the effect of global warming (URL1) and thus, understanding the mechanisms underlying abiotic stress response will be an urgent challenge in plant science (Hirayama and Shinozaki 2010; Wrzaczek *et al*,. 2011).

There have been many scientific efforts leading to great progression for our understanding of abiotic stress response mechanisms. In particular, studies in model plant *Arabidopsis thaliana* drew a general scheme of abiotic stress response in plant species.

Due to the variety of abiotic stress factors, it can be presumed that a strong specific cellular element is required for every individual stress response. Nevertheless, a remarkable common element takes place in the general response to all type of abiotic stresses. Substantially, all abiotic stress factors induce the generation of reactive oxygen species (ROS), however they are produced in different forms and in different subcellular locations. ROSs play a balanced role in metabolism. On the one hand, they are highly toxic and their expression must be kept under tight control. On the other hand, they serve as signals for gene expression, which leads to programmed cell death or scavenging protein production. Thus, the balance between the production and detoxification of ROS is critical to maintain cellular functions. (Genga *et al.*, 2011; Wrzaczek *et al*., 2011).

1.1.2.1 Drought stress

The ultimate goal in agriculture is to improve yield. As an environmental stress factor, drought or water deficit has severe effect on agricultural production. Drought has a direct impact on the cellular metabolism of plant, which causes a significant reduction in growth index and crop yield. In Figure 1.2, the current situation along with future estimation for drought severity on the world land is represented.

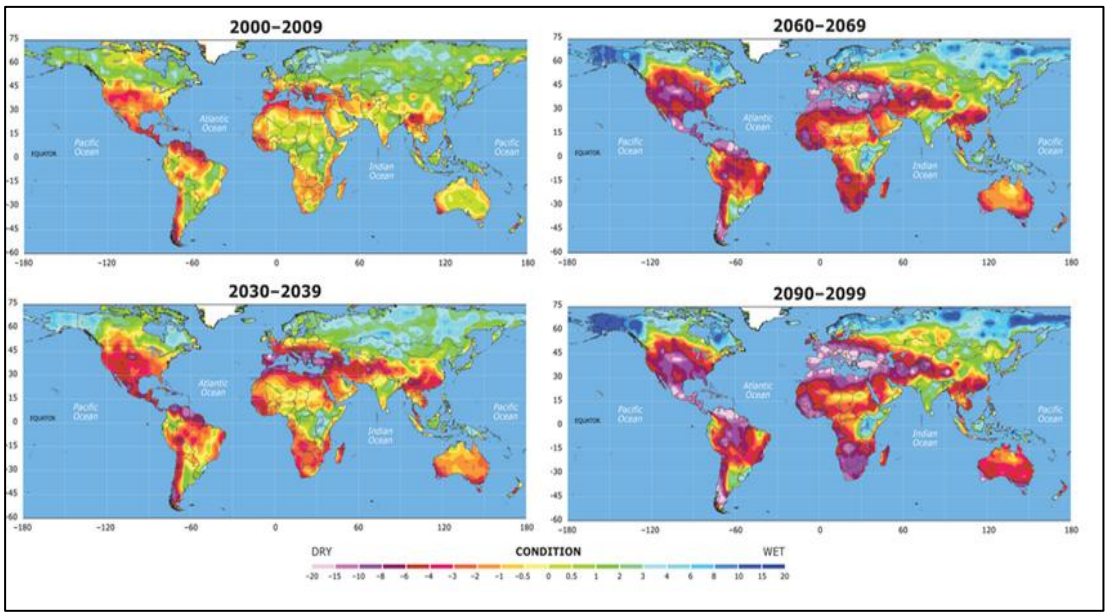


Figure 1.2: Current Palmer Drought Severity Index (PDSI) 2000-2009. A reading of -4 or below is considered extreme drought.

The existence of a complex network in the cell upon drought stress is highlighted by a number of studies. Changes in protein expression play the major role in this

complex network. The main strategy to improve crop yield is to integrate all the properties that are needed to sustain yield under drought stress and to accumulate the most powerful genes and proteins in elite genotypes without any harmful effect on the potential crop yield. This approach will provide the improvement of new plant cultivars that are stable and exhibit high yield potential under drought conditions.

Drought response begins with reprogramming in transcription and continues with many physiological changes. These physiological changes include closure of stomata, synthesis of osmolites and antioxidants, elimination of undesired reaction products (Bartels and Sunkar, 2005; Ergen and Budak, 2009; Ahuja *et al*., 2010, Ding *et al*., 2013). To date, a number of studies were carried out in comparative transcriptomics to identify stress-responsive genes in *Brachypodium* (Kantar *et al*., 2011; Padmalatha *et al*., 2012).

It is known that many transcripts undergo transcriptional, translational and post-translational modifications (Gygi *et al*., 1999). Depending on this issue, comparative proteomics is emerged as a powerful and promising tool to investigate stress response in plants (Ali and Komatsu, 2006; Peng *et al*., 2009; Gao *et al*., 2011; Shin *et al*., 2011; Abdalla and Rafudeen, 2012).

In plants, there are number of studies on transcriptome level to elucidate the mechanisms underlying stress response. However, alterations in mRNA levels do not always correlate well with protein levels in the cell due to the post-transcriptional and post-translational modifications. Therefore, transcriptome studies are insufficient for understanding the complex network of stress response, causing a lack of knowledge for the enlightenment of the mechanisms related to stress response.

1.2 Model Plants

Studying complex processes is achieved by the help of model systems. Model plants, in particular due to their compact genome, have become useful tools to study common problems in Plant Kingdom. In plant biology, *Arabidopsis thaliana*, *Oryza sativa* (rice) and *Brachypodium distachyon*, (‘*Brachypodium*’ hereafter) which represent well with desired attributes of a model organism are known common model species. The good synteny and orthology of *Brachypodium* with crop species, which provide food, fiber, feed and energy emphasize the attraction of *Brachypodium* as a

model plant (Brkijacic *et al*., 2011), especially for monocotyledonous plants (monocots) (Draper *et al*., 2001).

1.2.1 Model for monocots: *Brachypodium distachyon*

Flowering plants are subdivided into two major groups including monocots and dicots. Evolutionary divergence of monocots and dicots took place approximately 150 million years ago (Vogel and Bragg, 2009). Grass family members including wheat, oat and rye that possess one cotyledon have monocot-specific characteristics, sunflower and bean like plants with two cotyledons have dicot-specific properties. The difference of these two major groups arises from the seed, root and leaf structure and germination features. *Arabidopsis thaliana*, as a dicotyledonous plant (dicot) has been utilized broadly as a model plant during last 30 years and satisfied numerous scientific needs in plant science (Gutterson and Zhang, 2004; Zhang *et al*., 2006; Zilberman *et al*., 2007; Century *et al*., 2008). Although *Arabidopsis* is a suitable model plant for dicots, it has limited applications on researches specific for monocots (Brkijacic *et al*., 2011). In order for well understanding the mechanisms and processes, which are not conserved in monocots and dicots, a closely related model plant is required due to the clear differences and divergence of some processes across these subdivisions.

Within the monocots, grasses, which include temperate cereal crops such as oat, rye, wheat and barley, and especially forage grasses have significance on human diet, along with animal feed and they are eligible for using as sustainable energy source. Consequently, the importance of grasses on agriculture and economy is extensive and critical (Brkljacic *et al*., 2011; Ozdemir *et al*., 2008).

Brachypodium (from the Greek *brachys* "short" and *podion* "a little foot") is a convenient model plant for temperate cereals (Figure 2.2) with its well suited attributes to be a adaptable model species for grasses. *Brachypodium* has a compact genome (272 Mbp), easy genetic transformation, 5 pair of chromosomes (2n=10) in diploid accessions, short generation time (~12 weeks), small stature, lack of seed shattering, ability to self-pollinate and easily growing under simple environmental conditions (Draper *et al*. 2001, Ozdemir *et al*. 2008). It has 26,552 coding genes. A number of applications such as mapping studies, mutant analysis, and studies of natural diversity require the large numbers of different genotypes. Availability of

gene pool diversity, self-fertilization and lack of outcrossing of *Brachypodium* are advantegous characteristics for breeding and sustaining homozygous lines for these kinds of applications (Filiz *et al*., 2009). Polyploid accessions of *Brachypodium*, like those in wheat, also may be useful for polyploidy studies. Furthermore, it has one of the most compact genomes of grass family members, which leads to be chosen as a candidate species among grasses for genome sequencing (Vogel and Bragg, 2009) and the availability of whole genome sequence of *Brachypodium* (The International *Brachypodium* Initiative, 2010).

Brachypodium is a member of the subfamily Pooideae of the grasses (Poaceae), which also includes the grain crops barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereal* L.), and oat (*Avena sativa* L.). The close relationship of *Brachypodium* to these agriculturally and economically important cereals (Figure 1.3) makes *Brachypodium* attractive as a model plant for research of crop species, even more attractive than rice (*Oryza sativa*) (Opanowicz *et al*., 2008).

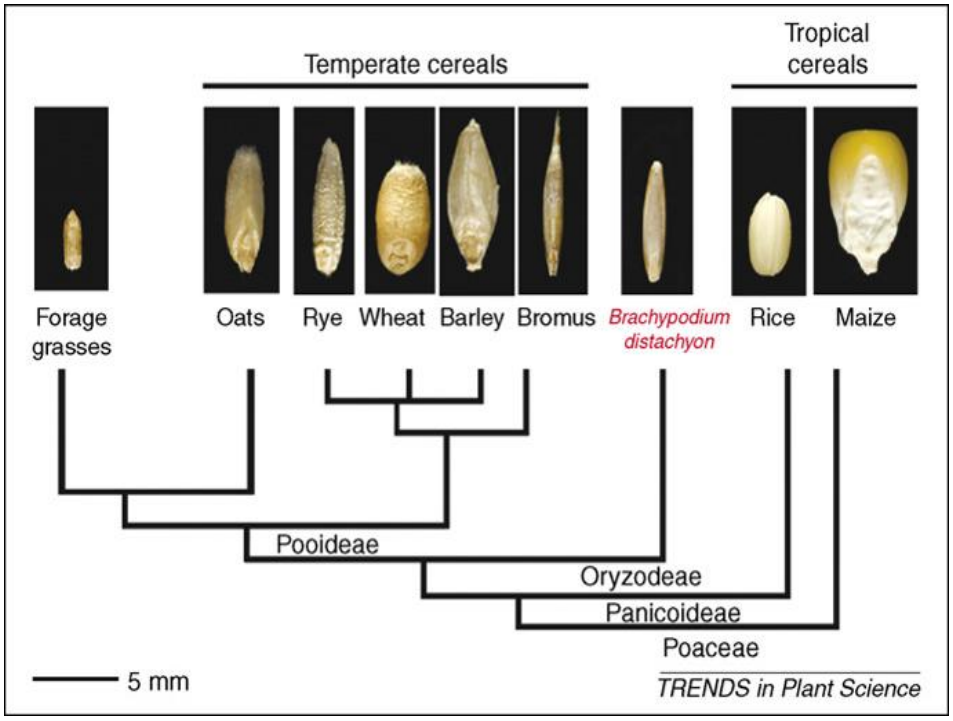


Figure 1.3: Phylogenetic position of *Brachypodium* (Opanowicz *et al*., 2008).

Similar to the ancestral range of small grain cereals, *Brachypodium* is native to the Mediterranean, the Middle East, South-West Asia and North-East Africa (Figure 2.4) (Opanowicz *et al*. 2008).

Domestication of plants is an evolutionary process, which promotes phenotypic transition enabling the improvement of plant yield and productivity for human wellfare. However, domestication of plants by artificial selection results in the exposure of many traits to genetic erosion caused by the loss of their ancestral genes. This genetic erosion alters physiological, developmental or morphological characteristics of plants. *Brachypodium*, as a wild type species, could serve much to understand biological mechanisms underlying these traits, which may have been lost during domestication, in comparison to its domesticated relatives such as wheat, oat and rye (Opanowicz *et al*. 2008, Bourguiba *et al*. 2012, Verelst *et al*. 2013, Tanackovic *et al*. 2014). In crop species, there is a significant reduction in gene variation for example 34% in soybean, 38% in maize, 70-90% in wheat compared to their wild relatives (Bourguiba *et al*. 2012). *Brachypodium*, as a wild species, is capable of showing a strong variation for adaptation in terms of survival mechanism compared to domesticated cereals due to the absence of genetic erosion caused by human selection. Studies highlighted that *Brachypodium* shows high diversity in a number of agriculturally important traits and in this respect, it is an advantageous species to understand the mechanisms underlying biological processes (Opanowicz *et al*. 2008, Bourguiba *et al*. 2012, Verelst *et al*. 2013).

1.2.2 *Brachypodium* vs. other model plants

Plant scientists have widely adopted the small weedy species *Arabidopsis thaliana* as a universalized model plant. Depending on its natural biological attributes, *Arabidopsis* has risen up as a highly robust model system. However, *Arabidopsis* is not that suitable to deal with many fronts of cereal biology due to the 150-million-year biological separation between dicots and monocots. For example, cell walls of grasses and dicots differ significantly from each other in terms of major structural polysaccharides, linkage form of these polysaccharides, and the abundance and importance of pectin, proteins and also phenolic compounds. Depending on the distantly related phylogenetic position of *Arabidopsis* to Poaceae family, a grass model system is needed for future studies on crop species (Vogel and Bragg, 2009). The differences between *Arabidopsis* and grass species in some areas like mycorrhizal associations, grass plant architecture, grain characteristics, intercalary meristems, and developmental properties highlights that *Arabidopsis* is not a suitable model system for the investigation of grass species. The extensive importance of

grasses as food, feed and, nowadays, as fuel, is a robust sign of the requirement to develop a truly tractable model system for the grass Family (Koornneef and Meinkee, 2010).

Rice is another model plant, which has been promoted for grass species. Although, rice would seem to meet expectations instead of *Arabidopsis*, it does not represent all relevant attributes for temperate grasses. The sequenced genome and large research network of rice, which advance the development of bioinformatics tools, provide an advantage to be a model system for grasses. However, its long life cycle, large genome size and demanding growth requirements make it less desirable and a poor choice for high-throughput genomic experiments when compared to *Brachypodium* (Brkljacic *et al*. 2011). Due to the fact that rice is a semi-aquatic tropical grass, its applicability as a model for temperate grass species is limited, in particular on freezing resistance and vernalization studies (Vogel and Bragg 2009). Based on these issues and drawbacks of other model plant species, *Brachypodium* increasingly have gained favor as a model system for grass species, especially for the temperate grasses.

Table 1.1: Comparison of *Brachypodium* with other cereals and *Arabidopsis* (Tatli, 2015).

	Brachypodium distachyon	***Arabidopsis thaliana***	***Triticum aestivum***	***Zea mays***	***Oryza sativa***	***Hordeum vulgare***
Chromosome number	10(2n)	10(2n)	42(2n)	20(2n)	24(2n)	14(2n)
Genome size (1C)	272 Mb	164 Mb	16700 Mb	2400 Mb	441 Mb	5000 Mb
Reproductive strategy	Self-fertilizing	Self-fertilizing	Self-fertilizing	Cross-polination	Self-fertilizing	Self-fertilizing
Life cycle (weeks)	10-18	10-11	12 (spring)	10+	20-30	16+
Height at maturity (m)	0.3	0.2	Up to 1	Up to 2	1.2	Up to 1.2
Transformation	Facile	Facile	Possible	Facile	Facile	Facile
Growth requirements	Very simple	Very simple	Simple	Simple	Specialized	Simple

1.3 Proteomics in Plant Science

Development of high-throughput separation and identification techniques has provided scientists to examine plant cellular mechanisms in a more complex way via “omics” approaches which include genomics, transcriptomics, proteomics and metabolomics. Proteins are directly involved in plant cellular interaction and metabolism, thus they are crucial components of stress response.

Proteome is defined as all proteins in a spesific tissue at a given time. Unlike the genome, which is only one for an organism, there are numerous proteomes depending on the growth and developmental stage, tissue, cell type of an organism. Furthermore, various protein products can be risen from one gene through post-transcriptional (e.g. alternative RNA splicing, RNA editing) and post-translational modifications (e.g. phosphorylation, acetylation, methylation, ubiquitination). Hence, an organism can synthesize distinct proteins in several orders higher than the total number of genes encoded by genome. Extensive variety of proteomes makes plants to possess an efficient tool for modulation of its response to environmental conditions. Understanding the mechanism of stress response is a complex process that needs all the outputs of multi-omics approaches: genomics, transcriptomics, proteomics and metabolomics.

In plants, from translational proteomics view, the development of abiotic stress tolerance involves three main steps: i) identification of stress-responsive proteins (proteomics), ii) confirmation of the role of identified proteins in stress response mechanism and evaluation of stress response, iii) application of confirmed data to modern plant domestication studies. These steps and their contribution to safe, nutritious, affordable food supply are represented on Figure 1.4.

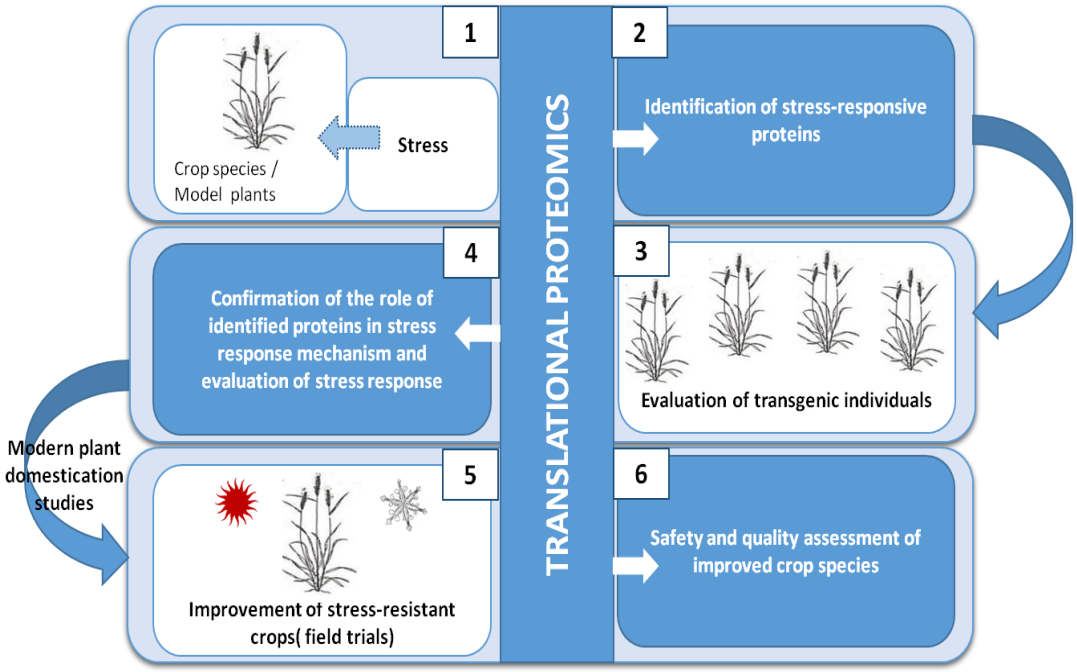


Figure 1.4: Safe, nutritious, affordable food supply

To date, some of the proteins, which were confirmed to be associated with abiotic stress tolerance using transgenic plants, are shown to be involved in signal transduction (Rab7 and GF14) (George and Parida, 2011), prevention mechanisms from oxidative stress (DHAR,GST) (Lee *et al*., 2007), protection process of proteins (HSP70, Rab21, ME-LeaN4, Lea3) (Montero-Barrientos *et al*., 2008; Qian *et al*., 2014) and in biotic/abiotic stress crosstalk (PR-10a) (Hanafy *et al*., 2013). Less supported data also showed that there are some promising proteins for crop improvement, which are need to be validated in transgenic plants, such as HSP-70 stabilizing proteins and HSFs (for the protection of proteins), OsRMC (stress signaling), Mn-SOD and specific APX isoforms (oxidative stress protection) and PsbP, chloroplastic ATP synthase, GAPDH and ZmICDH (improvement of photosynthesis/metabolism) (Abreu *et al*., 2013).

In *Brachypodium*, stress response studies are largely focused on genomic and transcriptomic research. In the study of Feng *et al*. (2015), drought tolerance ability of brassinosteroid intensitive 1 (BRI1) knocked-down mutants was enhanced with the elevated expression of drought-responsive genes, indicating that the importance of brassinostreoids in drought tolerance as a direct regulator in the expression of important drought-responsive genes in *Brachypodium*. Micro RNAs (miRNA) are another striking subject of plant stress response studies. A large number of studies (Unver and Budak, 2009; Zhang *et al*., 2009; Baev *et al*., 2011; Bertolini *et al*., 2013; Budak and Akpinar, 2011; Jeong *et al*., 2013; Zhang *et al*., 2009) have been performed to identify and characterize abiotic stress-responsive micro RNAs in *Brachypodium*. Thus, these identified and characterized miRNAs have become potential targets for developing abiotic stress resistance in *Brachypodium* individuals.

Stress response studies are not only limited with stress-responsive miRNAs. Researchers also identified stres-responsive transcription factors as key players in defense and abiotic stress signaling mechanism controlled in *Brachypodium* (Mochida *et al*., 2011; Tripathi *et al*., 2012; You *et al*., 2015). Bayramov and Guliyev (2014) showed the importance of Rubisco activase (RCA) gene expression at different developmental stages for continued CO_2 fixation under drought and salt stresses. Colton-Gagnon *et al*. (2013) monitored phenotypical characteristics along with profiling VRN1 gene expression as well as soluble sugars and proline contents

and found out that some diploid accessions of *Brachypodium* had a facultative growth habit.

As a transcriptomic study, Verelst *et al*. (2013) studied the physiological and molecular effects of drought stress on leaves as well as developing robust and reproducible drought stress protocol. All these information mediated to draw a general picture of stress-response mechanism of *Brachypodium* from genomic and transcriptomic perspective.

So far, the proteome studies on *Brachypodium*, which draws attention with its phylogenetic relationship to important crops, focused on the identification of protein content in grain to compare with other grass family members (Guillon vd., 2012; Larre vd., 2010; Wang vd., 2012; Hammami vd., 2011). However, the studies on stress response of *Brachypodium* at protein level are limited with two studies. Filiz *et al*. (2014) screened superoxide dismutases, which are antioxidant proteins catalyzing dismutation of free radicals to hydrogen peroxide and dioxygen, thus involved in stress response mechanism. On proteome level, proteome and phosphoproteome changes induced by salt stress were carried out by Lv *et al*. (2014) on *Brachypodium* leaves, which resulted in the identification of 60 differentially expressed unique proteins.

1.3.1 Two-dimensional difference gel electrophoresis (2D-DIGE)

Two-dimensional gel electrophoresis simultaneously separate thousands of proteins, based on a denaturing environment. In the first dimension, proteins are separated depending on their isoelectric points. The isoelectric point is defined as the specific pH at which the net charge of the protein is zero. The theoretical information behind this phenomena is that: under the electric field, in a pH gradient proteins will migrate to the position where its net charge is zero. Following isoelectric focusing (IEF), the proteins are separated according to their molecular weight by traditional SDS Polyacrylamide gel electrophoresis (SDS-PAGE) (Beckett, 2012).

The usage of multiplex fluorescently labeled proteins on the same gel has become a breakthrough in 2D gel electrophoresis. This innovation is referred to as 2D difference gel electrophoresis (2D-DIGE). The fluorescent dyes used in 2D-DIGE are modified cyanine dyes. Labeling approaches include minimal labeling, where the dye binds to a limited number of lysine residues and saturation labeling, where the

dye binds to all cysteine residues. A standard workflow for minimal CyDye labeling protocol is represented in Figure 1.5.

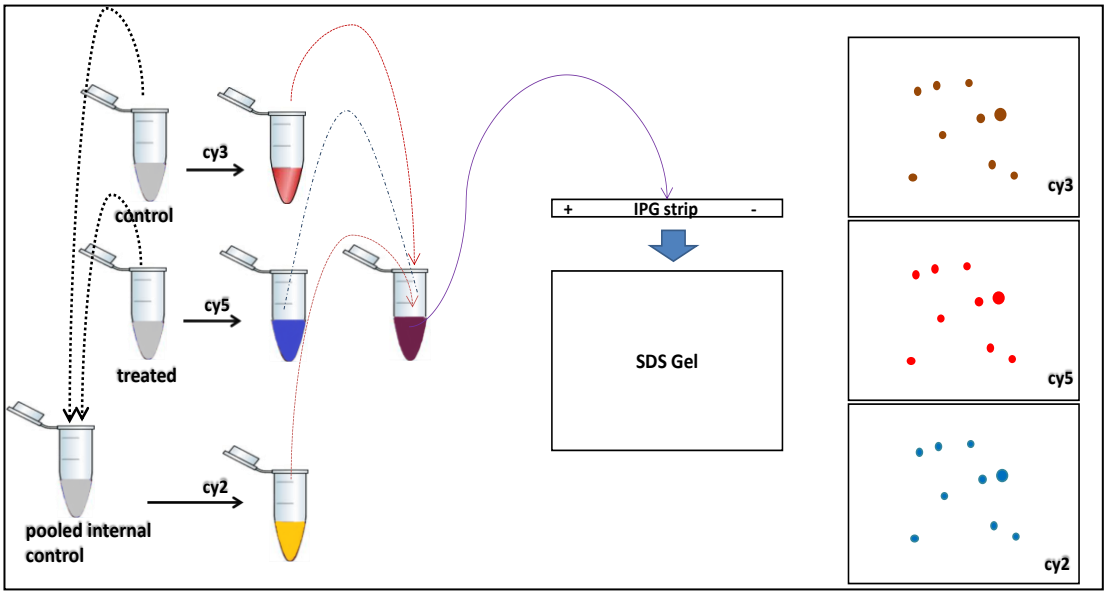


Figure 1.5: Workflow of minimal CyDye labeling protocol.

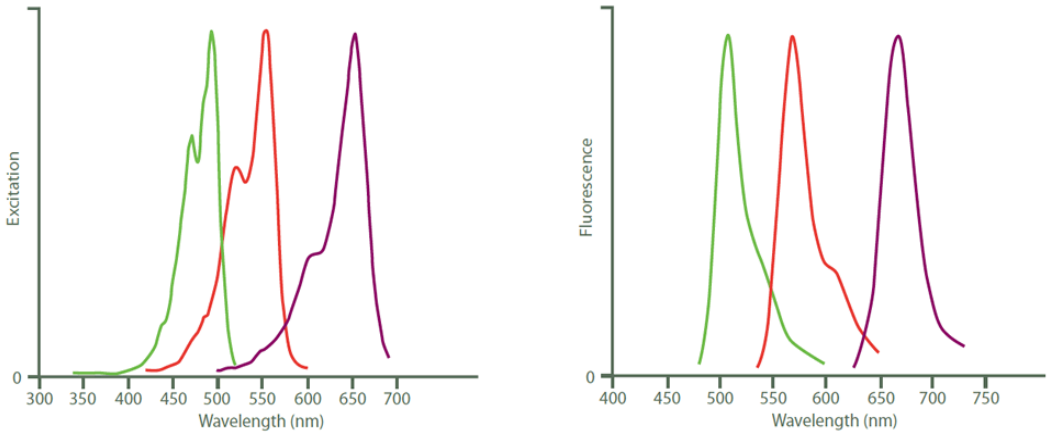


Figure 1.6: Excitation (left) and emission (right) spectra of Cy2 (green), Cy3 (red) and Cy5 (purple) (Beckett, 2012).

The important aspect of the DIGE technology is its ability to label two or more samples with different dyes and separate them on the same gel, eliminating gel-to-gel variability. This makes spot matching and quantitation much simpler and more accurate. Cy2 is used for a normalization pool created from a mixture of all samples in the experiment. This Cy2-labeled pool is run on all gels, allowing spot matching and normalization of signals from different gel. Excitation and emission spectra of Cy dyes are given in Figure 1.6. CyDyes are spectrally resolvable cyanine dyes carrying an N-hydroxysuccinimidyl ester reactive group that covalently binds the ε-

amino groups of lysine residues in proteins. CyDye DIGE fluors are supplied as a N-hydroxy succinimidyl ester, which reacts with primary amino groups, typically the terminal amino group of lysine side chains (Beckett, 2012).

1.3.2 Mass spectrometry

In proteomics pipeline, determination of differentially expressed spots from a series of 2D gels is followed by identification of these proteins. Mass spectrometry is a technique, which allows for the identification of proteins within an organism by representing detailed knowledge including amino acid sequences, peptide molecular weights, and post-translational modifications.

MALDI-TOF is able to identify the proteins by means of peptide-mass fingerprinting (PMF) method of which the principle is based on endo-protease mediated digestion to generate peptides, such as trypsin. Subsequently, MALDI-TOF mass spectrometer measures these peptides by their molecular masses and further these sequences are matched with a corresponding theoretical list of peptide masses within a database for a particular protein.

Bioinformatic tools (MASCOT), which contain information regarding their function and metabolic pathways, are utilized, thus the determined proteins by mass spectrometry are analyzed with databases such as Uniprot and KEGG (Aebersold and Mann, 2003).

1.4 Aim of the Study

In this study, the ultimate goal is to identify alterations in cellular pathways linked to drought stress in *Brachypodium distachyon.* To do that, semi-quantitative description of changes in protein repertoire of *Brachypodium* leaves after exposing individuals to time-dependent drought stress (4, 8 and 12 day application of drought) was aimed. To compare and contrast the responses of the leaf proteome of *Brachypodium*, a proteomic approach based on two-dimensional difference gel electrophoresis (2D-DIGE) and combined tandem mass spectrometry (MS) experiments were carried out. Untreated samples belonging to different time points were compared with drought-treated samples.

The identified proteins in this study could serve to delineate molecular pathways associated with drought tolerance in this model organism and also as potential biomarkers for the selection of drought-tolerant *Brachypodium distachyon* plants as well as its relatives like wheat, barley and rye. Furthermore, identified proteins could aid for the contribution of the studies on development of drought-resistant crop species, which are close relatives of *Brachypodium distachyon*..

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and commercial kits

All chemicals and commercial kits used in this research are listed in Table 2.1.

Table 2.1: List of chemicals and commercial kits.

Chemical/Kit	Trademark	Catalog No
2-Mercaptoethanol	Sigma-Aldrich	M6250
Acetone	Sigma-Aldrich	320110
Acrylamide/Bis Solution 40%	BioRad	1610148
Amonium acetate	SIAL	A7262
Ammonium persulphate (APS)	Sigma-Aldrich	A3678
CHAPS hydrate	Sigma-Aldrich	C3023
Cy2 dye for 2D electrophoresis, 25 nmol	Lumiprobe	BL-3A041
Cy3 dye for 2D electrophoresis, 25 nmol	Lumiprobe	BL-3B041
Cy5 dye for 2D electrophoresis, 25 nmol	Lumiprobe	BL-3C041
Dimethylformamide (DMF), labeling grade	Lumiprobe	BL-13050
Dithiothreitol (DTT)	Fermentas	K0311
Ethylenediamine tetra acetic acid (EDTA)	Sigma-Aldrich	E6758
Glycine	Chem Cruz	SC-29096
L-lysine	Sigma-Aldrich	BCBK 3837V
Methanol	Merck	1.06009.2511
Mineral oil	BioRad	163-2129
Phenol Tris-HCL saturated	Sigma-Aldrich	P4557
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	P7626
Potassium chloride (KCl)	Sigma-Aldrich	P9541
RNAlater stabilization solution	Ambion	AM7021
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L3771
Sucrose	Sigma-Aldrich	S0389

Table 2.1 (cont.): List of chemicals and commercial kits.

Chemical/Kit	Trademark	Catalog No
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T9281
Tris	MultiCell	600-127LG
Urea	Prolabo	28877.292
2-D Quant Kit	GE-Healthcare	80-6483-56
Electrode wicks	BioRad	165-4071
ReadyPrepTM 2-D Cleanup Kit	BioRad	163-2130
ReadyPrep™ 2-D Starter Kit	BioRad	163-2105
ReadyStrip™ IPG Strips 17 cm 3-10 pH nonlinear	BioRad	163-2009
Brilliant Blue G	Sigma-Aldrich	B0770
Proteomics Grade Water	BioRad	163-2001
Ammonium sulphate	Carloerba	420777
Phosphoric acid	Merck	M.100546.0100

2.1.2 Equipments

All equipments used in this study were listed in Table 2.2.

Table 2.2: List of equipments.

Equipment	Trademark
Precision scales	Precisa-BJ610C
Visualization	ChemiDoc- BioRad
Isoelectric focusing	Protean IEF Cell, BioRad
Vortex	DragonLab MX-F
Second dimension large format electrophoresis system	Protean II Xi Cell, BioRad
Micropipettes	Gilson
Power supply	CS Cleaver MP-300V
pH Meter	Mettler Toledo Five-Easy
Microcentrifuge	Beckman Coulter Allegra 25R, ScanSpead
IEF Focusing tray with lid	17 cm, BioRad
Equilibration tray with lid	17 cm, BioRad
Microplate spectrophotometer	Bio-RAD Benchmark Plus
Oven	Memmert UN 55
Shaker	HeidolphDuomax 1030, Sartorious CERTOMAT SII

2.2 Methods

2.2.1 Growth of *Brachypodium* plants and drought stress treatment

Brachypodium seeds used in this study were kindly provided by Prof. Dr. Metin Tuna, Namık Kemal University, Department of Field Crops, Tekirdağ, Turkey.

Brachypodium seeds were stratified at 4 ºC between wetted filter papers in petri plates and keep in the dark for 7-10 days. After cold treatment, the seeds were put in petri plates under light at room temperature for 5 days. The germinated seeds were transfered first to peat-soil mixture in the viols and grow them until the emergence of third leaf, and then transfer the plantlets into plastic pots. The plants were grown under a controlled environment (16/8 h light/dark photoperiod at 25/22 °C, relative humidity 60%-70%, and a photosynthetic photon flux of 320 μmol m^{-2} s^{-1} at canopy height provided by fluorescent lamps) in the greenhouse. The soil was treated with 200 mg kg^{-1} N ($Ca(NO_3)_2$), 100 mg kg^{-1} P (KH_2PO_4), 20 mg kg^{-1} S (K_2SO_4), 5 mg kg^{-1} Fe (Fe-EDTA) and 2.5 mg kg^{-1} Zn ($ZnSO_4$) for every 20 days to supply basal fertilization.

For drought treatment, experimental group was withheld from water, while control group were irrigated daily. Fresh leaf samples were randomly collected at the 4^{th}, 8^{th} and 12^{th} day of drought treatment and also from control group, compared to the days of the collected sample. Basically, each treatment day had its own control and treatment sample. Experimental design for sampling step was represented in Figure 2.1. Each treatment day had its own control sample. Following sampling, leaves were taken into RNA later (Ambion) solution to prevent protease activity and then stored at -20°C for further use.

2.2.2 Relative water content measurement

For the evaluation of plant water status at the end of the stress treatment, relative water content (RWC) was measured according to the protocol of Barrs and Weatherly (1962). At the end of 12^{th} day, both from stress treated and untreated *Brachypodium* plants, leaves were cut and weighed to obtain the fresh weight (FW). Then leaves were hydrated by floating on water in a closed petri dish for 4 hours at

room temperature at dark. After hydration, leaves were taken out of water and dried between filter papers and weighed to determine turgid weight (TW). Samples were then dried in an oven at 80°C for 24 hours and weighed to obtain dry weight (DW). The RWC was determined as follows: RWC = (FW – DW)/(TW – DW) x 100. The final leaf RWC was the mean value taken from three individual seedlings. Statistically significant differences relative to the control were calculated by Student's t test.

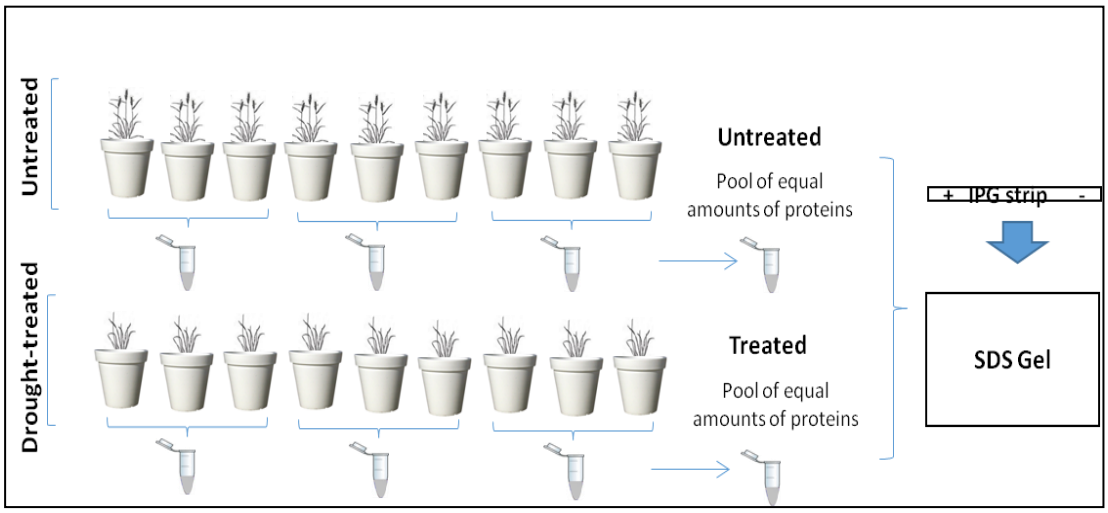


Figure 2.1: Experimental set-up for sampling of each treatment day.

2.2.3 Total protein extraction

Extraction of proteins was performed according to the protocol of Faurobert *et al.* (2006), with slight modifications.

Approximately 500 mg of fresh plant tissue was ground to a fine powder with pre-chilled mortar and pestle in liquid nitrogen. Ground tissue was suspended in 4 mL of extraction buffer which was freshly prepared (500 mM Tris-HCL, 50 mM EDTA, 700 mM sucrose, 100 mM KCL, pH 8.0) and 2% 2-mercaptoethanol and 1 mM PMSF were added just before extraction. Incubated for 10 min on ice. EDTA inhibits metalloproteases and polyphenol oxidases by chelating their bound metal ions. PMSF irreversibly inhibits serine proteases. 2-mercaptoethanol is a reducing agent that prevents protein oxidation. The presence of KCl is related to its "salting in" effect, improving the solubility and then the extraction of proteins.

Equal volume of Tris-buffered phenol was added and incubated for 10 min at room temperature. To separate insoluble material (in the pellet), for aqueous and organic phases, the sample was centrifuged for 10 min at 5500 g and 4°C. The phenolic

phase, which is on the top of the tube is carefully recovered to avoid contact with the interphase and transferred to a new tube. (The trick here is to use sucrose in the extraction buffer to invert the phases. The upper phenol phase contains cytosolic and membrane proteins.)

The upper phenol phase was then back-extracted with 4 mL of extraction buffer, shaken for 3 min, vortexed. Centrifugation for phase separation was repeated for 10 min at 4°C and 5500 g. The phenol phase still on the top of the tube was carefully recovered and poured into a new tube, 3 or 4 vol of precipitation solution, 0.1 M ammonium acetate in cold methanol, were added, shaken by inverting and incubated overnight at -20°C.

Following precipitation step, proteins were pelleted by centrifugation for 10 min at 5500 g and 4°C. Pellet was washed 3 times with cooled precipitation solution and finally with cooled acetone. After each washing step, the sample was centrifuged for 5 min at 5500 g and 4°C. Finally, pellet was dried to remove acetone.

The dried protein pellet was resuspended in rehydration buffer, composed of 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% ampholyte. The suspension was incubated and then centrifuged to remove any insoluble material.

Protein concentration was determined by a 2-D Quant Kit (GE Healthcare Life Sciences) using bovine serum albumin (BSA; 2mg/ml) as a standard. The quantified protein samples were stored in aliquots at -80°C until they were used for two-dimensional difference gel electrophoresis (2D-DIGE). Three biological replicates were conducted for the protein extraction of each treatment.

2.2.4 Two dimensional difference gel electrophoresis (2D-DIGE)

2.2.4.1 Sample preparation

Samples were desalted/cleaned with ReadyPrep 2D Cleanup kit (Bio-Rad, USA) according to manifacturer's instructions. Final protein pellet was resuspended in rehydration buffer. Measured amounts of the proteins, both before and after clean-up procedure, were represented in Table 2.4.

Individual protein samples (180 µg of each sample) were labeled with the appropriate CyDye (Cy3 or Cy5) according to DIGE minimal labeling procedure of GE Life Sciences (Sweden). Experimental set up for this experiment was shown in

Table 2.3. A pooled sample, prepared by mixing equal amounts of protein for all samples in the experiment and acting as an internal standard, was labelled with the Cy2 dye. The internal standard ensures that all proteins present in the samples are represented, allows gel-to-gel matching and in-gel analyses of relative protein spot intensities.

The labeling reaction was performed on ice for 30 min, and then, terminated by the addition of lysine for 10 min on ice to quench any unreacted dye. The Cy2, Cy3 and Cy5 labeled samples were mixed together and the volume was adjusted to 360 μL with rehydration buffer followed by loading of the samples into gel strips made pH gradient (IPG strip) and incubating for 1 h to rehydrate strips.

Table 2.3: Experimental design for minimal labelling CyDye protocol.

Gel	Time point (day)	Cy2	Cy3	Cy5
1	4	Pooled internal standard	Untreated	Drought-treated
2	4	Pooled internal standard	Drought-treated	Untreated
3	4	Pooled internal standard	Untreated	Drought-treated
1	8	Pooled internal standard	Untreated	Drought-treated
2	8	Pooled internal standard	Drought-treated	Untreated
3	8	Pooled internal standard	Untreated	Drought-treated
1	12	Pooled internal standard	Untreated	Drought-treated
2	12	Pooled internal standard	Drought-treated	Untreated
3	12	Pooled internal standard	Untreated	Drought-treated

Table 2.4: Protein amounts of extracted samples.

Time point (day)	Before clean-up (mg) untreated	Before clean-up (mg) treated	After clean-up (mg) untreated	After clean-up (mg) treated
4	1.237	1.341	0.750	0.764
4	1.345	1.268	0.768	0.703
4	1.210	1.179	0.659	0.636
8	1.351	1.298	0.782	0.738
8	1.423	1.343	0.853	0.804
8	1.278	1.341	0.692	0.707
12	1.116	1.267	0.684	0.701
12	1.235	1.201	0.725	0.718
12	1.426	1.343	0.902	0.894

2.2.4.2 First dimension: isoelectric focusing

Following sample preparation, isoelectric focusing was performed using a PROTEAN IEF cell (Bio-Rad) according to the manufacturer's instructions. Briefly, active rehydration was carried out at 30 V for 12 h, followed by 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3000 V for 1 h, and then focusing at 8000 V until 80,000 Vh at 20 °C.

2.2.4.3 IPG strip equilibration

Equilibration step is necessary to allow the SDS molecules to associate with the proteins and produce the anionic complexes that have a net negative charge.

After isoelectric focusing, the strips (17 cm, pH 3-10 nonlinear) were equilibrated with an equilibration solution (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.2% bromophenol blue) containing 1% DTT for 15 min, with a second equilibration step of 15 min with the same equilibration buffer containing 2.5% w/v iodoacetamide.

2.2.4.4 Second dimension: SDS-PAGE

Equilibrated IPG strips were placed on top of the SDS-PAGE gel and sealed with 0.5% w/v agarose. As a result of the electric force the proteins migrate out of the IEF gel and into the SDS gel, where they separate according to their molecular weights. The SDS-PAGE step was performed in Protean xi cell (BioRad) electrophoresis system at a constant current setting by 20 mA/gel until the bromphenol blue tracking dye move out of the gel

2.2.4.5 Visualization and data analysis

The gels were scanned using ChemiDoc Imaging System (BIORAD, USA) to image each gel to detect the Cy2-, Cy3- and Cy5-labelled proteins. Image analysis was performed with PD Quest Advance 8.1.2 (BIORAD, USA). The experimental molecular weight of each protein was estimated by means of comparison with the protein marker, and the experimental isoelectric point was determined by its migration on the immobilized pH gradient strip. The abundance of each spot was estimated based on density (set as 2 fold change).

Relative comparison of the intensity abundance between control and drought-treated (three replicate samples for each group) was performed using the Student's t test. Only those with significant and biological reproducible changes were considered as differentially expressed protein (DEP) spots.

2.2.4.6 Preparation of preparative gel

After determination of DEP spots, preparative gel was prepared. Gel was fixed with fixative solution (40% methanol, 10% acetic acid) for overnight on a shaker. Following fixation, gel was stained with colloidal commasie (containing Coomassie brilliant blue (CBB) R-250 staining, phosphoric acid and ammonium sulphate) for 8 h and destained with 25% methanol solution. Gel was washed with ultrapure water and DEP spots were excised for further step.

2.2.5 Protein identification by mass spectrometry and data analysis

Selected spots excised from preparative gel were sent to Kocaeli University, Faculty of Medicine, Department of Medical Biology for MALDI- TOF/TOF analysis to identify DEP spots. Tryptic digestion of spots was performed using In-gel tryptic digestion kit (Invitrogen, Thermo Fisher Scientific) according to manufacturer's instructions. Identification of the spots was performed via MALDI-TOF/TOF. MS/MS spectra were searched against the NCBI *Brachypodium* protein database (26,035 entries in total; downloaded on July 16, 2011) using MASCOT version 2.1 (Matrix Science, London, UK). The protein score confidence interval percentage and total ion score confidence interval percentage were both set above 95%, and the significance threshold was $p < 0.05$ for the MS/MS. 13 protein spots were identified by Mass Spectrometry analysis.

2.2.6 Bioinformatic analysis of identified proteins

Proteins were examined using AgriGO for Gene Ontology (GO) annotation and enrichment analysis to determine subcellur localizations and biological functions of identified proteins. To determine cellular pathways and thus investigate effect of the alterations in these pathways to drought outcome, proteins were searched in Kyoto Encyclopedia of Genes and Genomes (KEGG) *Brachypodium* database and mapped to the *Brachypodium* specific pathways with KEGG Mapper.

The R Project for Statistical Computing Program was used to generate HeatMap and hierarchical clustering of DEP spots in the treatment group, to represent time-dependent changes. While each column represented a day of treatment, each row represented the change of a DEP spot using color coding based on the relative ratio. The logarithms of fold change values were taken to represent the up- and down-regulated proteins indicated in red and green, respectively.

3. RESULTS

3.1 Morphological Changes and Relative Water Content of *Brachypodium*

Morphological changes caused by drought treatment are depicted in the representative photographs of control and drought-treated plants taken at different time points of the treatment (4, 8 and 12 days) (Figure 3.1). At the end of the stress treatment, drought caused strong reductions in plant height as compared to control.

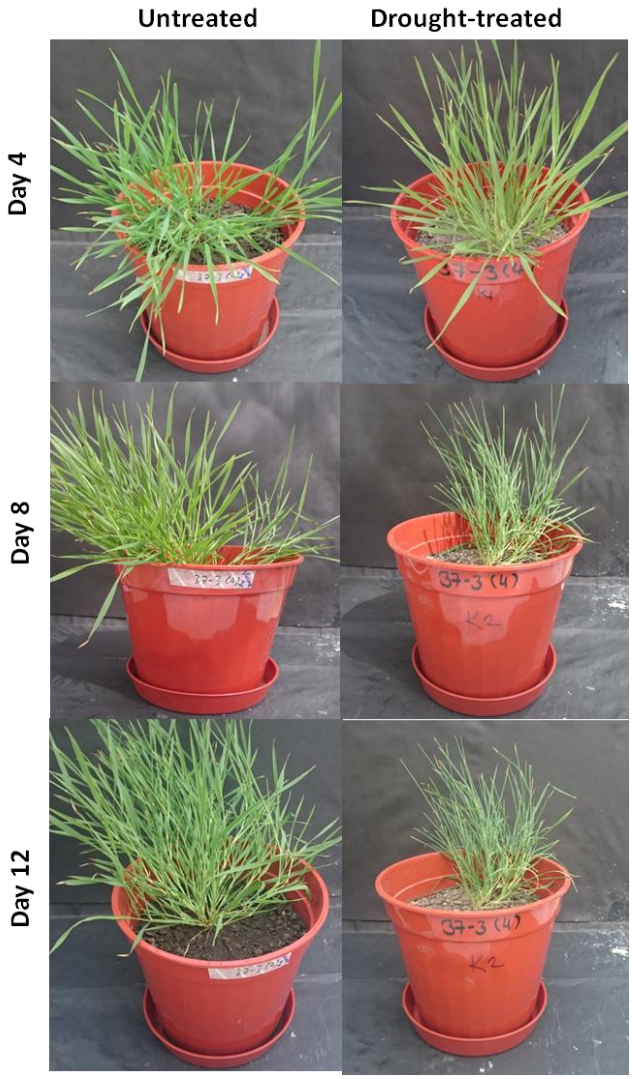


Figure 3.1: Representative *Brachypodium* plants after 4, 8 and 12 days of withholding water. Each pot contains one plant.

As a physiological measurement, relative water content (RWC) was used to quantify water status of *Brachypodium* individuals at the end of the 12-day-drought treatment. To study the effect of both leaf water potential and osmotic adjustment, RWC is used as an estimation method for cellular hydration. The method estimates water hold of the leaf at the time of the measurement, when comparing to water hold at full turgidity, and the water deficit in the leaf is measured. The values of weighed samples for relative water content estimation were indicated in Table 3.1, along with p value calculated via Student's *t* test. Three biological replicates were conducted for this experiment.

Table 3.1: Measurements used for relative water content calculation.

	Fresh Weight (g) (Mean)	Turgid Weight (g) (Mean)	Dry Weight (g) (Mean)	RWC%	P value for student's t test
Untreated	0.47 ± 0.05	0.50 ± 0.04	0.10 ± 0.01	91.60	
Drought-treated	0.53 ± 0.04	0.75 ± 0.06	0.22 ± 0.02	58.54	**0.002***

*Significant at $p < 0.050$

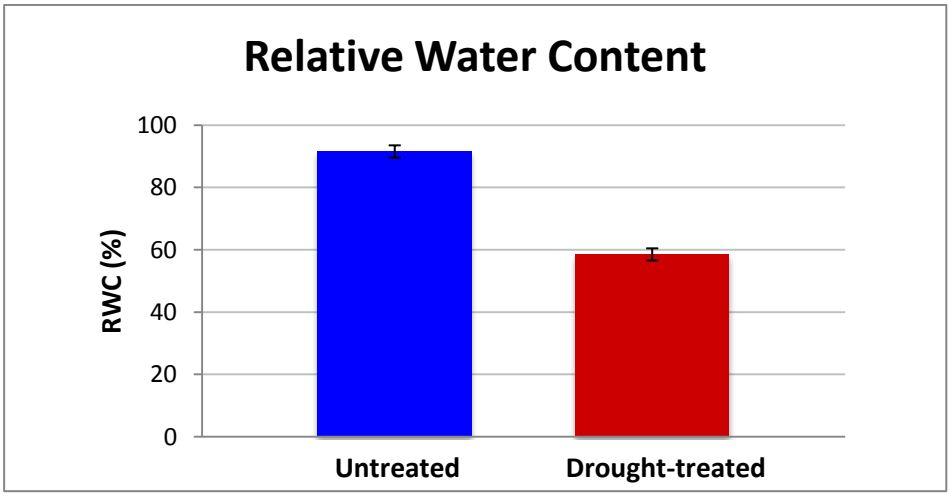


Figure 3.2: Leaf relative water content analysis. 'Drought-treated' represents RWC of leaves of *Brachypodium* treated with water withheld for 12 days. Error bars represent standard deviation of three biological replicates.

As shown in Figure 3.2, there was a statistically significant difference between relative water content of untreated and drought-treated leaf samples at the end of 12-day of drought treatment. Relative water content of drought treated individuals showed approximately 40% decrease upon drought stress.

3.2 Differential Proteomic Analysis of the *Brachypodium* Leaves Upon Drought Stress

To identify proteins whose expression significantly changed upon drought stress, a proteomic approach was carried out based on 2D-DIGE and MS/MS. To discuss time-dependent proteome changes, total protein was extracted from *Brachypodium* leaves at three different time points (4^{th}, 8^{th} and 12^{th} day of drought treatment) and resolved by 2D-DIGE, within the pH range from 3 to10 and mass range from 10 to 180 kDa.

In order to quantify the differences in protein abundance due to drought treatment, gel replicates were subjected to comparative software-assisted image analysis using PDQuest Advance 8.1. Spot detection on Cye-dyed gels (Figure 3.3) revealed a total of 497 distinct spots in *Brachypodium* protein repertoire.

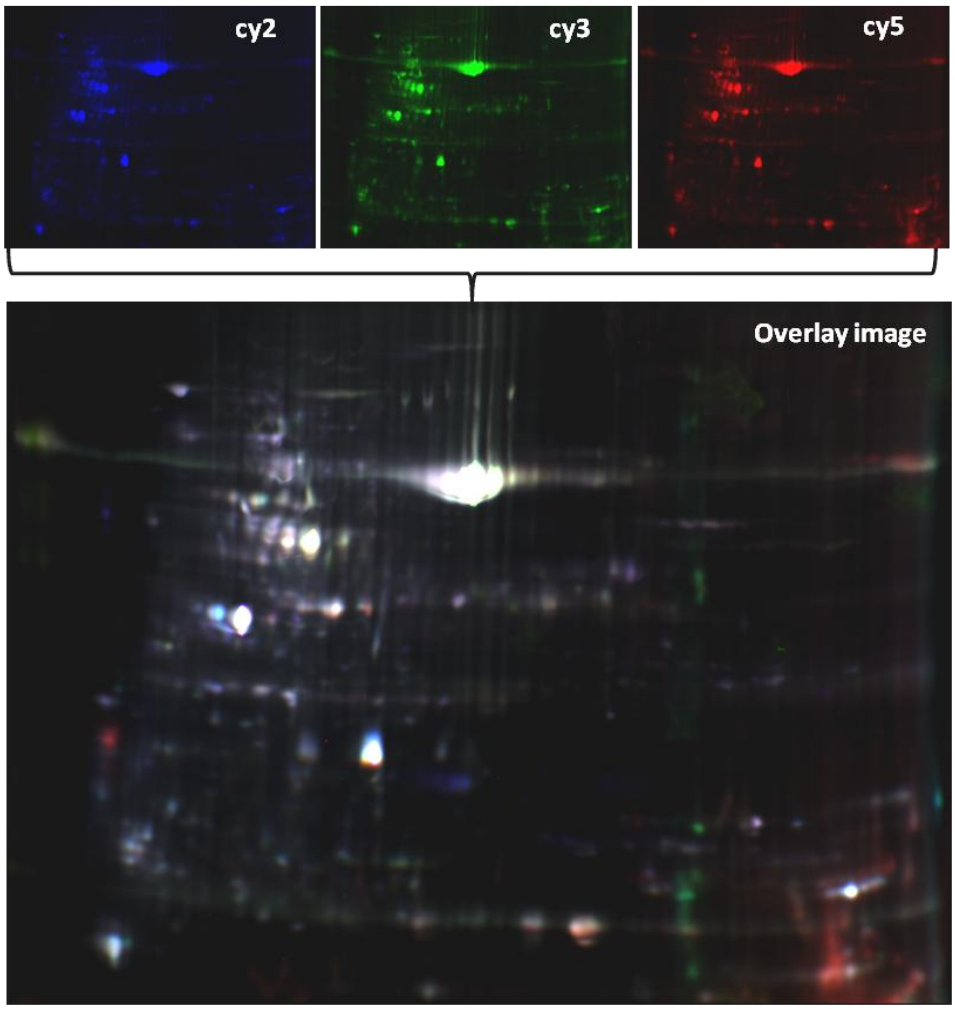


Figure 3.3: Representative Cy-dyed gel. While Cy3 represents untreated samples, Cy5 represents drought-treated samples, Cy2 is the internal control.

Among 497 spots, statistical evaluation of relative spot densities allowed us to detect 37 differentially expressed proteins (DEPs) upon drought treatment (2-fold, $p < 0.05$). Differential spots which were excised from the preparative gel stained by colloidal commasie (Figure 3.4) were subjected to peptide fragmentation analysis for the identification of proteins. Mascot search against the Uniprot database for green plants revealed the identity of 13 proteins from 37 spots (Figure 3.5). The number of the 13 identified DEP spots correspond to position numbers are listed in Table 3.2, along with their molecular weight, pI values and involved biological processes.

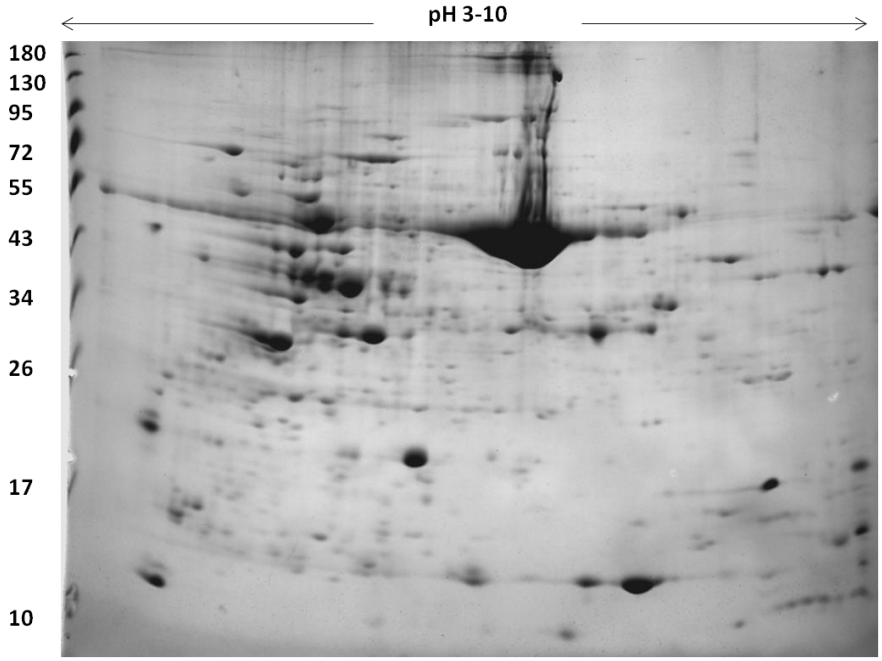


Figure 3.4: Coomassie blue stained preparative gel of *Brachypodium* leaves. 500 µg protein were separated on a 17 cm, pH 3-10 nonlinear strip, followed by separation on 12% SDS-PAGE gel.

Gene ontology (GO) enrichment and KEGG pathway analyses revealed the function and distribution of DEP spots (Figure 3.6). The biological functions of differentially regulated proteins included roles in photosynthesis, protein folding, antioxidant mechanism and metabolic processes. The proteomic data thus revealed a total of 13 proteins for all treatment days (including 4, 8 and 12), differentially regulated between drought and control conditions and the majority of these proteins were located in chloroplast.

Table 3.2: Differentially exprressed protein (DEP) spots identified by MALDI-MS/MS analysis.

ID	Protein description	MW kDa	Prot. Score	pI	Covarage %	Biological process
BRADI1G58160.1	Oxygen-evolving enhancer protein 2, chloroplastic	27.3	139	8.84	31	Photosynthesis
BRADI1G56580.1	Oxygen-evolving enhancer protein 1, chloroplastic	35.2	474	5.89	27	Photosynthesis
BRADI5G07190.1	Transketolase, chloroplastic	72.9	158	5.47	15	Catalytic activity
BRADI4G24367.1	Fructose bisphosphate aldolase	37.8	156	5.98	22	Glycolytic process
BRADI5G09650.1	2-cys peroxiredoxin	23.3	280	5.48	37	Antioxidant
BRADI4G39470.1	Stromal 70 kDa heat shock-related protein, chloroplastic	64.9	374	4.37	21	Protein folding
BRADI1G69680.1	Superoxide dismutase Cu/Zn	15.2	138	5.75	19	Antioxidant
BRADI5G05900.1	HSP 70, mitochondrial	72.5	286	5.95	17	Protein folding
BRADI2G44856.1	Carbonic anhydrase, chloroplastic	35.1	77	8.93	17	Metabolic process
BRADI3G14120.3	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	36.5	437	6.67	56	Oxidoreductase activity
BRADI1G09300.2	Serine hydroxymethyl transferase 1, mitochondrial	57.0	386	8.72	27	Catalytic activity
BRADI4G09120.2	Ribulose bisphosphate carboxylate/oxygenase activase B	47.2	431	7.59	32	ATP-binding
BRADI4G09120.2	Rubisco small subunit	19.4	136	8.52	24	Photosynthesis

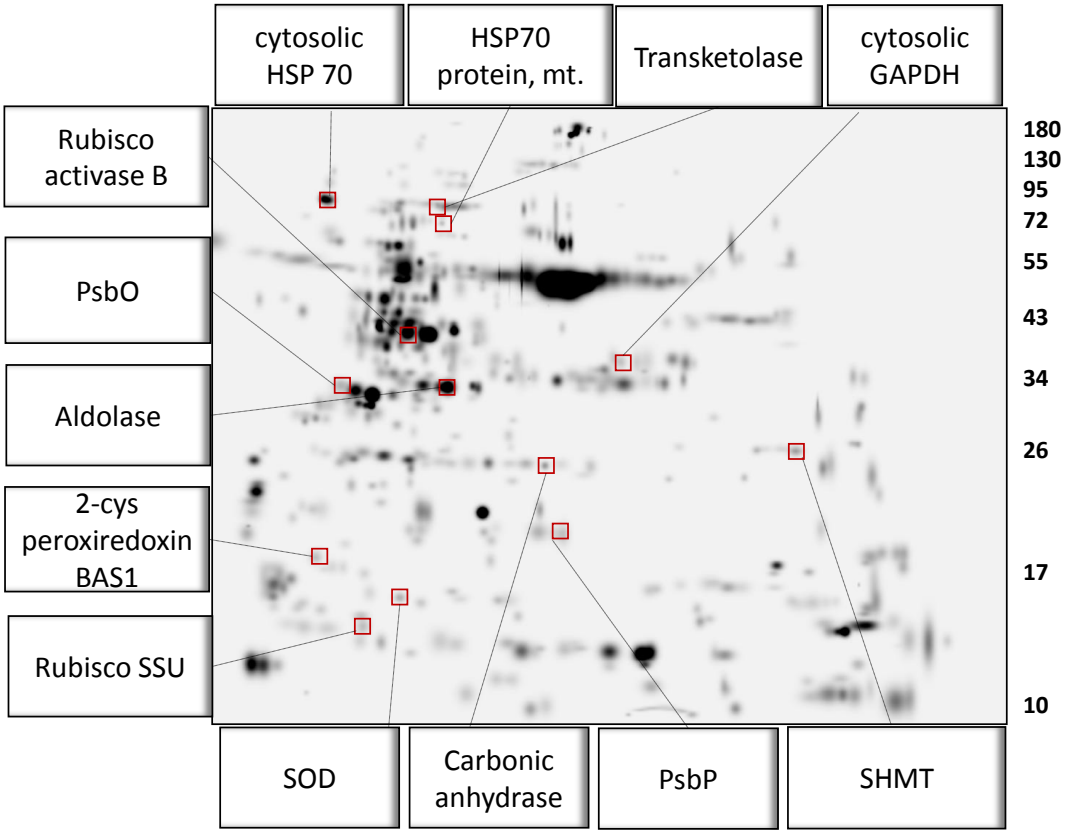


Figure 3.5: Master gel with differentially expressed spots.

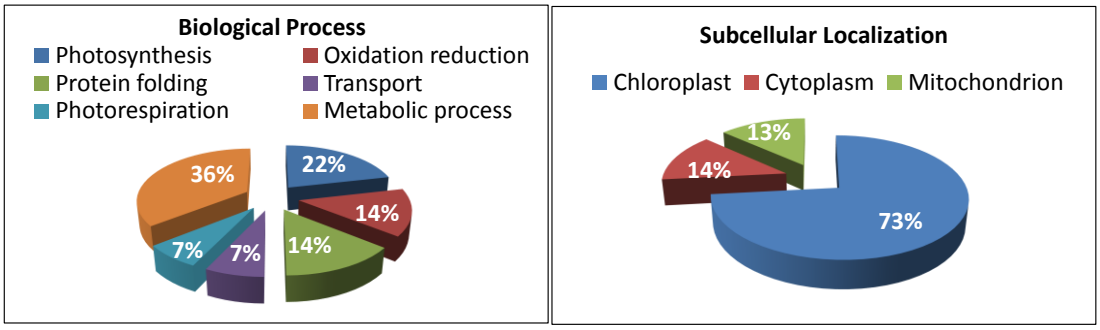


Figure 3.6: Gene Ontology (GO) term enrichment analysis of identified proteins.

3.2.1 Chloroplast-located proteins

Reduction of photosynthetic activity, accumulation of organic acids and osmolytes, and changes in carbohydrate metabolism are typical physiological and biochemical responses to drought stres (Ji *et al.*, 2012). In this study, a large proportion of the proteins whose abundance changed significantly under drought are associated with photosynthesis. In the leaf quantitative proteomic analyses, 8 of the identified differentially expressed proteins are located in chloroplast; oxygen evolving enhancer protein 1, oxygen evolving enhancer protein 2, transketolase, ribulose bisphosphate carboxylate/oxygenase activase B, ribulose bisphosphate carboxylase

small chain, fructose bisphospahte aldolase, carbonic anhydrase and 2-cys peroxiredoxin BAS 1.

The proteomic analysis revealed structural components of the oxygen-evolving complexes as being significantly upregulated upon drought treatment. Oxygen evolving enhancer protein 1 and 2 are subunits of oxygen evolving system of photosystem II, involved in the stabilization of the PSII complex. Both of them showed a similar pattern in time-dependent application of drought and showed significant increase at the 8th day ($p = 0.046$, Figure 3.7).

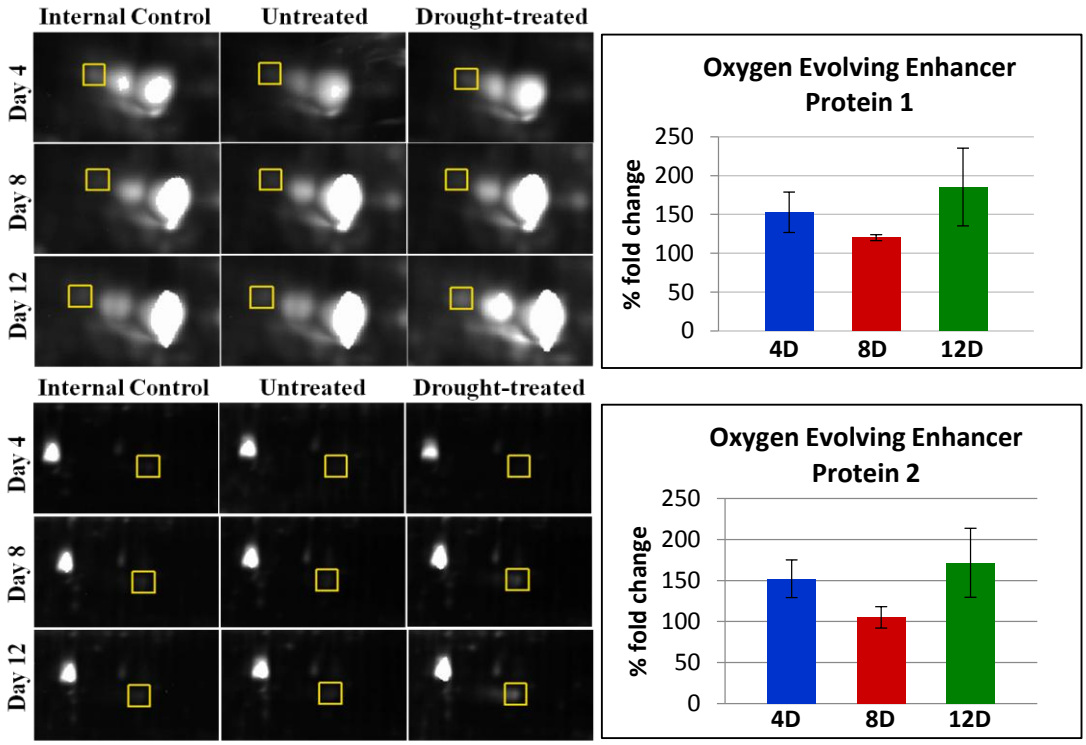


Figure 3.7: Protein spots of oxygen evolving enhancer protein 1 and 2 (PsbO, PsbP) associated with the response of *Brachypodium* leaves to dehydration.

Transketolase which is an enzyme of the Calvin cycle of photosynthesis in plants showed significant increase at the 8th day of drought treatment ($p = 0.029$, Figure 3.8). However, under drought, the elevation in its level goes back to the normal state. In addition to its role in Calvin cycle, transketolase also participates in the oxidative pentose phosphate pathway to produce erythrose-4-phosphate. One of its substrates, fructose-6-phosphate is also the beginning point for starch synthesis.

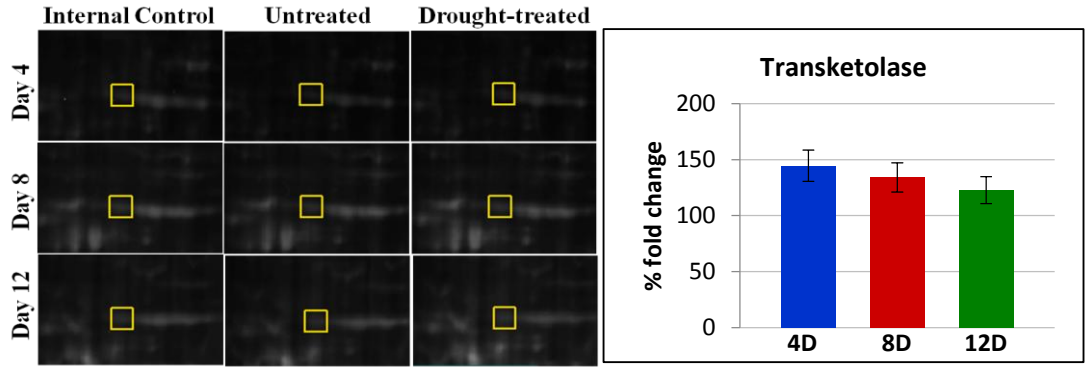


Figure 3.8: Protein spots of transketolase associated with the response of *Brachypodium* leaves to dehydration.

Ribulose bisphosphate carboxylase small chain is a bifunctional enzyme that catalyses both the carboxylation and oxygenation of ribulose-1,5-bisphosphate and fixing carbon dioxide as the first step of the Calvin cycle. In this study, it was noted that the abundance of RuBisCO small subunit decreased in response to drought stress, significantly at the 4^{th} ($p = 0.041$), 8^{th} ($p = 0.038$) and 12^{th} ($p = 0.037$) day of treatment (Figure 3.9).

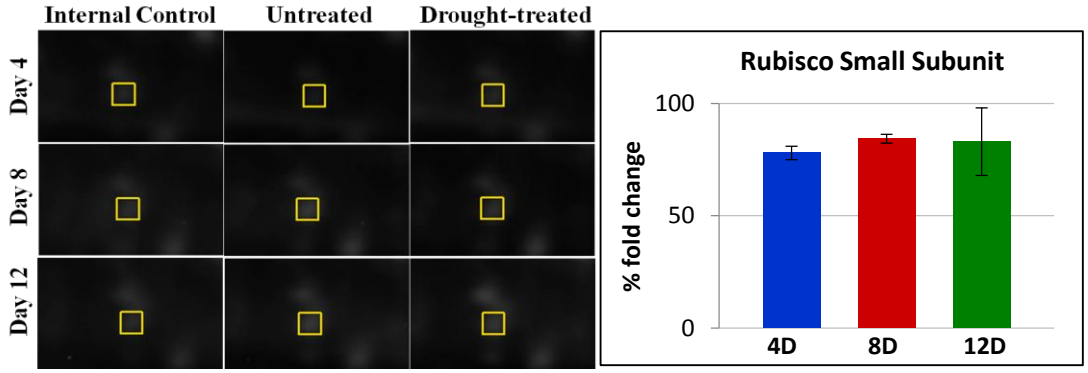


Figure 3.9: Protein spots of RUBISCO small chain associated with the response of *Brachypodium* leaves to dehydration.

Rubisco activase is a protein located in chloroplast and controls the switching of Rubisco conformation from inactive to active and thus acts as a molecular chaperone. In *Brachypodium*, Rubisco activase B decreased by the increased duration of drought significantly at the 8^{th} day ($p = 0.003$, Figure 3.10).

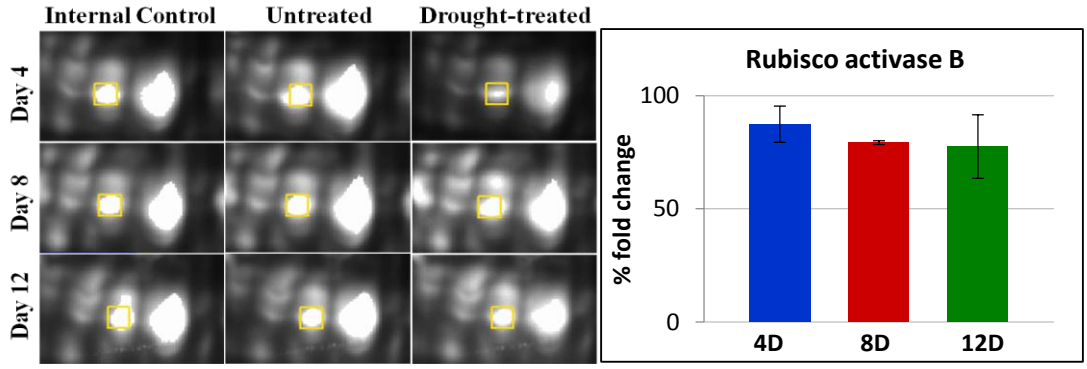


Figure 3.10: Protein spots of RUBISCO activase B associated with the response of *Brachypodium* leaves to dehydration.

Fructose-bisphosphate aldolase is an enzyme of both glycolysis and (in the opposite direction) Calvin cycle of CO_2 fixation. Two different forms of aldolase are present in higher plants: cytoplasmic and plastidic. The change of aldolase began with slight up-regulation at the 4th day of treatment and continued to decrease with time and reached the minimum level significantly at the 12th day of treatment ($p = 0.041$, Figure 3.11).

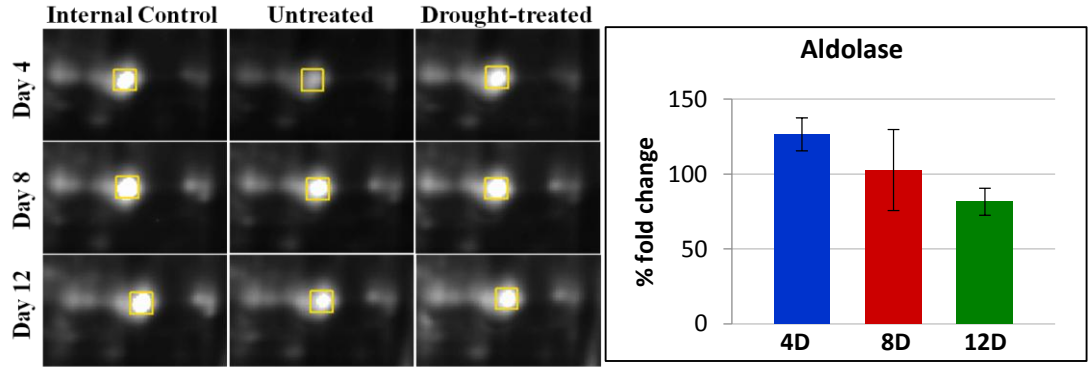


Figure 3.11: Protein spots of fructose-bisphosphate aldolase associated with the response of *Brachypodium* leaves to dehydration.

Drought-treated plants showed significant up-regulation of Carbonic anhydrase, which is a zinc-containing metalloenzyme**,** in the 12th day of treatment ($p = 0.035$, Figure 3.12). It functions in reversible hydration of CO_2. Increased expression of carbonic anhydrase can play a role in the buffering capacity of plant cells suffering from high concentrations of HCO_3 and CO_3^{-2} and facilitating CO_2 diffusion.

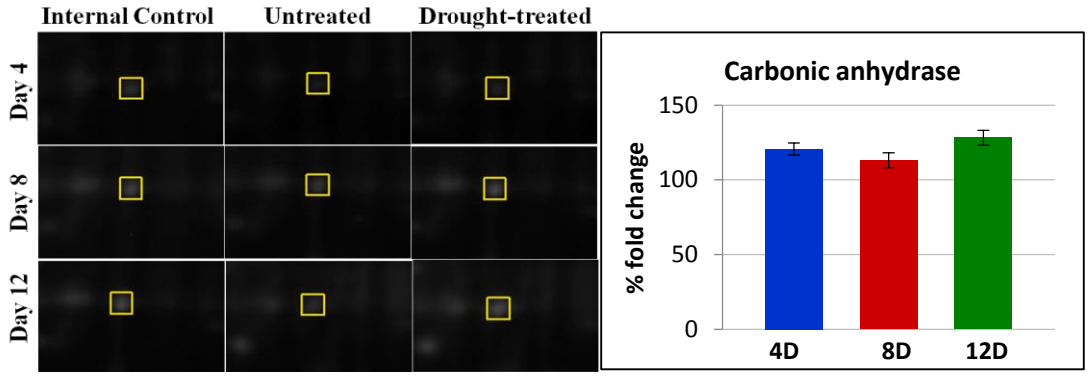


Figure 3.12: Protein spots of Carbonic anhydrase associated with the response of *Brachypodium* leaves to dehydration.

2-Cys peroxiredoxin BAS1 is involved in the detoxification of hydroperoxides. In *Brachypodium* leaves 2-Cys peroxiredoxin Bas 1 showed significant increase in the 12th day of drought treatment (p = 0.019, Figure 3.13).

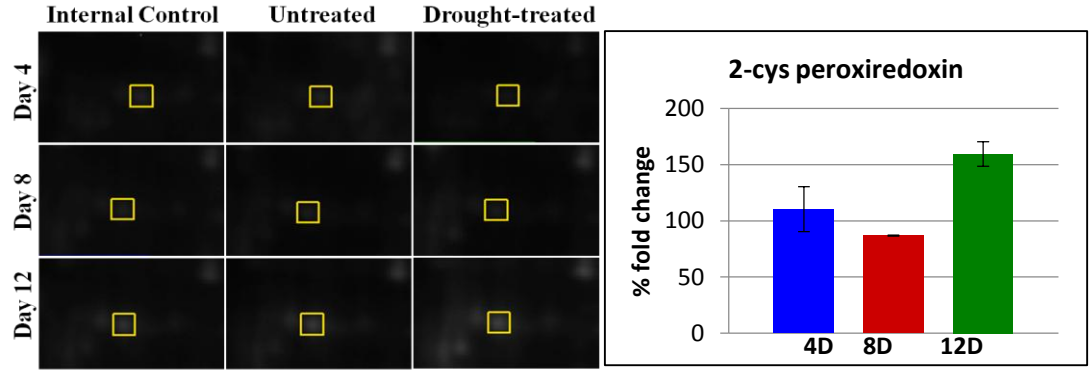


Figure 3.13: Protein spots of 2-cys peroxiredoxin associated with the response of *Brachypodium* leaves to dehydration.

3.2.2 Mitochondria-located proteins

In plants, drought stress can have an effect on the enzymes involved in respiratory carbon metabolism, other chloroplastic enzymes. Serine hydroxymethyltransferase (SHMT) functions in the photorespiratory pathway in catalyzing the interconversion of serine and glycine and involved in controlling cell damage caused by abiotic stress, such as high light and salt and the hypersensitive defense response of plants. It was noted that SHMT was upregulated in the 8th and significantly 12th day (p = 0.040) of drought treatment of *Brachypodium* leaves (Figure 3.14), correlating well with the findings of Moreno *et al.* (2005) and Zhou *et al.* (2012).

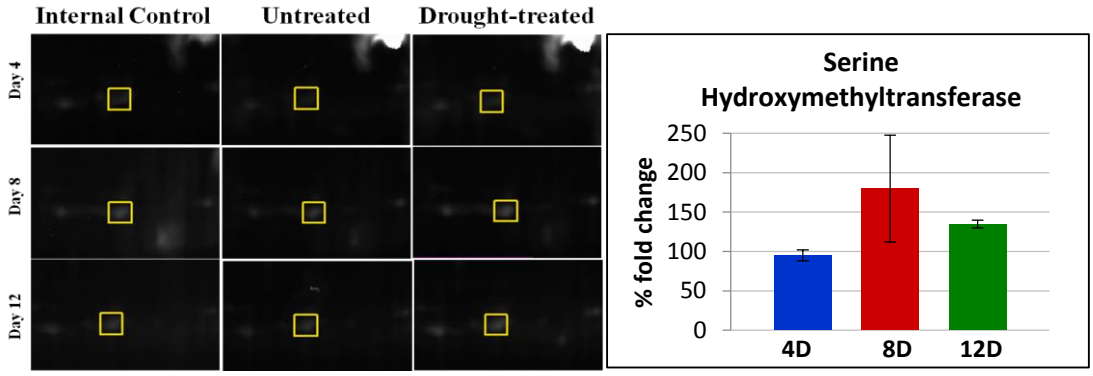


Figure 3.14: Protein spots of Serine hydroxymethyltransferase anhydrase associated with the response of *Brachypodium* leaves to dehydration.

As well as functioning as molecular chaperones, HSP family members function in protein disaggregation and protein degradation. Removal of potentially detrimental polypeptides caused by misfolding, denaturation or aggregation is important to maintain cellular homeostasis. HSP-70 was found to be upregulated in drought-treated *Brachypodium* leaves in the 12th day of treatment ($p = 0.036$, Figure 3.15), correlating with the findings of Ashoub *et al.* (2013).

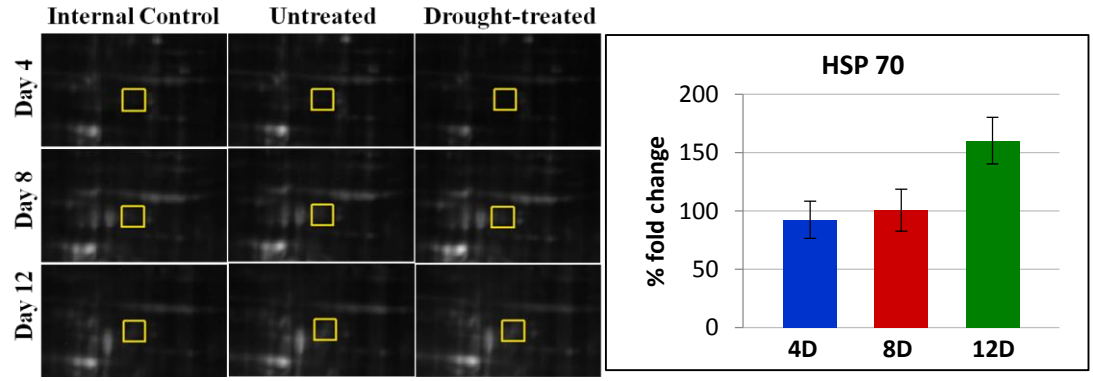


Figure 3.15: Protein spots of HSP-70 associated with the response of *Brachypodium* leaves to dehydration.

3.2.3 Cytosolic proteins

Glucose-6-phosphate dehydrogenase is a key enzyme, which regulates carbon flux through the pentose phosphate pathway. It is also a key enzyme that catalyses the first step of the oxidative pentose phosphate pathway. The main function of the pentose phosphate pathway is to provide NADPH and other intermediates, such as pentose and erythrose 4-phosphate.

Increased levels of GAPDH were observed under drought stress, especially in the 12th day of treatment ($p = 0.032$, Figure 3.16). There is supporting evidence for the

activation of ATP-generating pathways under different stresses, presumably to cope with a higher demand for ATP to maintain homeostasis under stress conditions.

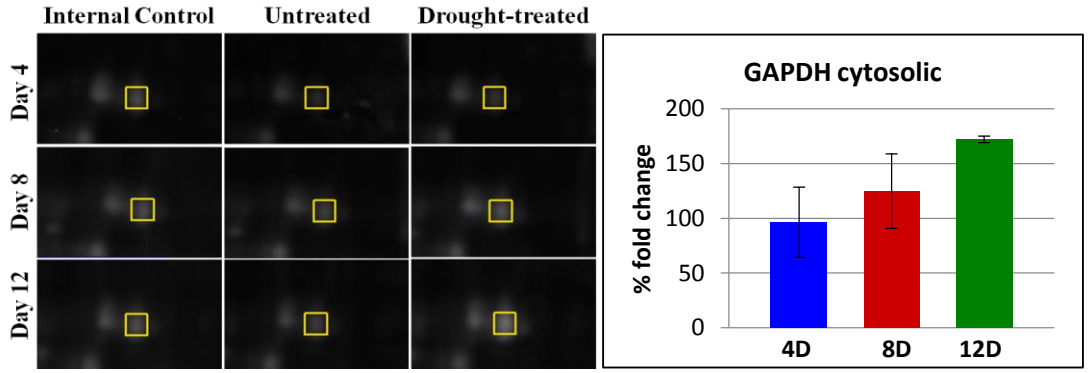


Figure 3.16: Protein spots of cytosolic GAPDH associated with the response of *Brachypodium* leaves to dehydration.

Drought stress induces the formation of reactive oxygen species (ROS) which result in lipid peroxidation, protein denaturation and nucleic acid damage with severe outcomes on cellular elements. ROS are scavenged chemically by antioxidant molecules or enzymatically by SOD and ascorbate peroxidase (APX) which scavenge O_2^- and H_2O_2, respectively. SOD is the first line of defense against oxidative stress. In this study, Cu-Zn SOD was up-regulated significantly in the 8th day of drought treatment ($p = 0.010$, Figure 3.17).

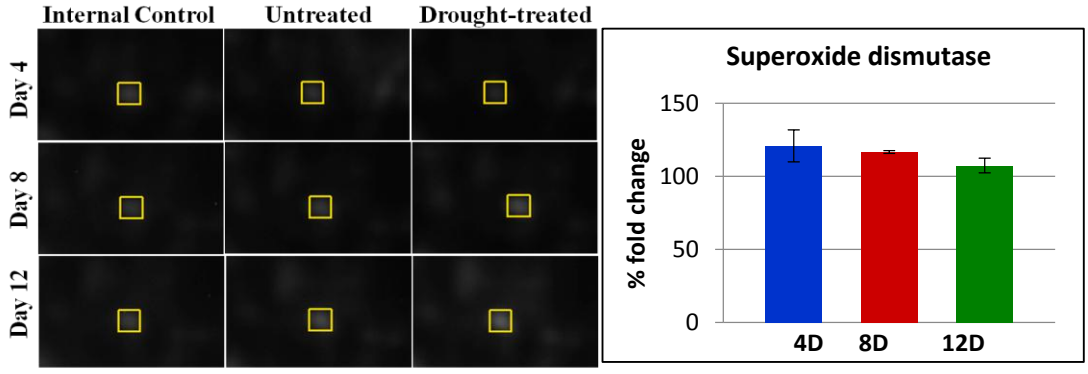


Figure 3.17: Protein spots of Superoxide dismutase associated with the response of *Brachypodium* leaves to dehydration.

Time-dependent fold changes are represented in HeatMap in Figure 3.18. Red bars indicate down-regulation of the protein, green bars indicate up-regulation of the protein. SSP numbers of DEP proteins are represented. All calculated p values of t test were represented in Table 3.3.

Table 3.3: P values of T-test of DEPs according to the day of treatment.

	4th day of treatment	8th day of treatment	12th day of treatment
Oxygen evolving enhancer protein 1	0.095	**0.046***	0.057
Oxygen evolving enhancer 2	0.080	**0.028***	0.135
Rubisco small chain	**0.041***	**0.038***	**0.036***
SOD	0.099	**0.011***	0.144
Rubisco activase B	0.150	**0.003***	0.064
Transketolase	0.138	**0.029***	0.103
2-cys peroxiredoxin	0.297	**0.028***	**0.019***
Fructose bisphosphate aldolase 2	0.095	0.480	**0.041***
Carbonic anhydrase	0.087	0.087	**0.035***
GAPDH cytosolic	0.414	0.249	**0.031***
HSP 70	0.309	0.480	**0.036***
SHMT	0.247	0.179	**0.040***

*Significant at $p < 0.050$

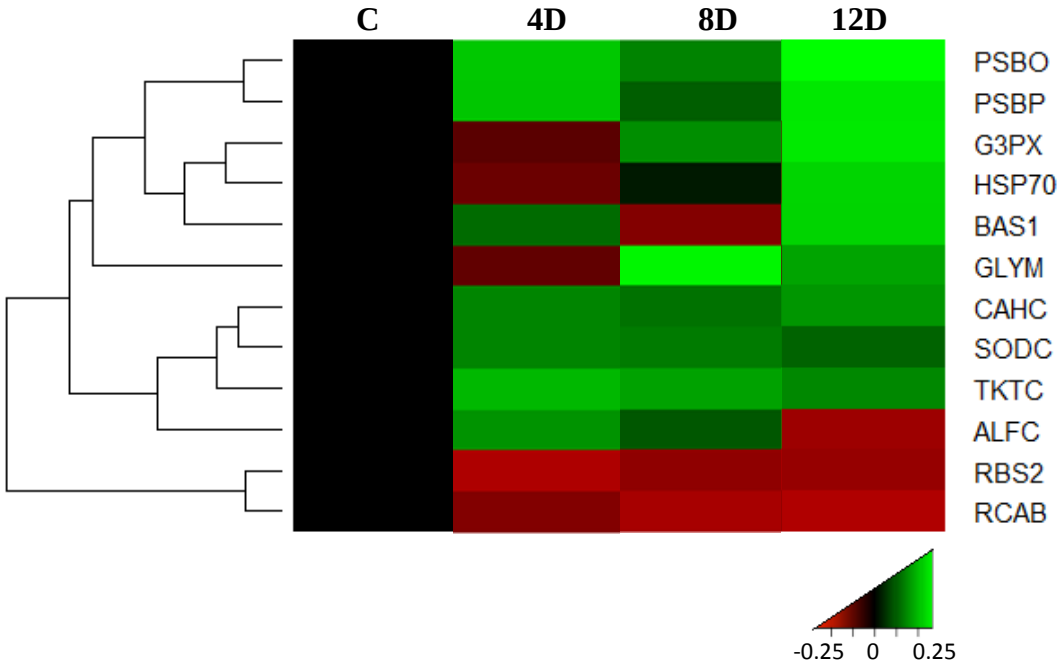


Figure 3.18: Hierarchical clustering of DEP spots in the treatment group. Each column represents a day of treatment. Each row represents the change of a DEP spot using color coding based on the relative ratio. The up- and down-regulated proteins are indicated in red and green, respectively.

Functional association network of the identified proteins was represented in Figure 3.19. Nodes are either colored (if they are directly linked to the input) or white (nodes of a higher iteration/depth).

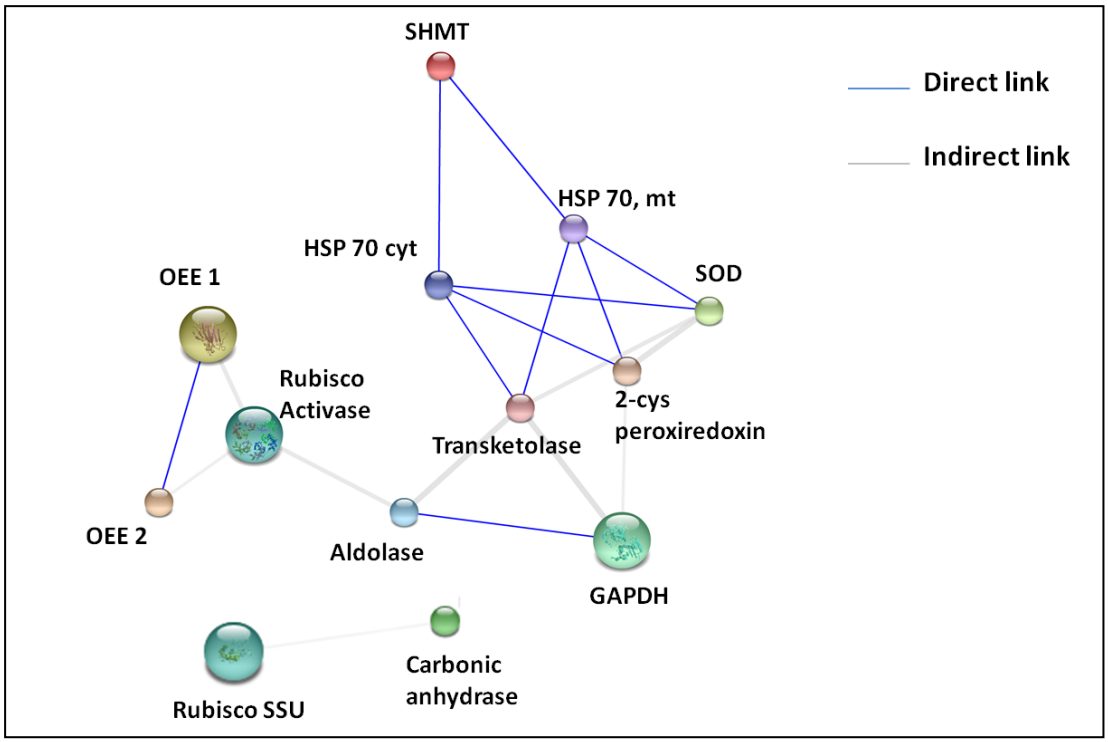


Figure 3.19: String analysis of associated proteins from *Brachypodium* leaves that changed with water withheld.

4. DISCUSSION

Plant stress response is a dynamic process which depends on intensity and duration of stress condition and can be divided into several stages. Response begins with an alarm phase, which results in a reduction in tolerance level when stress causes a shock to a non-acclimated plant. Then it continues with an acclimation phase leading to the establishment of a new state in plant metabolism under stress, resulting in an increase in the level of stress tolerance. Finally it is followed by an exhaustion phase, which occurs if stress continues and plant fails to maintain stress-induced homeostasis. The level of plant stress tolerance decreases during the exhaustion phase. Each phase of plant stress response corresponds to a different proteome composition (Kosova *et al*., 2011). In the current study, through a proteomic approach, a comprehensive analysis of drought stress response and defense in *Brachypodium distachyon* leaves was studied with a time-dependent manner for the first time. Plants respond to drought stress through a complex mechanism, which involves various cross-talk pathways. Studies about wheat, which is a close relative of *Brachypodium*, showed that the most affected molecular events in these cells upon drought stress are photosynthesis and energy metabolism (Komatsu *et al*., 2014). Our study represented a similar general picture of stress defense and response mechanisms in *Brachypodium* especially which mainly include photosynthesis and energy production. The proteins related to ROS scavenging and protein folding also involved in drought stress response and defense.

Inthis study, decreased level of both Rubisco oxygenase and Rubisco indicates the depression of Calvin cycle and as a consequence, the photosynthetic electron transport chain becomes reduced, which leads to the formation of reactive oxygen species and peroxides. These molecules give damage to the photosynthetic proteins. Peroxiredoxins and superoxide dismutases are part of antioxidant defense. To scavenge ROS and peroxides and thus reduce the cellular damage, superoxide dismutase and 2-cys peroxiredoxin levels show an increase, suggesting that an antioxidant system was induced in *Brachypodium* in response to dehydration. It was

noted that 2-Cys peroxiredoxin BAS1 could be induced by oxidative stress in *Arabidopsis* (Horling *et al*., 2003) to decrease oxidative damage to chloroplast proteins. Accumulation of Superoxide dismutases in response to drought and salinity was also observed in many studies (Ke *et al*., 2009; Rasoulnia *et al*. 2011; Salekdeh *et al*., 2002). Meanwhile, to regulate and stabilize photosystem II complex, the levels of oxygen-evolving complexes increase. Decreased activity of Rubisco leads to accumulation of HCO_3 and CO_3^{-2} and consequently cause a change in the pH of chloroplast stroma. Increased expression of carbonic anhydrase, which functions in reversible hydration of CO_2, presumably plays a role in the buffering of plant cells suffering from high concentrations of HCO_3 and CO_3 (Figure 4.1).

On the other hand, Rubisco is also responsible for the initiation of photorespiration system and the inactivation of rubisco results in photorespiration deficiency and thus reactive oxygen species accumulation in mitochondria. Serine hydroxy methyl transferase (SHMT) begins to accumulate in mitochondria to control the damage caused by drought stress, indicating that photorespiration is another part of the drought response mechanism. As a consequence of stress condition, many proteins are misfolded, and these proteins cannot be assembled and function as normal. To prevent the aggregation of denatured proteins upon drought stress, increased levels of molecular chaperones were observed both in mitochondria and cytosol (Lv *et al*., 2014).

In cytosol, GAPDH expression increases for the activation of ATP-generating pathways, presumably to cope with a higher demand for ATP to maintain homeostasis under drought stress. There are other studies that showed the up-regulation of cytosolic GAPDH protein under drought and salt stresses (Aranjuelo *et al*., 2011; Pang *et al*., 2010). Researchers overexpressed a stress-inducible GAPDH gene from oyster mushroom into potato plants and achieved to enhance tolerance to salt stress (Jeong *et al*., 2001) Therefore, it is relevant to analyze the effect of overexpression of GAPDH in other crops, in particular regarding tolerance to drought or other abiotic stresses.

As a consequence, for the first time, the model plant *Brachypodium* was studied in a comprehensive analysis at translational level under drought stress. This study highlighted that there is a significant degree of overlapping between metabolic alterations induced by drought stress. There are a number of previous data of

quantitative proteomic changes in other species associated with drought response that support the findings of this study. These findings could potentially broaden our view and provide a deeper knowledge into the complex mechanisms underlying stress response.

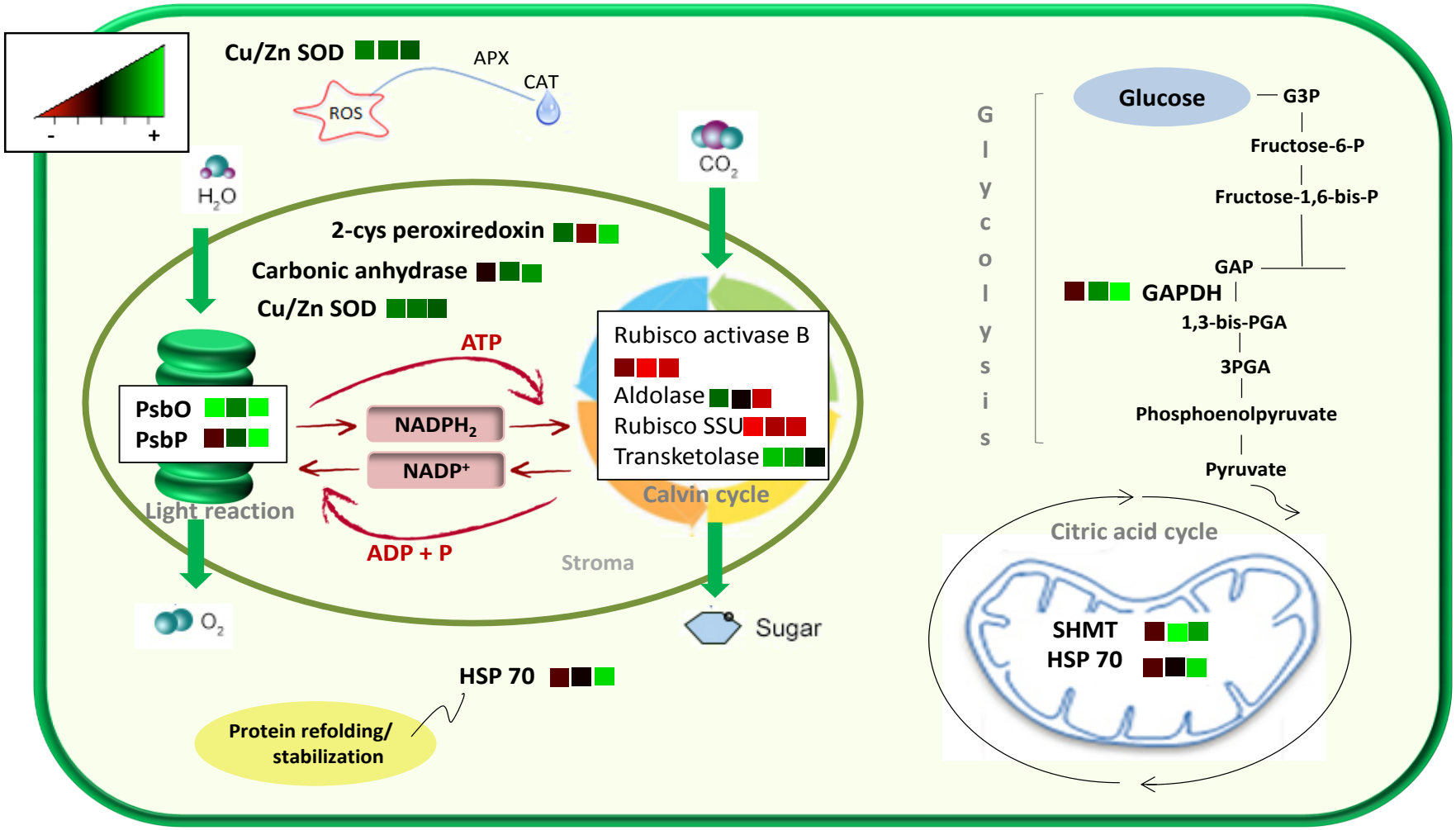


Figure 4.1: Schematic representation of identified proteins in response to drought stress in this study.

5. CONCLUSION

In summary, for the first time, the model plant *Brachypodium* was studied in a comprehensive analysis at translational level under drought stress. Time-dependent proteome analysis of drought stress treatment displayed the protein expression characters for drought stress response and defense in *Brachypodium*.

Changes in protein repertoire of *Brachypodium distachyon* leaves after exposing individuals to time-dependent drought stress (4, 8 and 12 day application of drought) highlighted some specific metabolic pathways to their functions and their role and relation in drought response. In total, 13 DEPs were identified through proteomic analyses. The biological functions of differentially regulated proteins included roles in photosynthesis, protein folding, antioxidant mechanism and metabolic processes. The proteomic data thus revealed a total of 13 proteins for all treatment days (including 4, 8 and 12), differentially regulated between drought and control conditions and the majority of these proteins were located in chloroplast.

The identified proteins in this study could serve as potential biomarkers for the selection of drought-tolerant *Brachypodium distachyon* plants as well as its relatives belong to the Grass family, Poaceae. Furthermore, identified proteins will contribute on the studies on development of drought-resistant crop species such as wheat, oat and barley, which are close relatives of *Brachypodium distachyon*.

REFERENCES

Aebersold, R., Mann, M. (2003) Mass-spectrometry based proteomics. Nature 422:198-207.

Ali, G.M., Komatsu, S. (2006) Proteomic analysis of rice leaf sheath during drought stress. J. Proteome Res. **5**:396–403.

Abdalla, K. O., & Rafudeen, M. S. (2012). Analysis of the nuclear proteome of the resurrection plant Xerophyta viscosa in response to dehydration stress using iTRAQ with 2DLC and tandem mass spectrometry. J Proteomics, 75(8), 2361-2374. doi: 10.1016/j.jprot.2012.02.006.

Abreu, I. A., Farinha, A. P., Negrao, S., Goncalves, N., Fonseca, C., Rodrigues, M. vd. (2013). Coping with abiotic stress: proteome changes for crop improvement. J Proteomics, 93, 145-168. doi: 10.1016/j.jprot.2013.07.014.

Ahuja, I., de Vos, R. C., Bones, A. M., & Hall, R. D. (2010). Plant molecular stress responses face climate change. Trends Plant Sci, 15(12), 664-674. doi: 10.1016/j.tplants.2010.08.002.

Aranjuelo I, Molero G, Erice G, Avice JC, Nogues S. (2011). Plant physiology and proteomics reveals the leaf response to drought in alfalfa (Medicago sativa L.). J Exp Bot 62: 111–23.

Baev, V., Milev, I., Niaydenov, M., Apostolova, E., Minkov, G., Minkov, I., Yahubyan, G. (2011). Implementation of a *de novo* genome-wide computational approach for updating *Brachypodium* miRNAs, Genomics, 97: 282-293.

Barr, H.D. and Weatherley, P.E. (1962). A re-examination of the relative turgidity technique for estimating water deficit in leaves. Aust. J. Biol. Sci. 15:413-428.

Bartels, D. and Sunkar, R. (2005). Drought and salt tolerance in plants, Critical Reviews in Plant Sciences, 24:23-58.

Bayramov, S., Guliyev, N. (2014). Changes in Rubisco activase gene expression and polypeptide content in *Brachypodium distachyon*, Plant Physiology and Biochemistry, http://dx.doi.org/10.1016/j.plaphy.2014.01.013.

Beckett, P. (2012) The basics of 'D-DIGE. Methods Mol Biol. 854:9-19.

Bertolini, E. *et al*. (2013). Addressing the Role of microRNAs in Reprogramming Leaf Growth during Drought Stress in *Brachypodium distachyon,* Molecular Plant, 6: 423–443.

Brkljacic, J. et al. (2011)**.** Brachypodium as a model for the grasses: today and the future, Plant Physiology, 157: 3-13.

Budak, H. and Akpinar, A. (2011). Dehydration stress-responsive miRNA in *Brachypodium distachyon*: evident by genome-wide screening of microRNAs expression, OMICS: A Journal of Integrative Biology, 15: 791-799.

Century, K., Reuber, T.L. and Ratcliffe, O.J. (2008). Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products, *Plant physiol*., 147:20–29.

Colton-Gagnon, K. et al. (2013). Comparative analysis of the cold acclimation and freezing tolerance capacities of seven diploid *Brachypodium distachyon* accessions, Annals of Botany, doi:10.1093/aob/mct283.

Ding, Y., Tao, Y., Zhu, C. (2013). Emerging roles of microRNAs in the mediation of drought stress response in plants, Journal of Experimental Botany, published online.

FAO, (2009)**.** Increasing crop production sustainably the perspective of biological processess. Global perspective studies unit, food and agriculture organization of the united nations.

Faurobert, M., Pelpoir, E. and Chaïb, J. (2006). Phenol Extraction of Proteins for Proteomic Studies of Recalcitrant Plant Tissues, Plant Proteomics: Methods and Protocols, Humana Press: New Jersey.

Fraire-Velázquez, S., Rodríguez-Guerra, R. and Sánchez-Calderón, L., (2011). Abiotic and biotic stress response crosstalk in plants, Abiotic Stress Response in Plants—Physiological, Biochemical and Genetic Perspectives, InTech, Rijeka, Croatia, pp. 3–26.

Feng, Y., Yina, Y., Feia, S. (2015). Down-regulation of BdBRI1, a putativebrassinosteroid receptor geneproduces a dwarf phenotype with enhanced drought tolerance in *Brachypodium* distachyon

Frey T. J., Weldekidan T., Colbert T., Wolters P. J. C. C., Hawk J. A. (2011). Fitness evaluation of Rcg1, a locus that confers resistance to *Colletotrichum graminicola* (Ces.) G.W. Wils. using near-isogenic maize hybrids. *Crop Sci.* 51 1551–1563.

Filiz, E., Ozdemir, B.S. and Budak, F. et al. (2009). Molecular, morphological, cytological analysis of diverse *Brachypodium* inbred lines, *Genome*, 52:876-890.

Gao, L., Yan, X., Li, X., Guo, G., Hu, Y., Ma, W., & Yan, Y. (2011). Proteome analysis of wheat leaf under salt stress by two-dimensional difference gel electrophoresis (2D-DIGE). Phytochemistry, 72(10), 1180-1191. doi: 10.1016/j.phytochem.2010.12.008.

Genga, A. et al., 2011. Plant Metabolomics: A Characterization of Plant Responses to Abiotic Stresses, Abiotic Stress in Plants - Mechanisms and Adaptations, pp. 309-340.

Gutterson, N. and Zhang, J.Z. (2004). Genomics applications to biotech traits:a revolution in progress?, Curr. Opin. Plant Biol., 7:226–230.

Gygi, S.P., Rochon, Y., Franza, B.R. ve Aebersol, R. (1999). Correlation between protein and mRNA abundance in yeast. Molecular and Cellular Biology, 19:1720-1730.

Hirayama, T. and Schinozaki, K. (2010). Research on plant abiotic stress responses in the post-genome era: past, present and future, *The plant journal*, 61:1041–1052.

Horling, F., Lamkemeyer, P., Ko¨ nig, J., Finkemeier, I., Kandlbinder, A., Baier, M., and Dietz, K.-J. (2003) Divergent light-, ascorbate-, and oxidative stress-dependent regulation of expression of the peroxiredoxin gene family in Arabidopsis. Plant Physiol. 131, 317–325.

Jeong MJ, Park SC, Byun MO. (2001) Improvement of salt tolerance in transgenic potato plants by glyceraldehyde-3 phosphate dehydrogenase gene transfer. Mol Cells 12:185–9.

Jeong, D.H. et al., (2013). Parallel analysis of RNA ends enhances global investigation of microRNAs and target RNAs of Brachypodium distachyon, *Genome biology*, 14:145-167.

Kantar, M., Lucas, S. J., & Budak, H. (2011). miRNA expression patterns of Triticum dicoccoides in response to shock drought stress. Planta, 233(3), 471-484. doi: 10.1007/s00425-010-1309-4.

Ke YQ, Han GQ, He HQ, Li JX. (2009) Differential regulation of proteins and phosphoproteins in rice under drought stress. Biochem Biophys Res Commun 379:133–8.

Komatsu, S., Kamal, A.H.M., Hossain, Z. (2014) Wheat proteomics: proteome modulation and abiotic stress acclimation. Front. Plant Sci. 684:1-19.

Koornneef, M. and Meinke, D. (2010). The development of Arabidopsis as a model plant, *The plant journal*, 61:909-921.

Kosova, K., Vitamvas, P., Prasil, I. T., & Renaut, J. (2011). Plant proteome changes under abiotic stress--contribution of proteomics studies to understanding plant stress response. J Proteomics, 74(8), 1301-1322. doi: 10.1016/j.jprot.2011.02.006.

Lv, D. W., Subburaj, S., Cao, M., Yan, X., Li, X., Appels, R. vd. (2014). Proteome and phosphoproteome characterization reveals new response and defense mechanisms of Brachypodium distachyon leaves under salt stress. Mol Cell Proteomics, 13(2), 632-652. doi: 10.1074/mcp.M113.030171.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490–498.

Mochida, K., Yoshida, T., Sakurai, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L.P. (2011). In Silico Analysis of Transcription Factor Repertoires and Prediction of Stress-Responsive Transcription Factors from Six Major Gramineae Plants, *DNA research*, 18: 321-332.

Moreno, JI., Martin, R. and Castresana R. (2005) Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory

pathway influences resistance to biotic and abiotic stress. The Plant Journal, 41:451–463.

Nouri, M. Z., & Komatsu, S. (2013). Subcellular protein overexpression to develop abiotic stress tolerant plants. Front Plant Sci, 4, 2. doi: 10.3389/fpls.2013.00002.

Opanowicz, M., Vain, P., Draper, J., Parker, D., Doonan, J.H. (2008). Brachypodium distachyon, making hay with a wild grass, Trend in Plant Science, 13:172-177.

Ozdemir, B.S., Hernandez, P., Filiz, E., Budak, H. (2008). Brachypodium genomics, Plant Physiology, Volume 208.

Padmalatha, K. V., Dhandapani, G., Kanakachari, M., Kumar, S., Dass, A., Patil, D. P. vd. (2012). Genome-wide transcriptomic analysis of cotton under drought stress reveal significant down-regulation of genes and pathways involved in fibre elongation and up-regulation of defense responsive genes. Plant Mol Biol, 78(3), 223-246. doi: 10.1007/s11103-011-9857-y.

Pang QY, Chen SX, Dai SJ, Chen YZ, Wang Y, Yan XF. (2010) Comparative proteomics of salt tolerance in Arabidopsis thaliana and Thellungiella halophila. J Proteome Res 9: 2584–99.

Peng, Z., Wang, M., Li, F., Lv, H., Li, C., & Xia, G. (2009). A proteomic study of the response to salinity and drought stress in an introgression strain of bread wheat. Mol Cell Proteomics, 8(12), 2676-2686. doi: 10.1074/mcp.M900052-MCP200.

Rasoulnia A, Bihamta MR, Peyghambari SA, Alizadeh H, Rahnama A. (2011) Proteomic response of barley leaves to salinity. Mol Biol Rep 38:5055–63.

Ryu, J.Y. et al., (2014). Molecular and functional characterization of cold-responsive C-repeat binding factors from *Brachypodium distachyon*, *BMC Plant biology*, 14:1-15.

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J. (2002) Proteomic analysis of rice leaves during drought stress and recovery. Proteomics 2:1131–45.

Schulze,E.D., Beck,E. and Müller-Hohenstei, K. (2005). Stress Physiology, *Plant ecology*, Germany pp: 8.

Shin, Kwang-Hyun; Kamal, Abu Hena Mostafa; Cho, Kun; Choi, Jong-Soon; Yu, Jin; Nam-Chon, Paek; Lee, Yin Won; Lee, Jong Kwan; Park, Jong-Chul; Kim, Heung-Tae and Woo, Sun Hee. (2011) Defense proteins are induced in wheat spikes exposed to Fusarium graminearum [online]. Plant Omics, 4: 270-277.

Tanackovic, V. et al. (2014). The deposition and characterization of starch in *Brachypodium distachyon*, *Journal of experimental botany*, 65:5179-5192.

Tatli, O. (2015). Targeted metabolomic analysis of central carbon metabolism on model plant *Brachypodium distachyon* to elucidate physiological response to

drought stress (Published master's thesis). Bahçeşehir University, Istanbul, Turkey.

Tripathi, P. et al. (2012). The WRKY transcription factor family in *Brachypodium distachyon*, *BMC Genomics*, 13: 270-290.

Unver, T. and Budak, H. (2009). Conserved microRNAs and their targets in model grass species *Brachypodium distachyon*, Planta, 230:659–669.

URL1 < http://www.ipcc.ch > Retrieved December 28, 2015,.

Velthuizen H, Huddleston B, Fischer G, Salvatore M, Ataman E, Nachtergaele FO, et al. (2007). Mapping biophysical factors that ınfluence agricultural production and rural vulnerability. Environment and Natural Resources Series No. 11, Rome: FAO.

Verelst et al. (2013). Molecular and Physiological Analysis of Growth-Limiting Drought Stress in Brachypodium distachyon Leaves, Molecular Plant, 6:311-322.

Vogel, J.P. and Bragg, J. (2009). Brachypodium distachyon, a new model for the Triticeae. *Genetics and genomics of the Triticeae. Plant genetics and genomics: crops and models*, Berlin: Springer, 7:427–449.

Wei, B. et al., (2009). Novel microRNAs uncovered by deep sequencing of small RNA transcriptomes in bread wheat (Triticum aestivum L.) and *Brachypodium distachyon* (L.) Beauv, *Funct integr genomics*, 9:499–511.

Wrzaczek, M. et al. (2011). Reactive Oxygen in Abiotic Stress Perception - From Genes to Proteins, *Abiotic Stress Response in Plants – Physiological, Biochemical and Genetic Perspectives*, InTech, Rijeka, Croatia, pp. 27-38.

You, J., Zhang, L., Song, B., Qi, X., Chan, Z. (2015). Systematic Analysis and Identification of Stress-Responsive Genes of the NAC Gene Family in *Brachypodium* distachyon, PLoS ONE, 10(3): e0122027.

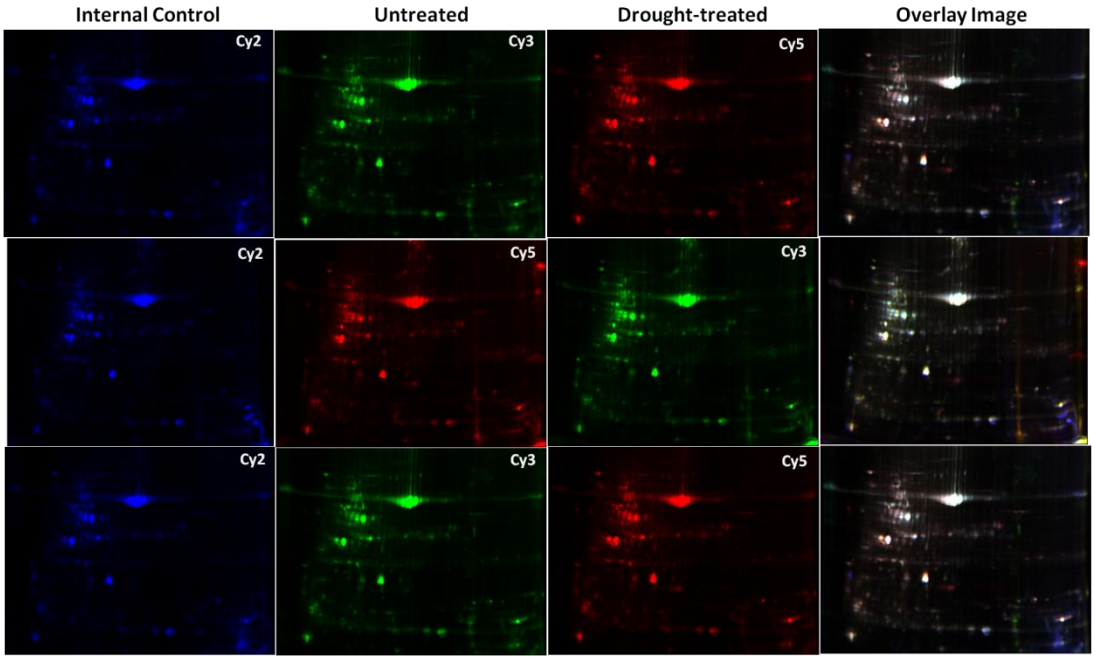
Zhang, X. et al. (2006). Genome-wide high-resolution mapping and functional analysis of DNA methylation in Arabidopsis, Cell,126:1189–1201.

Zhang, J., Xu, Y., Huan, Q., Chong, K. (2009). Deep sequencing of *Brachypodium* small RNAs at the global genome level identifies microRNAs involved in cold stress response, *BMC Genomics*, 10:449-465.

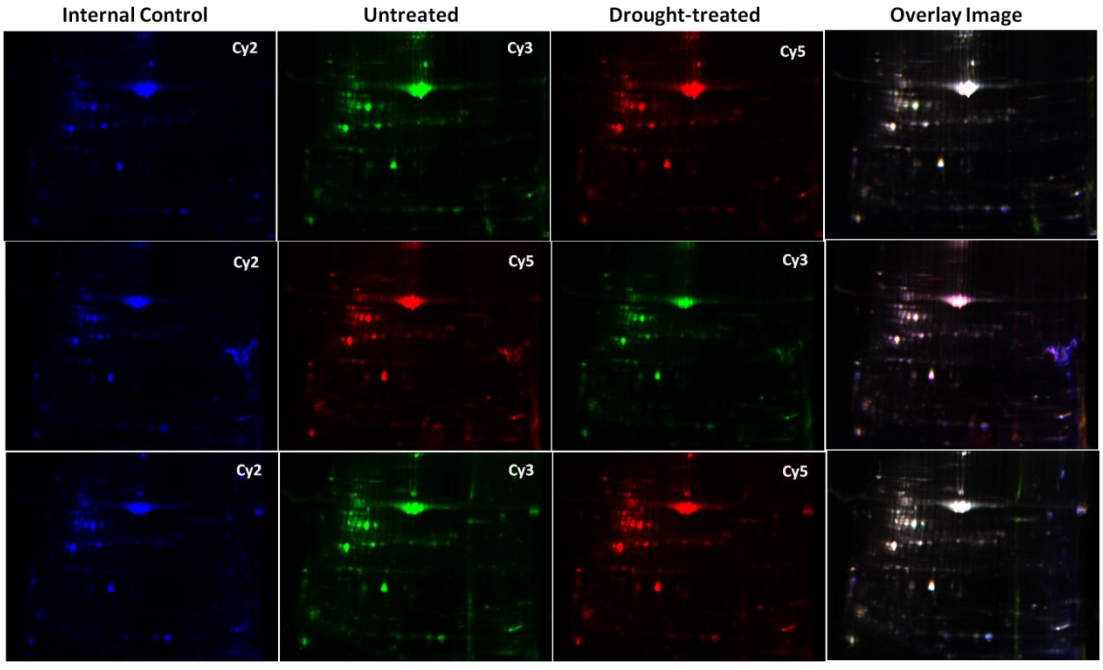
Zilberman, D. et al. (2007). Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription, Nature Genet., 39:61–69.

APPENDICES

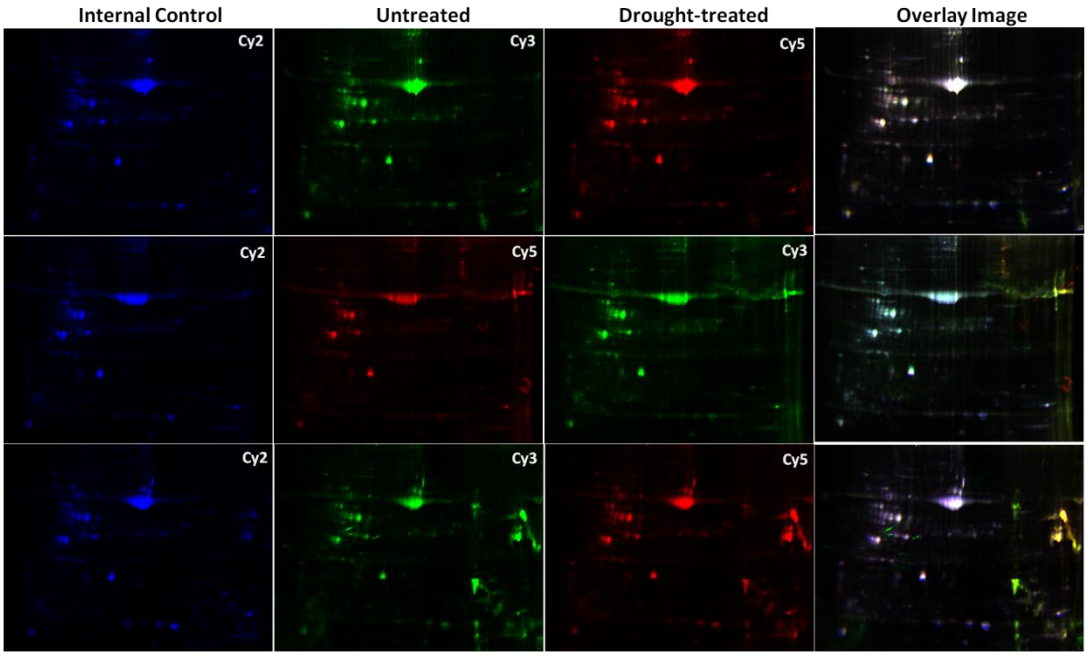
Appendix A. Replicates of 2D-DIGE gels belong to the experimental and control group for 4th day of treatment.



Appendix B. Replicates of 2D-DIGE gels belong to the experimental and control group for 8th day of treatment.



Appendix C. Replicates of 2D-DIGE gels belong to the experimental and control group for 12th day of treatment.



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